Common noncoding UMOD gene variants induce salt-sensitive hypertension and kidney damage by increasing uromodulin expression

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Hypertension and chronic kidney disease (CKD) are complex traits representing major global health problems1,2. Multiple genome-wide association studies have identified common variants in the promoter of the UMOD gene3–9, which encodes uromodulin, the major protein secreted in normal urine, that cause independent susceptibility to CKD and hypertension. Despite compelling genetic evidence for the association between UMOD risk variants and disease susceptibility in the general population, the underlying biological mechanism is not understood. Here, we demonstrate that UMOD risk variants increased UMOD expression in vitro and in vivo. Uromodulin overexpression in transgenic mice led to salt-sensitive hypertension and to the presence of age-dependent renal lesions similar to those observed in elderly individuals homozygous for UMOD promoter risk variants. The link between uromodulin and hypertension is due to activation of the renal sodium cotransporter NKCC2. We demonstrated the relevance of this mechanism in humans by showing that pharmacological inhibition of NKCC2 was more effective in lowering blood pressure in hypertensive patients who are homozygous for UMOD promoter risk variants than in other hypertensive patients. Our findings link genetic susceptibility to hypertension and CKD to the level of uromodulin expression and uromodulin’s effect on salt reabsorption in the kidney. These findings point to uromodulin as a therapeutic target for lowering blood pressure and preserving renal function.

Current understanding of the complex genetic architecture of hypertension and CKD stems from the identification of mutations causing rare inherited disorders10,11 and of several susceptibility loci through population-based association studies12–14. However, deciphering the biological mechanisms underlying these genetic associations has proven to be a major challenge.

Recent genome-wide association studies (GWAS) in more than 200,000 individuals of European ancestry have identified susceptibility variants for renal function, CKD and hypertension in the UMOD gene encoding uromodulin3–9. Uromodulin (or Tamm-Horsfall protein) is the most abundant urinary protein and is specifically produced and secreted by the epithelial cells lining the thick ascending limb (TAL) of the loop of Henle in the kidney15. Studies in Umod knockout mice revealed that uromodulin may protect against urinary tract infection16 and kidney stones17 and modulate electrolyte tubular transport18. Recent evidence suggests that uromodulin regulates the activity of the sodium-potassium-chloride transporter (NKCC2) and the renal outer medullary potassium channel (ROMK), the two main ion transporters involved in NaCl reabsorption by the TAL segment19,20. Mutations in UMOD have been associated with rare dominantly inherited disorders causing kidney damage and CKD21. The observation that susceptibility variants in the UMOD gene have a high frequency (about 0.8) in the general population and confer about 20% increased risk for CKD and 15% for hypertension emphasizes the pressing need to understand the nature of their associated risk and how they affect uromodulin function22.

Given the localization of the most significant (lead) single nucleotide polymorphisms (SNPs) identified by GWAS in a linkage disequilibrium block that includes the UMOD gene promoter (Fig. 1a), we hypothesized that these variants could be associated with an effect on gene expression. We tested this hypothesis in vivo by measuring UMOD transcript levels in nephrectomy samples from individuals homozygous for either the risk or protective alleles at lead variants

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rs12917707 and rs4293393, which are both localized in the UMOD promoter. Carriers of the UMOD promoter risk variants showed twofold higher UMOD expression in kidney samples compared to carriers of the protective haplotype (Fig. 1b). We confirmed the association of UMOD promoter risk variants with higher uromodulin expression by showing a similar dose-dependent increase in urinary uromodulin levels in a large population-based cohort (SKIPGHO) (Fig. 1c).

In silico analysis revealed that among the lead SNPs from GWAS, rs4293393 maps to a highly conserved region of the UMOD promoter. Consensus alignments of human (Hs), gorilla (Gg), gibbon (Nl), macaque (Mm), cow (Bt) and dog (Cf) UMOD promoter sequences. The intensity of the blue color shading corresponds to nucleotide conservation (the darker the color, the higher the degree of nucleotide conservation). The rs4293393 SNP is predicted to lie within a glucocorticoid response element; the protective allele would disrupt this predicted binding site.

In vitro analysis of the relative effects of the protective (C) and risk (T) alleles of SNP rs4293393 on the transcriptional activity of UMOD promoter as assessed by a luciferase reporter assay in three types of kidney cells: mTAL, (highly differentiated mouse primary TAL cells retaining uromodulin expression), immortalized MKTAL (mouse kidney TAL) and HEK293 (human embryonic kidney). The data are from four independent experiments. The schematic shows the luciferase reporter constructs used, in which a 3.7-kb promoter fragment containing either the C or T allele was cloned upstream of the firefly luciferase (LUC) gene in the pGL3-Basic reporter vector (Basic corresponds to the promoterless vector). Data are expressed as means ± s.e.m. **P < 0.01; ***P < 0.001 (ANOVA followed by Bonferroni’s test).

