Meta-GWAS Reveals Novel Genetic Variants Associated with Urinary Excretion of Uromodulin

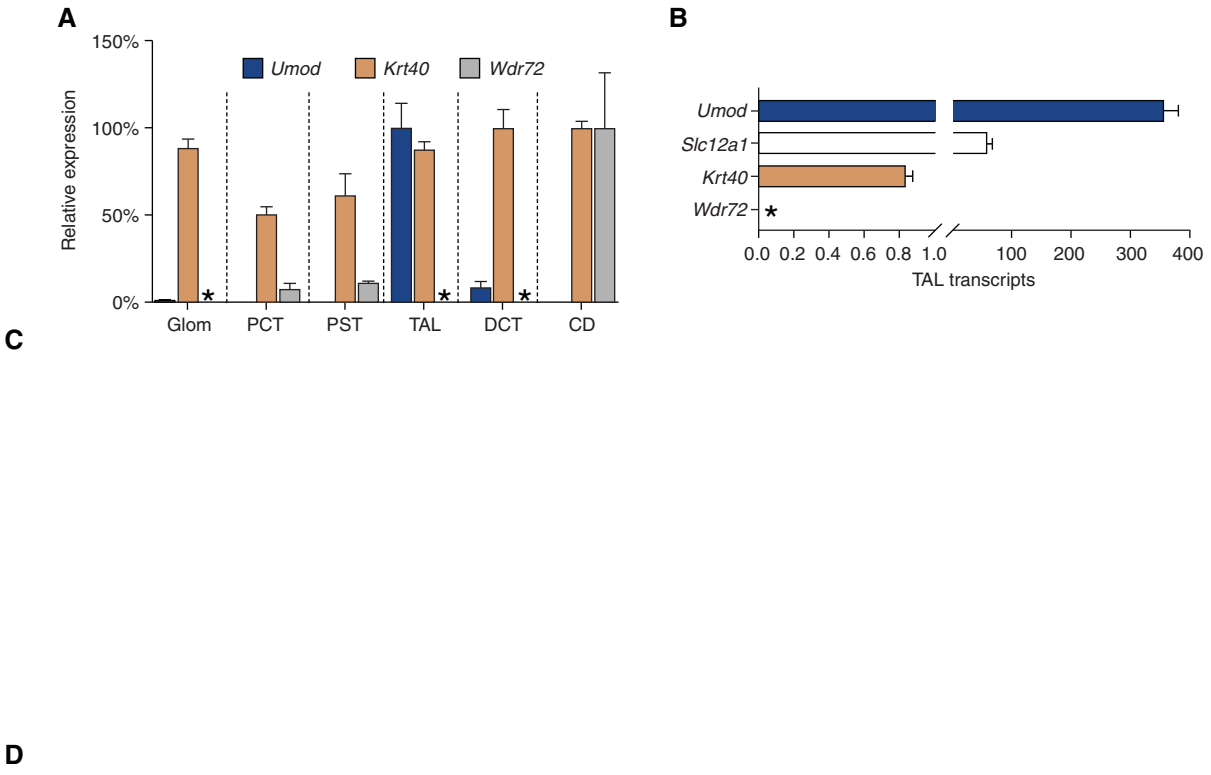

Figure 3. Segmental distribution of UMOD, KRT40, and WDR72 in the mouse kidney. (A) The mRNA levels of *Umod*, *Krt40*, and *Wdr72* in isolated mouse nephron segments were analyzed by SYBR green quantitative PCR. Quantification of targeted genes was done in comparison with *Gapdh*, which was used as housekeeping gene (n=4 pools for each segment). The nephron segments were validated by enrichment in specific markers.[6,18] (B) Relative expression of *Krt40*, *Wdr72*, *Slc12a1*, and *Umod* transcript levels in isolated TALs from C57BL/6J mice as assessed by SYBR green quantitative PCR. Values are expressed as 2^(CtGapdh- CtGene of interest) × 10^2. Bars indicate average±SEM n=4 TAL fractions. Asterisk (*), not detected (A and B). (C) Representative immunofluorescence staining for UMOD (UMOD, green) and KRT40 or WDR72 (red) on paraffin-embedded kidney sections from wild-type mice, showing colocalization of the UMOD and KRT40 signals in the TAL. No staining for WDR72 is detected in UMOD-positive segments. Nuclei are counterstained with DAPI (blue). Scale bar: 25 μm. (D) Representative immunofluorescence staining for UMOD (UMOD, green) and KRT40 or KRT39 (red) on paraffin-embedded kidney sections from a normal human kidney. KRT40 is localized in both UMOD-positive and negative tubules, whereas no signal for KRT39 is detected. Nuclei are counterstained with DAPI (blue). Scale bar: 25 μm.