As uromodulin is expressed in the TAL, a tubular segment that has been implicated in rare inherited disorders characterized by defective complex hormonal regulation. Overall, these results demonstrate that the UMOD promoter risk variant rs4293393, probably acting together with other variants within the same linkage disequilibrium block, is associated with higher uromodulin expression.

To model this effect in vivo, we took advantage of a transgenic mouse line expressing hemagglutinin (HA)-tagged wild-type uromodulin (Tg(Umod)416Lura, here referred to as TgUmodwt mice). We also generated a line homozygous for the transgene (TgUmod+/+ mice). Transgenic animals from both lines were viable, apparently healthy and indistinguishable from control nontransgenic mice (here referred to as control mice). The presence of the transgene caused a dose-dependent increase in uromodulin expression and secretion (Supplementary Fig. 3a–c). Notably, uromodulin was expressed at approximately 80% higher amounts in TgUmod+/+ mice relative to control mice, comparable to the effect on uromodulin expression in subjects homozygous for UMOD risk variants. Similarly to endogenous uromodulin, the transgenic protein was expressed exclusively in TAL segments of the nephron (Supplementary Fig. 3d–f; see also ref. 23).

As uromodulin is expressed in the TAL, a tubular segment that has been implicated in rare inherited disorders characterized by defective
Uromodulin overexpression leads to hypertension and renal damage. (a) Box-and-whisker plots showing systolic blood pressure at baseline in control and Umod transgenic mice at the indicated ages (n = 6–10 mice per group). Bars represent minimum and maximum values. (b) Average value and distribution of heart weight relative to tibia length (heart/tibia) in 16-month-old control and TgUmodwt/wt mice (left) and heart histology showing left ventricular hypertrophy in TgUmodwt/wt mice (right) (H&E 1x magnification). Each dot represents an individual mouse. (c) Average systolic blood pressure in 14-month-old control and TgUmodwt/wt mice on standard (1% NaCl) or low-sodium (0.01% NaCl) diet (n = 7 mice per group). Data are expressed as means ± s.e.m. (d) Top, representative renal histological images of 16-month-old control (Ctrl) and TgUmodwt/wt mice. Kidneys from TgUmodwt/wt mice show numerous dilated tubules (top row) mostly filled by casts (top and bottom rows) (PAS, scale bars, 100 µm). Bottom, quantification of the histological analysis (only parameters reaching statistical significance are shown) (n = 5 mice per group). Data are expressed as means ± s.d. (e) Top, representative renal tissue from elderly subjects homozygous for the protective or risk variants shows normal interstitial compartment (top row) and mild focal tubular damage with increased thickness of the tubular basement membrane (bottom row) (n = 9), whereas tissue from subjects homozygous for the risk variants shows dilated tubules with detachment of the tubular epithelium (top row) and presence of tubular casts (bottom row) (n = 15) (PAS, scale bars, 100 µm). Bottom, quantification of the histological analysis (only parameters reaching statistical significance are shown). Data are expressed as means ± s.d. (f) Tubular casts in renal tissue from a TgUmodwt/wt mouse are present in TALs (uromodulin-positive) or more distal tubules (uromodulin-negative) and are formed mostly by uromodulin (arrowhead) or by PAS-positive uromodulin-negative (arrows) material. Scale bar, 100 µm. (g) Transcript levels (qRT-PCR) of renal damage markers Lcn2 and Havcr1 (encoding Kim-1) and chemokines Ccl2 and Ccl5 in kidneys from 16-month-old mice (n = 5 per group). Data are expressed as means ± s.e.m. (h) Representative immunoblot images and quantitative data (n = 13 mice per group) showing levels of Lcn2 and Kim-1 in kidney lysates from 16-month-old control and TgUmodwt/wt mice normalized to the loading control, β-actin. Data are expressed as means ± s.d. *P < 0.05; **P < 0.01; ***P < 0.001 determined by unpaired t-test (a, left), (b, c and h), ANOVA followed by Bonferroni’s test (a, right) and g) or Mann-Whitney test (d and e).
Figure 3 Increased activation of Nkcc2 co-transporter and Spak kinase in TgUmod/wt mice. (a) Representative immunoblot images and quantitative data (n = 10–13 mice per group) showing levels of phosphorylated (Thr96 and Thr101) Nkcc2 (p-Nkcc2) and total Nkcc2 in kidney lysates from 16-month-old mice. Data are expressed as means ± s.e.m. of 4 independent experiments. #, unspecific signal. Calnexin was used as a loading control. (b) Nkcc2 mRNA (qRT-PCR) in total kidney extracts from 16-month-old mice (n = 5 per group). Data are expressed as means ± s.e.m. (c) Change in sodium excretion (left) and systolic blood pressure (SBP, right) in 16-month-old TgUmod/wt and control mice 2 h after treatment with furosemide (n = 8 Ctr mice, n = 6 TgUmod/wt mice (sodium excretion); n = 5 mice per group (SBP)). Data are expressed as means ± s.e.m. (d) Representative immunoblot images and quantitative data showing levels of phosphorylated and total Nkcc2 and uromodulin (UMOD) in HEK293 cells stably expressing Nkcc2 and transfected with either wild-type (WT UMOD) or soluble (Sol. UMOD) uromodulin. Ctr, mock transfected cells. β-actin was used as a loading control. Densitometric analysis (means ± s.d. of three independent experiments) is shown. (e) Quantification of Nkcc2 activity as assessed by dpH/Δt−1. Data are from eight independent experiments and are expressed as means ± s.e.m. (f) Representative immunoblot images and quantitative data (n = 4 mice per group) showing levels of phosphorylated and total Spak and Osr1 in kidney lysates from 16-month-old control and TgUmod/wt mice. Bars indicate means ± s.d. ** P < 0.01; *** P < 0.001 determined by ANOVA followed by Bonferroni's test (a, b, d and e) or unpaired t test (c and f).