protein levels (Figure 3, A–C). In isolated TAL segments, the expression of KRT40 was at least two orders of magnitude lower than that of UMOD and NKCC2 (*Slc12a1*) (Figure 3B). *In situ* hybridization evidenced a weak, selective expression of *Krt40* in *Umod*-positive segments of the mouse kidney, with no signal for *Krt39* (Supplemental Figure 10). Immunostaining confirmed a signal for KRT40 in UMOD-positive tubules, particularly at the apical pole of cells lining the TAL, whereas no colocalization between UMOD and WDR72 was observed (Figure 3C). Both

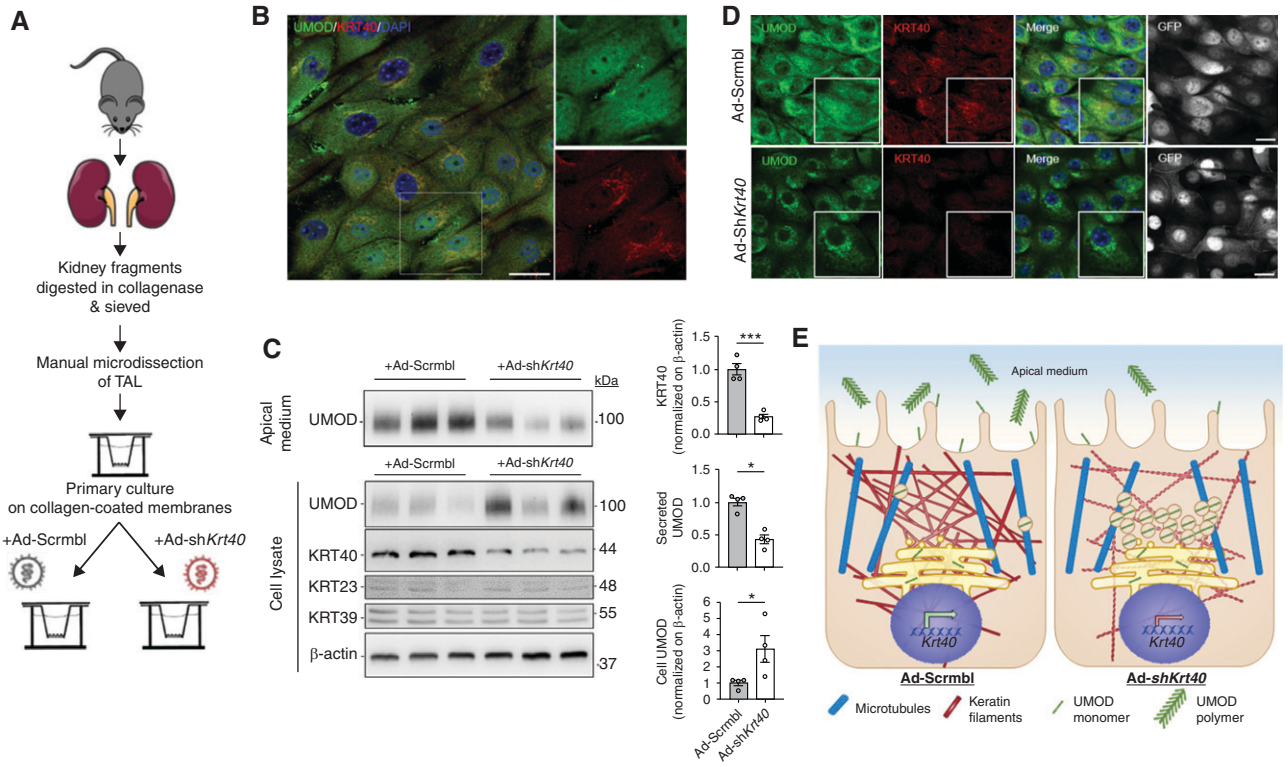

Figure 4. Effect of KRT40 modulation on UMOD processing in mTAL cells. (A) Schematic diagram illustrating the protocol to generate differentiated primary cell cultures (mTAL cells) from mouse kidney.[38] (B) Representative immunofluorescence staining for UMOD (UMOD, green) and KRT40 (red) on mTAL cells. Nuclei are counterstained with DAPI (blue). Scale bar: 25 μm. (C) Representative Western blot of secreted (apical medium) and cellular UMOD in mTAL cells. The apical medium and whole cell lysates were collected 5 days after treatment with Ad-sh*Krt40* or Ad-Scrmbl. *Krt40* downregulation resulted in an increase of intracellular UMOD, and a reduced release in the apical medium. β-actin was used as a loading control. Densitometry analysis for KRT40, secreted and cellular UMOD signals are shown relative to Ad-Scrmbl. Bars indicate mean±SEM. Unpaired two-tailed *t* test (KRT40) or Mann–Whitney test (cellular and secreted UMOD), $^{*}P<0.05$; $^{***}P<0.001$, $n=4$. (D) Representative immunofluorescence staining for UMOD (UMOD, green) and KRT40 (red) on mTAL cells after transduction with Ad-sh*Krt40*. Accumulation of UMOD is observed in the perinuclear compartment of *Krt40* silenced cells. Nuclei are counterstained with DAPI (blue). Both adenoviral vectors express GFP (gray). Scale bar: 25 µm. (E) Model showing the potential link between variants in *KRT40* and the excretion of UMOD. Specific *KRT40* variants (*e.g.*, the minor, C allele of rs8067385) may affect the expression of KRT40 in TAL cells, affecting the cytoskeleton, and altering the processing and apical excretion of UMOD in the urine.

KRT40 and WDR72 were detected in AQP2-positive segments of the mouse kidney (Supplemental Figure 11). In the human kidney, KRT40 was detected in both UMOD-positive and negative tubules, whereas KRT39 did not show any signal (Figure 3D).

Modulation of KRT40 Expression Influences UMOD Excretion by mTAL Cells