more distal segments (Fig. 2f). Evidence of renal damage in transgenic mice also included increased renal expression of established markers of tubule damage (Lcn2, encoding lipocalin 2 (Lcn2), and Haurc1, encoding kidney injury molecule-1 (Kim-1)) and the chemokines Ccl2 and Ccl5 (Fig. 2g, h), and transgenic mice also had substantial microalbuminuria (data not shown). There was also a significant dilation of glomerular capillary loops in these mice (Fig. 2d and Supplementary Fig. 5a). There were no signs of interstitial vascular remodeling in the kidney that could be ascribed to chronic hypertension (Supplementary Fig. 5b), in line with the fairly rapid BP response induced by changes in dietary salt (see above).

In the context of the normal renal function of Umod transgenic mice, which showed normal or slightly decreased renin expression (Supplementary Fig. 6), we hypothesized that the salt-sensitive hypertensive phenotype of the transgenic mice could be caused by abnormal activation of Nkcc2, the main sodium transporter in the TAL. Consistent with this hypothesis, we found a significantly higher level of phosphorylation on Nkcc2 at activating sites (Thr96 and Thr101) in TgUmod/wt mice relative to control mice (Fig. 3a). The increase in phosphorylated Nkcc2 levels was linearly correlated with Umod gene dosage (P < 0.01, ANOVA post hoc test for linear trend). The specificity of this effect for phosphorylation on Nkcc2, rather than on Nkcc1, which can also be recognized by the antibodies used in this experiment, is supported by the almost exclusive localization of the phosphorylated protein signal on the apical membrane of TAL cells, which lacks Nkcc1, and by the 40-fold higher global expression of Nkcc2 as compared to Nkcc1, in the kidney (Supplementary Fig. 7a, b). In line with a post-translational mechanism for the regulation of Nkcc2, Nkcc2 transcript levels were not different between transgenic and control mice (Fig. 3b). Romk expression and membrane localization were similar in transgenic and control mice (Supplementary Fig. 8a–c).

To test the functional importance of Nkcc2 to the hypertensive phenotype of Umod transgenic mice, we assessed the response of these mice to furosemide, a loop diuretic that specifically targets Nkcc2. Treatment of TgUmod/wt mice with a single dose of furosemide induced both a significantly enhanced natriuretic effect and a significant reduction of blood pressure (Fig. 3c). The enhanced response of transgenic mice compared to control mice to furosemide treatment is probably due to increased Nkcc2 activity and increased sodium reabsorption in the TAL rather than to adaptive downregulation of NaCl reabsorption in more distal nephron segments, as control and transgenic mice had comparable levels of sodium-chloride symporter (Ncc) (distal convoluted tubules) and epithelial sodium channel (ENaC) (collecting ducts) (Supplementary Fig. 8d, e), as well as similar levels of aldosterone (Supplementary Fig. 8b).

We next investigated the direct effect of uromodulin on Nkcc2 activation in kidney cells stably expressing Nkcc2 and transiently transfected with either human wild-type uromodulin or with a soluble uromodulin isoform truncated at the glycosylphosphatidylinositol (GPI)-anchoring site (S614X)29. Expression of wild-type uromodulin led to a significant increase in Nkcc2 activity and increased sodium reabsorption in the TAL rather than to adaptive downregulation of NaCl reabsorption in more distal nephron segments, as control and transgenic mice had comparable levels of sodium-chloride symporter (Ncc) (distal convoluted tubules) and epithelial sodium channel (ENaC) (collecting ducts) (Supplementary Fig. 8d, e), as well as similar levels of aldosterone (Supplementary Fig. 8b).
which is expressed mainly in the TAL and which acts as a negative regulator of Nkcc2 phosphorylation, but not that of full-length Spak, was reduced in transgenic mice (Supplementary Fig. 7d,e).

Finally, to investigate whether UMOD variants could have a role in modulating blood pressure in humans, we took advantage of a well-characterized cohort of naive (never-treated) hypertensive subjects (MI_HPT cohort) stratified a posteriori for their rs4293393 SNP status (Supplementary Table 2). Baseline mean diastolic blood pressure was significantly higher in hypertensive individuals homozygous for the risk allele relative to individuals that were heterozygous or were homozygous for the protective allele (Fig. 4a). For a subset of these subjects, data from furosemide tests were also available. Patients homozygous for the risk allele showed an increased diuretic response, with a significantly higher increase of natriuresis over baseline values (Fig. 4b) and a more marked drop in blood pressure, with a significant difference in the decrease of DBP and a similar trend for SBP (P = 0.06) (Fig. 4c and Supplementary Table 2).

Despite the limitations of this study, which involved retrospective analysis of a relatively small-sized cohort, these results suggest that the mechanism causing hypertension in transgenic mice (i.e., increased Nkcc2 activity linked to overexpression of uromodulin) may contribute to hypertension in humans.

This work identifies a causal role for a major risk locus for CKD and hypertension, which had been identified in multiple GWAS. Studies of rare monogenic disorders have contributed considerably to our current knowledge of the functional relationship between NaCl handling in the kidney and blood pressure regulation. Loss-of-function mutations impairing sodium reabsorption have been associated with salt wasting and reduced blood pressure in monogenic diseases affecting the TAL (Bartter’s syndrome) or more distal segments (Gitelman’s syndrome and pseudohypoaldosteronism type 1). Thus far, increased sodium reabsorption leading to hypertension has been linked exclusively to mutations affecting these more distal segments (Liddle’s syndrome and pseudohypoaldosteronism type 2). The new gain-of-function mechanism described here completes the paradigm that links renal transport of NaCl with blood pressure regulation. This mechanism could be widely relevant to human hypertension, given the high frequency of UMOD risk variants in the general population (Supplementary Table 3). Further prospective studies will be necessary to confirm our findings in patients with hypertension and to elucidate the contribution of increased sodium transport in the TAL to hypertension.

Through evidence obtained in mouse and cellular models, this study establishes the importance of uromodulin in modulating NaCl handling in the TAL. Notably, both the expression and urinary excretion of uromodulin are increased by high sodium intake in rats and humans. Here, we demonstrate that UMOD promoter activity is modulated by glucocorticoids, which are known to have a central role in ion homeostasis and blood pressure regulation and have been shown to act on TAL cells.

Both in aging Tg^UMOD^wt/wt mice and in elderly individuals homozygous for UMOD promoter risk variants, upregulation of uromodulin expression was associated with the presence of focal renal lesions, tubular dilation and casts, despite normal kidney function. The focal kidney damage observed in Tg^UMOD^wt/wt mice seems unlikely to be secondary to chronic hypertension, as no such lesions were detected in other rodent models of hypertension. Rather, these lesions, as well as the upregulation of Lcn2 and Kim-1 expression, are reminiscent of changes observed in aging kidneys. These results suggest that uromodulin overexpression is unlikely to lead to renal failure per se, but could predispose to CKD. In this scenario, CKD, whose incidence increases with age, would be triggered by additional conditions harming the kidney. This hypothesis is supported by recent evidence that the association of UMOD promoter risk variants with CKD is stronger in the older age groups with additional comorbid conditions.

Our study shows that common variants in a gene associated with a rare monogenic disorder may play a causal role in complex traits in the general population. UMOD risk variants are present in a high frequency in all ethnic groups studied (Supplementary Table 3), suggesting the action of selective pressure. Selective pressure for a disease-associated variant is atypical but not unprecedented; for example, APOL1 variants are strongly associated with kidney disease in African Americans but increase resistance to Trypanosoma brucei rhodesiense infection. We speculate that selective pressure might have favored UMOD variants leading to high expression and urinary levels of uromodulin due to its protective effect in urinary tract infections and its stimulation of renal salt reabsorption. However, with increased life expectancy, better hygienic conditions and higher salt intake, these same variants could now be exerting a deleterious effect. Accordingly, therapies targeting uromodulin expression or function may be relevant for controlling blood pressure and preserving renal function.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
M.T. and S.J. characterized the mouse model and carried out immunofluorescence and immunoblot analyses on mouse tissue; J.L. carried out expression studies for salt transporters; M.T. and C.S. performed RNA extraction and qRT-PCR analysis on mouse and human kidneys; M.T., S.J. and G.R. performed blood pressure measurements; S.J. and H.D. carried out plasma and urine analyses on mice; L.R., H.D. and M.T. did bioinformatics analysis; H.D. carried out in vitro analysis on the UMOD promoter; B.G. performed studies based on primary TAL cells. The SKIPOGH1 investigators provided the population-based cohort used for urinary uromodulin determination (H.D. and O.D.); P.M., C.L. and F.T. contributed to the hypertensive patient (MI_HPT) cohort patient recruitment and assessment; P.M. and C.L. designed and performed the study on human hypertensive patients; L.C. performed DNA extraction and genotyping on human samples; K.L. contributed in designing the in vitro experiments on Nkcc2 phosphorylation and activity that were performed by S.D.; G.D.A. and M.P.R. supervised the histology work on mouse and human kidneys; G.D.A., M.P.R. and M.I. carried out histological assessment; M.T. and M.C.L. designed and performed the study on human hypertensive patients; L.R., O.D. and M.T. wrote the manuscript. All authors critically reviewed and approved the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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Patient ambulatory blood pressure monitoring and furosemide test. Patients in the MI_HPT cohort \((n = 471)\) underwent 24-h ambulatory blood pressure monitoring (Spacelab 90207; Spacelab Medical) on a day chosen for typical weekly activity. Recordings were performed every 10 min during waking hours (daytime) and every 30 min during nighttime.