The codistribution of KRT40 and UMOD led us to test whether the level of KRT40 expression may modulate the processing and excretion of UMOD by TAL cells. This hypothesis was supported by the existence of at least two exonic variants in high LD with the index *KRT40* variant rs8067385, predicted to be damaging/deleterious by SIFT and PolyPhen2 (Supplemental Table 8). Furthermore, in the GTEx portal, the minor, C allele of rs8067385 is associated with a significant, dose-dependent decrease in the expression of KRT40 in a variety of epithelial tissues including the testis, pancreas, esophagus, and colon (no eQTL data for kidney medulla tissue available) (Supplemental Figure 12).

Characterization of mTAL cells verified that KRT40 and UMOD were both endogenously expressed (Figure 4, A and B). Transduction of mTAL cells with an adenovirus expressing a short hairpin RNA against mouse *Krt40* (Ad-sh*Krt40*) induced a specific silencing of KRT40, compared with cells treated with a scramble adenovirus (Ad-Scrmbl) (Figure 4C). In these conditions, the downregulation of KRT40 was reflected by a significant accumulation of UMOD and a sharp decrease in the amount of excreted UMOD in the apical medium of mTAL cells (Figure 4C). Confocal microscopy indicated the silencing of KRT40 in mTAL cells resulted in perinuclear accumulation of

UMOD, contrasting with the control signal in cells transduced with Ad-Scrmbl (Figure 4D). The trafficking defect induced by KRT40 downregulation was confirmed by Z-stack image analysis, with a perinuclear staining for UMOD contrasting with the diffuse signal observed in control conditions (Supplemental Figure 13A). The silencing of KRT40 had also an effect on the trafficking of ROMK (Supplemental Figure 13B), but did not modify the expression of TAL genes including *Slc12a1*, *Kcnj1*, *Hnf1b*, and *Muc1* in mTAL cells (data not shown). Taken together, these data suggest the expression of the cytokeratin KRT40 regulates the processing and excretion of UMOD in TAL cells (Figure 4E).

DISCUSSION

To gain novel insights into the mechanisms regulating UMOD excretion, we performed a meta-GWAS on urinary UMOD levels in 29,315 individuals of European ancestry, three times more than in our previous analysis.[19] We identified two novel, genome-wide significant loci, *KRT40* and *WDR72*, in addition to the previously known *UMOD-PDILT* locus to be associated with uUCR and uUMOD. Mechanistic studies in primary mTAL cells demonstrated that modulating the expression of KRT40 affects the processing and apical excretion of UMOD. These studies provide insights into the biology of UMOD and keratins, and into the links between the *UMOD-PDILT* locus and kidney function.

The *UMOD-PDILT* locus has been consistently among the strongest associated loci with eGFR and CKD.[14,40] The relevance of the *UMOD* variants, which are associated with the levels of UMOD in the kidney and urine, is immediate because the gene is kidney specific and involved in a spectrum of kidney diseases.[1,7,40] In our meta-analysis, the variant showing the strongest association with uUCR is rs13335818 (*P* value 3.86E−118), a synonymous variant within *UMOD*, in high LD (r^2 = 0.98) with the top SNP in our previous study, rs12917707, and with *UMOD* promoter variants associated with eGFR and CKD and with expression of UMOD.[7,40] In a previous study of genetic associations with urinary UMOD levels,[14] two independently associated variants in the *UMOD-PDILT* locus were identified in conditional analyses: rs77924615, mapping into an intron of the upstream gene *PDILT*, and rs34262842, mapping to an intron of *UMOD*. Similarly, our conditional analysis in a large cohort with individual-level genotype data identified two independent loci in that region, one in *UMOD* (SNP rs13335818 in high LD with rs34262842, r^2=0.94) and one in *PDILT* (rs11864909, in almost complete LD with rs77924615, r^2=0.98).

The lead SNP in *PDILT* from our meta-analysis, rs77924615, had the strongest association with CKD and eGFR in the GWAS performed by the CKDGen Consortium.[14] Of interest, the intronic *PDILT* rs77924615 maps to open chromatin regions identified from various kidney cell types. Because *PDILT* is not expressed in the human kidney and rs77924615 was significantly associated with both differential expression of *UMOD* in kidney tissue and urine UMOD levels (obtained in the GCKD cohort), it was considered a regulatory SNP.[14] Collectively, these results substantiate the independent association between *UMOD* and *PDILT* variants and the levels of UMOD in urine. A recent Mendelian randomization study clarified the causality between uUMOD levels and kidney function in individuals of European descent: genetically driven levels of UMOD have a direct, causal, and adverse effect on kidney function outcome in the general population.[44]

The *KRT40* locus on chromosome 17 is a novel, genome-wide significant locus associated with UMOD levels in the urine. The association at the *KRT40* locus is on the basis of multiple genome-wide significantly associated SNPs in high LD with the index SNP, rs8067385. Individuals homozygous for the minor, C allele of rs8067385 had lower levels of uUMOD compared with individuals homozygous for the G allele. Of note, the effect sizes of rs8067385 for uUMOD and uUCR are only marginally different, as indicated by Spearman's rank correlation analysis. The *KRT40* signal remained above the suggestive threshold ($P<5.17E-07$) in the meta-analysis on urinary UMOD corrected for eGFR, and in the VEGAS analysis. In contrast, the rs8067385 SNP in *KRT40* was not significantly associated with eGFR and plasma or urinary creatinine levels in the UK Biobank. Together, these results support the value of the *KRT40* signal in relation to urinary UMOD levels.

KRT40 encodes KRT40, a type I keratin that belongs to the family of intermediate filament-forming keratins that form the cytoskeleton in epithelial cells. Types I and II keratins form obligate heterodimers and are regulated in a pairwise fashion in epithelia, depending on the tissue, the differentiation state, and the biologic context.[45] *KRT40* belongs to a cluster of type I KRT genes located on chromosome 17q21.2, close to *KRT39*. As cytoskeletal proteins, keratins are involved in maintaining the physical integrity, mechanical stability, and shape of epithelial cells. They are also important for intracellular organization and transport within cells, for example, trafficking of proteins to the plasma membrane.[46] Keratins are considered as cytoprotective, undergoing dynamic upregulation in disease states, and potentially affecting migration, growth, proliferation, and protein synthesis.[47] Several inherited keratinopathies (*e.g.*, skin disorders) have been reported, but none involving *KRT40*.

Little is known about the role of keratins in the kidney. Recent studies evidenced robust changes in the expression and subcellular localization of KRT7–8 and KRT18–19 in response to kidney stress, with KRT18 in urine being a potential biomarker for tubular cell injury.[48] Our studies in mouse and human kidney reveal that KRT40 is weakly expressed in the TAL, where it colocalizes with UMOD. The prediction of missense variants in *KRT40* in LD with rs8067385 and the association in GTEx of the minor allele

of rs8067385 with a decreased expression of KRT40 in epithelial tissues suggest a possible loss of function of the *KRT40* variant. We tested this hypothesis in the mTAL cells, which endogenously express both KRT40 and UMOD. Specific silencing of KRT40 in mTAL cells was reflected by a sharp decrease in the apical excretion of UMOD, causing the intracellular accumulation of the protein. The silencing was also reflected by altered apical targeting of ROMK in these cells. That altered expression of KRT40 affects UMOD and ROMK processing in TAL cells may suggest a role of KRT40 on the polarized sorting of proteins to the apical membrane, affecting the release of UMOD in urine (Figure 4E).