Owing to the low frequency of the rs4293393 protective allele, we grouped patients carrying one \((CT, n = 152)\) or two \((CC, n = 9)\) copies of the protective variant and compared them with those homozygous for the risk variant \((TT, n = 310)\). The two groups did not significantly differ by age, sex, BMI or renal function (see Supplementary Table 2).

A subgroup of 165 patients underwent a furosemide test, as follows. After 2 h equilibration, we orally administered 25 mg of furosemide (Sanofi-Aventis). We collected urine immediately before and 4 h after furosemide administration. We measured BP every 60 min during the equilibration period and every 30 min after furosemide treatment. Reported values are the average of three measurements done every minute at each of the timepoints. As for ambulatory blood pressure monitoring, we grouped patients carrying one \((CT, n = 44)\) or two \((CC, n = 3)\) copies of the protective variant and compared them with homozygous for the risk variant \((TT, n = 118)\). The two groups did not significantly differ in age, sex, BMI or renal function.

We measured urinary \(\text{Na}^+\) by flame photometry, creatinine by picric acid test and plasma renin activity by commercial radioimmunoassay (Sorin Laboratories).

**Human urinary uromodulin measurements.** We measured urinary uromodulin concentration by ELISA using on a sheep anti-human uromodulin antibody (Meridian Life Science, K90071C, 5 \(\mu\)g ml\(^{-1}\)) as the capture antibody, a mouse monoclonal anti-human THP antibody (Cedarlane, CL1032A, 1 \(\mu\)g ml\(^{-1}\)) as the primary antibody and a goat anti-mouse IgG (H+L) horse-radish peroxidase–conjugated antibody (BioRad, 1721011, 1,200,000) as the secondary antibody\(^{44}\). We used human uromodulin (Millipore, stock solution 100 \(\mu\)g ml\(^{-1}\)) to establish the standard curve\(^{45}\) The uromodulin ELISA assay had a sensitivity of 2.8 ng ml\(^{-1}\), a linearity of 1.0, an interassay variability of 3.3% and an intra-assay variability of 5.5%. We measured urinary creatinine levels using Beckman Coulter Synchron System Creatinine Assay (Unicel DxC Synchron Clinical System)\(^{46}\) following the manufacturer’s instructions. We normalized uromodulin concentration to creatinine concentration (uromodulin-to-creatinine ratio) to compensate for variations in urine concentration.

**Transgenic mice.** We generated Tg\(\text{Umod}^{\text{wt/wt}}\) mice as described previously\(^{23}\). Briefly, we injected the FVB/N mouse strain with the transgenic uromodulin construct composed of a 2.9-kb fragment of the Umod gene promoter, the first noncoding exon, the first intron, the coding sequence from exon 2 to 11 and the untranslated region. An HA tag was inserted at the uromodulin N terminus. We generated \(\text{Tg}(\text{Umod}^{\text{wt/wt}})\) mice by breeding Tg\(\text{Umod}^{\text{wt/wt}}\) mice and verified transgene homozygosity by Southern blot analysis. The two transgenic lines are in pure FVB/N background. Control mice were nontransgenic animals in the same isogenic background. We carried out all animal procedures on female mice at San Raffaele Scientific Institute, Milan, Italy, and at Università Catholique de Louvain, Brussels, Belgium, according to protocols approved by the San Raffaele Institutional Animal Care and Use Committee and by the Belgian National Research Council Guide for the Care and Use of Laboratory Animals/Animal Ethics Committee, respectively.

**Plasma and urine collection and analysis.** We obtained urine and plasma from age- and gender-matched transgenic and control mice. Mice were housed in a light- and temperature-controlled room with ad libitum access to tap water and standard chow (Diet AO3, SAFE; 25/18 GR Mucedola Srl) or low-sodium chow (E15430-24, SSNIFF). We collected urine using individual metabolic cages (14 h overnight for baseline measurement and 2 h after furosemide administration, 10 mg per kg body weight) after appropriate training of the mice. We obtained blood by venous puncture or at the time of killing by decapitation. Sampling procedures were identical in all groups. We measured urinary electrolytes, creatinine and albumin, plasma electrolytes, creatinine (enzymatic determination), urea and uric acid on a Synchron CX5 analyzer (Beckman Coulter) and measured aldosterone using a validated Aldosterone

Online Methods

**MI_HPT cohort.** The MI_HPT cohort study was approved by the Comitato Etico Ospedale San Raffaele, the ethical committee of the San Raffaele Hospital. All participants provided informed written consent.