We detected a genome-wide significant association between variants in *WDR72* and the urinary UMOD level indexed to creatinine (uUCR). *WDR72* encodes a protein with eight WD40 (or β-transducin) repeats, which fold to form two circular, β-propeller structures, and an α-solenoid tail at the C-terminus. This combination of domains is conserved among membrane-coating proteins, which serve as a docking site for protein–protein interactions and stabilize membrane curvature.[49] WDR72 is highly expressed in the kidney, although we found it clustered in the collecting ducts. Recessive mutations in *WDR72* have been associated with amelogenesis imperfecta,[50] and distal renal tubular acidosis.[51,52] By GWAS, variants in *WDR72* have been associated with kidney function and CKD,[14,41,53] urine pH,[42] and risk of kidney stones.[42,43] In CKDGen, the index SNP at *WDR72* was associated with blood urea nitrogen.[14] Variants in *WDR72* are strongly associated with eGFR on the basis of serum creatinine or cystatin C and with BUN.[41] The fact that rs9672398 is significantly associated with eGFR and plasma creatinine levels in UK Biobank, that the *WDR72* locus does not reach any suggestive threshold in the meta-analysis using UMOD normalized for eGFR, and that WDR72 does not colocalize with UMOD suggest the *WDR72* signal, only detected for uUCR, is most likely related to its effect on eGFR.

Although limited by power, our candidate gene analysis revealed that a few common variants in genes causing rare Mendelian disorders targeting the TAL are weakly associated with UMOD levels. These results support the functional interactions operating in TAL cells, including the transcription factor HNF1-β, known to be an essential transcriptional regulator of UMOD,[1,9] and ROMK, which directly regulates processing and release of UMOD by TAL cells.[17] Analysis of the candidate genes from the loci associated with uUMOD and uUCR with a suggestive *P* value ($<$1.0E–05) revealed a number of genes expressed in the TAL/DCT, with encoded proteins playing roles in cell homeostasis, mitochondrial function and transport. Future studies will address the relevance and biologic mechanisms that underlie these genetic associations.

Our study combines the advantages of the largest to date meta-GWAS on urinary UMOD, measured with a robust assay in various types of cohorts, and complemented with detailed expression studies in mouse and human kidneys, and functional investigations in TAL cells. Limitations of this study include the availability of data only for individuals of European descent and the lack of replication due to limited availability of additional cohorts with available uUMOD measurements. We noted some variability of UMOD levels that were measured in different cohorts, even when using the same assay and apparently unrelated to sample processing and/or storage conditions.[19,24] Variations in the physiology excretion of UMOD have been reported, potentially linked to dietary habits, tubular transport activities, or level of residual kidney function.[1,16]

Common, independent variants in *KRT40*, *UMOD*, and *PDILT* influence the levels of UMOD in urine. The expression of the type I keratin KRT40 affects UMOD processing in TAL cells. These results advance our understanding of the biology of UMOD, the role of keratins in the kidney and substantiate the association of *UMOD-PDILT* variants with kidney function.

DISCLOSURES

A. Köttgen reports receiving honoraria from Sanofi Genzyme; reports being a scientific advisor or member of the American Kidney Fund, *American Journal of Kidney Diseases*, *Journal of the American Society of Nephrology*, *Kidney International*, and *Nature Reviews Nephrology*. C. Black reports having other interests/relationships through an honorary contract with the National Health Service. D. Conen reports consultancy agreements with Roche Diagnostics; reports receiving research funding from the Canadian Institutes of Health Research; and reports receiving honoraria from Bristol Myers Squibb/Pfizer. E. Wuehl reports being a scientific advisor to or member of the Alnylam Pharmaceuticals Advisory Board, Editorial Board Member of the *Journal of Hypertension* and *Pediatric Nephrology*, Executive Board Member of the German Hypertension League (Deutsche Hochdruckliga), and Vice-Chair COST Action HyperChildNET (EU Programme Horizon 2020). F. Madore reports receiving research funding from AstraZeneca, Bayer, Boehringer Ingelheim, GlaxoSmithKline, and Janssen; and reports being a scientific advisor or membership as Associate Editor for the *Canadian Journal of Kidney Health and Disease*. F. Schaefer reports having consultancy agreements with Akebia, Amgen, Alexion, Alnylam, Astellas, AstraZeneca, Bayer, Boehringer Ingelheim, Fresenius Medical Care, Otsuka, Roche, and Relypsa; reports receiving research funding from Fresenius Medical Care; reports receiving honoraria from Amgen, Gilead, Otsuka, Relypsa, and Roche; and reports being a scientific advisor or member of the Scientific Advisory Board activities for Alexion and Otsuka. M. Bochud reports receiving research funding from Merck Sharp & Dohme; reports receiving honoraria from various Swiss Federal Agencies (Swiss Federal Office of Public Health, Swiss Federal Office of Food Security and Veterinary Affairs); reports being a scientific advisor or member of scientific journals, such as Nutrients and Hypertension, Member of the Council of the Swiss Society of Public Health Plus, Member of the Council of the The National Institute for Cancer Epidemiology and Registration (NICER) Foundation (cancer epidemiology in Switzerland), representative of the University of Lausanne at the Swiss Academy of Medical Sciences; and reports other interests/relationships as a member of the Swiss Federal Commission on Nutrition, the Swiss Society of Hypertension, the Swiss Society of Nephrology, the Swiss Society of Nutrition, the Swiss Society of Public Health Plus. O. Devuyst reports having consultancy agreements with Alnylam,