In this study, we enrolled a cohort of 471 (391 males and 80 females) never-treated hypertensive patients referred to the Outpatient Clinic at San Raffaele Hospital\(^{41}\). Patients were between 20 and 65 years old and each had a body mass index <30 kg/m\(^2\) and systolic and diastolic blood pressure (SBP/DBP) >140/90 mm Hg and <160/110 mm Hg. We excluded from the study patients with chronic or acute pathologies, clinical history for ischemic cardiomyopathy, cardiac decompensation, cerebral vasculopathy, creatinine clearance <80 ml min\(^{-1}\), liver failure, diabetes, severe essential hypertension or secondary arterial hypertension. We also excluded subjects under antihypertensive or estrogen or progestin therapy or with a history of addiction and/or alcohol abuse. We gave patients dietary instructions including a list of suggested and forbidden foods and verified compliancy by assessing 24-h urinary sodium excretion.

**SKIPOGH cohort.** The Swiss Kidney Project on Genes in Hypertension (SKIPOGH) study was approved by Comité d’éthique de la recherche clinique, the ethical committee of Lausanne University Hospital, the Commission centrale d’éthique, the ethical committee of Geneva University Hospitals and the Kantonale Ethikkommission Bern, the ethical committee of University Hospital of Bern (Insepsiptal). All participants provided informed written consent.

The SKIPOGH study is a family-based cross-sectional study exploring the role of genes in blood pressure and kidney function regulation. We recruited participants from December 2009 until April 2013 in three centers (Bern, Geneva and Lausanne in Switzerland), as previously described\(^{42}\). Participants collected urine during the daytime, which had a median duration of 16 h (interquartile range, 2). The analysis of uromodulin urinary levels was performed for all available samples.

**Human kidney samples.** The study on adult human kidney samples was approved by the Comitato Etico Ospedale San Raffaele, the ethical committee of the San Raffaele Hospital. All participants provided informed written consent. Human renal samples were from individuals who underwent nephrectomy at the San Raffaele Hospital because of renal carcinoma. Samples of normal tissue were provided by the Comitato Etico Ospedale San Raffaele, the ethical committee of San Raffaele Hospital because of renal carcinoma. Samples of normal tissue were provided by the Comitato Etico Ospedale San Raffaele, the ethical committee of San Raffaele Hospital.

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EIA kit (Cayman Chemical) according to the manufacturer’s instructions and normalizing to urinary creatinine (Beckman Coulter Synchron System Creatinine Assay). We measured osmolality on a Fiske osmometer (Advanced Instruments).

Mouse blood pressure measurements. We measured systolic BP by the tail-cuff method (Physiograph Narco or BP-2000, Visitech Systems) on two different days in conscious animals after appropriate training of the mice. We averaged four to six successive measurements.

Tissue collection and preparation. To collect organs (kidney, heart, spleen, brain, adrenal glands, aorta and testis), the mice were killed by decapitation or cervical dislocation after anesthesia with Sevoflurane (Abbott). We immediately homogenized organs for protein or RNA extraction. We fixed renal and cardiac tissue in 4% paraformaldehyde before embedding in paraffin for histological, immunohistochemical and immunofluorescence analysis.

Cell cultures. We maintained human embryonic kidney (HEK) 293 cells in DMEM supplemented with 10% FBS (Invitrogen) and 1% penicillin/streptomycin. MKTAL cells are an immortalized TAL line isolated and characterized by Bourgeois et al.47. We cultured MKTAL cells in DMEM:F12 (1:1) with the SingleQuots Kit (Lonza) containing hydrocortisone, hEGF, FBS, epinephrine, insulin, triiodothyronine, transferrin and gentamicin/amphotericin. We obtained TAL primary cultures (mTAL) from microdissected tubules of the outer medulla of 5-week-old collagenase-treated C57BL/6 mouse kidneys according to the method described by Terryn et al.48. We selected TAL tubules on the basis of their morphology49 and cultured them on permeable filter supports for 14 d, allowing the formation of well-polarized confluent monolayers. These monolayers are characterized by morphological, functional and structural properties similar to those of the TAL segment in vivo, including a high level of endogenous uromodulin expression49. We kept cells at 37 °C in a humidified atmosphere containing 5% CO2.

Construc. We cloned a 3.7-kb fragment of the human UMOD gene promoter containing the risk allele at the SNP rs4293393 (nucleotide −551 C) by site-directed mutagenesis using the QuickChange kit (Stratagene, Agilent) following the manufacturer’s protocol. We confirmed all plasmids by sequencing. We cloned HA-tagged human wild-type uromodulin (WT UMOD) and truncated uromodulin soluble isoform (Sol. UMOD), which lacks the GPI-anchoring site (S614X), into the expression vector pcDNA3.1 (+) (Invitrogen), as previously described29.