Galapagos, Otsuka Pharmaceuticals, and Sanofi; reports receiving research funding from Otsuka Pharmaceuticals and Roche; reports being a scientific advisor or member of the editorial board of *CJASN*, *Kidney International*, *Nephrology Dialysis Transplantation*, *Pflügers Archiv*, *Peritoneal Dialysis International*, and *Orphanet Journal of Rare Diseases*. K. Eckardt reports consultancy agreements with Akebia, AstraZeneca, Bayer, Boehringer Ingelheim, Genzyme, Otsuka, Travere, and Vifor; research funding from Amgen, AstraZeneca, Bayer, Evotec, Fresenius, Genzyme, Shire, and Vifor; honoraria from Akebia, AstraZeneca, Bayer, Boehringer Ingelheim, Genzyme, Otsuka, Travere, and Vifor; and advisory or leadership role with KI and BMJ (editorial boards). All remaining authors have nothing to disclose.

FUNDING

This work was supported by the Swiss National Science Foundation (project grant 310030_189044), the University Research Priority Program Innovative Therapies in Rare Diseases of the University of Zurich (UZH), the Swiss National Centre of Competence in Research, Kidney Control of Homeostasis (Kidney.CH), and the TrainCKDis project, funded by the European Union's Horizon 2020 research and innovation programme under Marie Skłodowska-Curie grant agreement 860977 (to O. Devuyst) and by an Medical Research Council University Unit Programme grant MC_UU_00007/10 (QTL in Health and Disease) (to C. Hayward). CARTaGENE is supported by the Kidney Foundation of Canada and the Fonds de la Recherche du Québec-Santé. CoLaus received financial contributions from GlaxoSmithKline, the Faculty of Biology and Medicine of Lausanne, and the Swiss National Science Foundation (33CSCO-122661, 3200BO-111361/2, 3100AO-116323/1, and 310000-112552). The CROATIA_Vis, CROATIA_Korcula, and CROATIA_Split studies were funded by European Commission Framework 6 project EUROSPAN (contract LSHG-CT-2006-018947) and Republic of Croatia Ministry of Science, Education and Sports research grants (108-1080315-0302). The FHS is supported by the National Heart, Lung, and Blood Institute (FHS contract N01-HC-25195). The Genetic and Phenotypic Determinants of Blood Pressure and other Cardiovascular Risk Factors study was supported by the Liechtenstein Government, the Swiss Heart Foundation, the Swiss Society of Hypertension, the University Hospital Basel, the Hanela Foundation, the Mach-Gaensslen Foundation, Schiller AG, and Novartis. The GCKD study was supported by the German Ministry of Education and Research (Bundesministerium für Bildung und Forschung, grants FKZ 01ER 0804, 01ER 0818, 01ER 0819, 01ER 0820 and 01ER 0821) and the KfH Foundation for Preventive Medicine (Kuratorium für Heimdialyse und Nierentransplantation e.V.–Stiftung Präventivmedizin) and corporate sponsors (www.gckd.org). The work of A. Kottgen and M. Wuttke is funded by the Deutsche Forschungsgemeinschaft (German Research Foundation) Project-ID 431984000– SFB 1453. GS:SFHS received core support from the Chief Scientist Office of the Scottish Government Health Directorates (CZD/16/6) and the Scottish Funding Council (HR03006). INGI-Carlantino and INGI-Val Borbera were supported by Italian Ministry of Health grants RC 35/17 and D70-RESRICGIROTTO. The LBC1936 is supported by Age UK (Disconnected Mind project) and the Medical Research Council (MR/M01311/1, MR/K026992/1). The Viking Health Study– Shetland was supported by Medical Research Council University Unit Programme (MC_UU_00007/10, QTL in Health and Disease).