UMOD promoter activity in vitro. We tested the effect of rs4293393 protective and risk alleles on the transcriptional activity of the UMOD promoter in mTAL, MKTAL and HEK293 cells. Whereas mTAL cells retain high uromodulin expression consistent with their fully differentiated state, weak or no uromodulin expression was detected in MKTAL and HEK293 cells, respectively (qRT-PCR, data not shown).

We transiently transfected mTAL, MKTAL and HEK293 cells with 2 µg of a firefly luciferase reporter plasmid (pGL3-Basic, Promega), either promterless or carrying UMOD promoter fragments (see constructs paragraph) and 10 ng of a Renilla luciferase vector (prRL-SV40, Promega) using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions.

We evaluated luciferase activity 48 h after transfection with the Dual-Luciferase Reporter Assay System (Promega) using a GloMax 96 luminometer (Promega) with 10 s of integration time (the duration of measurement per well). Firefly luciferase activity was corrected for transfection efficiency using measurement of Renilla luciferase activity. We used the promterless pGL3-Basic vector as a negative control. To assess the response of the UMOD promoter to glucocorticoids, 24 h after transfection cells were treated with 1 µM dexamethasone (Sigma) and/or 10 µM RU-486 (Sigma) for 48 h. For each combination of UMOD allele and cell type, we carried out at least four independent transfections and assayed extracts in duplicate.

Nkcc2 cotransporter activity in vitro. We cloned a construct containing a cDNA for mouse Nkcc2 with a Myc tag fused to its N-terminus into the pTarget expression vector (Promega). To generate HEK293 cells stably expressing Nkcc2, we transfected the cells, selected for resistance to genetin (G418, 500 µg ml−1) and isolated single clones. We performed all experiments on the same clone. We carried out transient transfection of plasmds expressing full-length or truncated uromodulin or empty vector using Lipofectamine and Plus Reagent (Invitrogen) according to the manufacturer’s instructions. Equal expression of the two transfected uromodulin isoforms was verified by qRT-PCR (data not shown).

We measured Nkcc2 cotransporter activity as bumetanide-sensitive NH4 influx as previously described30. Briefly, we used the intracellularly trapped pH-sensitive dye BCECF to measured cytoplasmic pH (pH4) in cells grown to confluence on coverslips. We measured baseline pH4 in cells bathed at 37 °C in a CO2-free Hepes-Tris buffered medium. We then added NH4Cl (20 mM) to the medium to induce cellular alkalinization and measured the initial rate of intracellular pH recovery (dpH4. dr−1) over the first 20 s of recording. We used the dpH4 caused by NH4Cl addition to calculate the cell buffer capacity. Nkcc2 cotransporter activity is therefore expressed as dpH4. dr−1.

qRT-PCR. We used 50–200 µg of human renal tissue for total RNA extraction using the mirVana miRNA Isolation kit (Ambion, Life Technologies), following the manufacturer’s protocol.

We extracted total RNA from mouse whole kidney by homogenization in TRIzol reagent (Invitrogen) and reverse-transcribed extracted RNA using the iScript kit (BioRad) according to the manufacturer’s instructions. We analyzed expression of target genes by qRT-PCR on LightCycler 480 (Roche) using the qPCR Core kit for SYBR Assay (Eurogentec SA). We designed specific primers using Primer 3 (ref. 51) (primer sequences and PCR conditions available upon request). We determined amplification efficiency by dilution curves. We normalized UMOD expression in human samples to NKCC2 (SLC12A1), to account for TAL segment content in each renal tissue sample, and in mouse samples to Hprt1. We calculated the relative mRNA expression of genes of interest following the ΔΔCT method52.

Histology. We performed routine staining (PAS, Trichrome and H&E) according to standard techniques. We stained heart sections with H&E for evaluation by light microscopy, capturing digital images with a Mirax Midi digital camera (Zeiss).

We used PAS-stained renal sections (three sections per mouse or patient) for the quantification of histological features. The sections were independently examined and scored by two observers unaware of the mouse or patient genotype. Sections were viewed using a Zeiss Axioscope 40FL microscope (Carl Zeiss) equipped with an AxioCam MRC5 digital video camera. Images were recorded using AxioVision software 4.3 (Carl Zeiss). We assessed mesangial expansion and capillary loop dilation semiquantitatively (0, absent; 1, 1–50%; 2, 51–100% of the tuft area), as we did for interstitial inflammation and fibrosis, presence of tubular casts and tubular dilation (0, absent; 1, 1–30%; 2, 31–60%; 3, 61–100% of the whole section area). We quantified the presence of segmental and global glomerulosclerosis as percentage of the total number of glomeruli.

In mouse renal sections, we scored vascular damage as present or absent and quantitatively determined tunica media width in at least six vessels (average value of five or six measurements per vessel per organ (n = 5 per group).

Immunofluorescence and immunohistochemistry. Kidney sections (4–5 µm thick) were first deparaffinized by heating for 60 min at 56 °C and subsequently hydrated in the following solutions (5 min each): 2× xylene, 100% ethanol, 80% ethanol, 70% ethanol and cold tap water. Antigenic retrieval was obtained by incubation with heated citrate buffer (0.01 M, pH 6.0), twice for 2.5 min.

For immunofluorescence (IF), slides were blocked in either 10% bovine serum albumin or 10% donkey serum (1 h at room temperature) and incubated for 1 h at room temperature with primary antibody and for 1 h at room temperature with an appropriate secondary antibody.
with appropriate AlexaFluor-labeled secondary antibody (Life Technologies, 1:500). All slides were viewed using a DM 5000B fluorescence upright microscope (Leica DFC480 camera, Leica DFC Twain Software, 40× 0.75 lens; Leica Microsystems) or an LSM510Meta Confocal microscope (Zeiss) equipped with a 40× 1.4 Plan-Apochromat oil-immersion objective (Zeiss). We used identical acquisition parameters for the same antibody in different kidney sections.

Immunohistochemistry (IHC) was performed following standard protocols. Briefly, after antigen retrieval, sections were incubated for 15 min in 3% H2O2 to quench endogenous peroxidase activity. Sections were then blocked with 10% normal goat serum and incubated for 1 h at room temperature with primary antibody, followed by incubation for 30 min at room temperature with the biotinylated secondary antibody (Vector Laboratories, 1:300) and peroxidase-labeled streptavidin (InVitrogen). Peroxidase activity was detected with 3,5-diaminobenzidine (InVitrogen). Sections were analyzed using a Zeiss Axioscope 40FL microscope equipped with an Axiocam MRc5 digital video camera (Carl Zeiss).

Immunoblotting. We homogenized mouse tissues from 6- to 24-week-old animals at 4°C in lysis buffer (NaCl 150 mM, N-octyglycglycol 60 mM, NaF 10 mM, Na2VO4 1 mM, glycerophosphate 1 mM, Protease-Inhibitor Cocktail 1:1,000 (Sigma–Aldrich) and Tris-HE1020 m, pH 7.4). We obtained protein lysates from 16-month-old mice by solubilization of the tissue in lysis buffer, followed by sonication and centrifugation at 16,000g for 1 min at 4°C, after which we used the supernatant. Alternatively, mouse tissues were homogenized in TRIzol (Invitrogen) and proteins were extracted after centrifugation at 11,000g for 15 min at 4°C and collection of the organic phase that was then dialyzed against 0.1% SDS, precipitated with acetone and resuspended in PBS containing SDS 1%, NaF 10 mM, Na2VO4 1 mM, glycerophosphate 1 mM and Protease-Inhibitor Cocktail 1:1,000 (Sigma–Aldrich) (adapted from ref. 53). We analyzed the expression of Ncc and ENaC from kidney membrane preparations of 16-month-old mice as previously described. Briefly, we homogenized kidneys in ice-cold lysis buffer (200 mM mannitol, 80 mM HEPES, 41 mM KOH, pH 7.5) with protease and phosphatase inhibitor cocktails (Complete ULTRA and PhosSTOP, Roche), obtained membrane preparations by ultracentrifugation (100,000g, 1 h, 4°C) and solubilized in Laemmli buffer.

We obtained protein lysates from HEK cells 48 h after transfection by solubilizing the cells in lysis buffer (NaCl 0.4 M; EGTA 0.5 mM; MgCl2 1.5 mM, Heps 10 mM, pH 7.9; glycerol 5% (v/v); Nonidet P-40 0.5% (v/v) and protease inhibitors (Complete, Roche Diagnostics)). We assessed total and phosphorylated Nkcc2 levels following immunoprecipitation with anti-Myc antibody (Clontech, 631206, 1:200), followed by affinity purification using protein G–agarose beads (Dynal). The purified immunocomplex was washed three times in PBS (Invitrogen).

Urinary proteins were precipitated with acetone, resuspended in PBS and subsequently loaded onto gels with normalization to urinary creatinine levels. Protein lysates, immunoprecipitated proteins or urinary proteins were separated on 8–12% SDS-PAGE gel in nonreducing (total uromodulin) or in reducing (all other experiments) conditions and transferred onto nitrocellulose membrane (GE Healthcare). We performed western blotting (WB) following standard protocols. Statistical analyses. We performed comparisons between groups using a two-tailed unpaired Student’s t-test, two-tailed nonparametric Mann–Whitney test or one-way ANOVA followed by Bonferroni’s post hoc test (when three groups were compared). Only statistically significant pairwise comparisons are indicated in the figures. We used a mixed linear model to explore the association of UMOD rs4293393 with square-root–transformed daytime urinary uromodulin-to-creatinine ratio in order to better approximate a normal distribution of the residuals and to take familial correlations into account.

We expressed continuous measures as mean ± s.d. and averages of measurements as mean ± s.e.m. We set the significance level to P < 0.05.

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