ACKNOWLEDGMENTS

We are grateful for the willingness of the patients to participate in the study and CRediT Taxonomy. O. Devuyst and C. Hayward conceptualized the study; S. Aeschbacher, P. Awadalla, C. Black, S. Bergmann, M. Bochud, V. Bruat, A. Campbell, H. Campbell, M. Cocca, M. Concas, D. Conen, T. Corre, I. Deary, O. Devuyst, K.-U. Eckardt, G. Girotto, S. Harris, C. Hayward, J. Huffman, C. Joseph, I. Kolcic, A. Köttgen, F. Madore, M. Mariniello, J. Marten, M. Olden, E. Olinger, O. Polasek, D. Porteous, A. Robino, F. Schaefer, G. Schiano, S. Thèriault, S. Troyanov, S. Ulivi, J. Wilson, E. Wühl, M. Wuttke, an A. Yoshifuji were responsible for the data curation; S. Aeschbacher, P. Awadalla, C. Black, S. Bergmann, M. Bochud, V. Bruat, M. Cocca, M. Concas, D. Conen, T. Corre, I. Deary, O. Devuyst, K.-U. Eckardt, G. Girotto, S. Harris, C. Hayward, J. Huffman, C. Joseph, A. Köttgen, F. Madore, M. Mariniello, J. Marten, M. Olden, E. Olinger, A. Richmond, A. Robino, C. Sala, G. Schiano, S. Thèriault, S. Troyanov, S. Ulivi, J. Wilson, E. Wühl, M. Wuttke, and A. Yoshifuji were responsible for the formal analysis; H. Campbell, O. Devuyst, C. Hayward, and S. Troyanov were responsible for the funding acquisition; Y. Cheng, O. Devuyst, C. Hayward, C. Joseph, J. Lake, M. Mariniello, G. Schiano, S. Troyanov, and A. Yoshifuji were responsible for the investigation; T. Corre, O. Devuyst, C. Hayward, C. Joseph, J. Lake, M. Mariniello, J. Marten, M. Olden, G. Schiano, and A. Yoshifuji were responsible for the methodology; A. Campbell, H. Campbell, O. Devuyst, S. Harris, C. Hayward, and D. Porteous were responsible for the project administration; S. Aeschbacher, P. Awadalla, C. Black, S. Bergmann, M. Bochud, V. Bruat, A. Campbell, H. Campbell, Y. Cheng, M. Cocca, M. Concas, D. Conen, T. Corre, I. Deary, O. Devuyst, K.-U. Eckardt, G. Girotto, S. Harris, C. Hayward, J. Huffman, A. Köttgen, F. Madore, M. Olden, E. Olinger, O. Polasek, D. Porteous, A. Robino, C. Sala, F. Schaefer, S. Thèriault, S. Troyanov, S. Ulivi, J. Wilson, E. Wühl, and M. Wuttke were responsible for the resources; O. Devuyst provided supervision; O. Devuyst, C. Hayward, S. Troyanov, and A. Yoshifuji were responsible for the validation; O. Devuyst, C. Hayward, C. Joseph, J. Lake, M. Mariniello, and G. Schiano were responsible for the visualization; O. Devuyst, C. Hayward, C. Joseph, and A. Yoshifuji wrote the original draft; S. Aeschbacher, P. Awadalla, C. Black, S. Bergmann, M. Bochud, V. Bruat, A. Campbell, H. Campbell, Y. Cheng, M. Cocca, M. Concas, D. Conen, T. Corre, I. Deary, O. Devuyst, K.-U. Eckardt, G. Girotto, I. Kolcic, A. Köttgen, A. Robino, S. Harris, C. Hayward, J. Huffman, C. Joseph, J. Lake, F. Madore, M. Mariniello, J. Marten, M. Olden, E. Olinger, O. Polasek, D. Porteous, A. Richmond, C. Sala, F. Schaefer, G. Schiano, S. Thèriault, S. Troyanov, S. Ulivi, J. Wilson, E. Wühl, M. Wuttke, and A. Yoshifuji reviewed and edited the manuscript. We deeply acknowledge Professor John Starr (1960–2018), founding Director of the Alzheimer Scotland Dementia Research Centre, University of Edinburgh, Edinburgh, United Kingdom, who contributed in the early phase of this study. The help of Larissa Govers and Huguette Debaix (UZH, Zurich) is deeply appreciated. We thank Aleksander Edelman (Institut Necker Enfants Malades, Paris, France) for fruitful discussions on keratins. CARTaGENE: We thank the dedicated team at CARTaGENE for their diligent help. CoLaus: The computations for CoLaus imputation were performed in part at the Vital-IT center for high-performance computing of the Swiss Institute of Bioinformatics. M. Bochud is supported by the Swiss School of Public Health Plus. CROATIA-Korcula, CROATIA-Split, CROATIA-Vis: We would like to acknowledge the staff of several institutions in Croatia that supported the field work, including but not limited, to the University of Split and Zagreb Medical Schools, Institute for Anthropological Research in Zagreb, and the Croatian Institute for Public Health. We also thank all of the participants from the islands of Vis and Korcula and the city of Split. FHS: This research was conducted in part using data and resources from the FHS of the National Institutes of Health National Heart Lung and Blood Institute (NHLBI) and Boston University School of Medicine. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. GCKD: Genotyping was supported by Bayer Pharma AG. The GCKD study was/is funded by grants from the Federal Ministry of Education and Research (BMBF, grant number 01ER0804) and the KfH Foundation for Preventive Medicine. We are grateful for the willingness of the patients to participate in the GCKD study. The enormous effort of the study personnel of the various regional centers is highly appreciated. We thank the many nephrologists who provide routine care for the patients and collaborate with the GCKD study. GS:SFHS: We are grateful to all of the families who took part, the general practitioners and the Scottish School of Primary Care

for their help in recruiting them, and the whole Generation Scotland team, which includes interviewers, computer and laboratory technicians, clerical workers, research scientists, volunteers, managers, receptionists, healthcare assistants, and nurses. GS:SFHS is supported by the Wellcome Trust (216767/Z/19/Z). Genotyping of the GS:SFHS samples was carried out by the Genetics Core Laboratory at the Edinburgh Clinical Research Facility, University of Edinburgh, Scotland and was funded by the Medical Research Council UK and the Wellcome Trust (Wellcome Trust Strategic Award "STratifying Resilience and Depression Longitudinally" Reference 104036/Z/14/Z). INGI-Carlantino and INGI-Val Borbera: We would like to thank the people of Carlantino and of the Val Borbera Valley for the everlasting support. LBC: The authors thank all LBC study participants and research team members who have contributed, and continue to contribute, to ongoing LBC studies. Genotyping was funded by the Biotechnology and Biological Sciences Research Council (BB/F019394/1). VIKING: We would like to acknowledge the invaluable contributions of the research nurses in Shetland, the administrative team in Edinburgh and the people of Shetland. We thank the UK Biobank Resource, approved under application 19655. The authors would like to thank the Rivas lab for making the Global Biobank Engine resource available. DNA extractions and genotyping were performed at the Edinburgh Clinical Research Facility, University of Edinburgh. The GTEx Project was supported by the Common Fund of the Office of the Director of the National Institutes of Health, and by National Cancer Institute, National Human Genome Research Institute, National Heart, Lung, and Blood Institute, National Institute on Drug Abuse, National Institute of Mental Health, and National Institute of Neurological Disorders and Stroke. The data used for the analyses described in this manuscript were obtained from the GTEx Portal (V8) on December 14, 2020.

DATA SHARING STATEMENT

Information on the datasets and summary statistics are available in the Edinburgh Datashare repository, under the link https://doi.org/10.7488/ds/3012 created on April 7, 2021 with additional material under the link https://doi.org/10.7488/ds/3262 created on 16th December 2021. For the purpose of open access, the author has applied a Creative Commons Attribution (CC BY) licence to any Author Accepted Manuscript version arising from this submission.

SUPPLEMENTAL MATERIAL

This article contains the following supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2021040491/-/DCSupplemental.

Supplemental Table 1. Study sample characteristics for UMOD measurement.

Supplemental Table 2. Genotyping and imputation platforms.

Supplemental Table 3. List of candidate genes associated with rare Mendelian disorders affecting the TAL.

Supplemental Table 4. List of genes associated with diseases in specific kidney segments and candidate genes previously published by Olden *et al.* (2014).

Supplemental Table 5. Primers for quantitative RT-PCR analyses.

Supplemental Table 6. Heritability estimates for family-based cohorts.

Supplemental Table 7. Most significant SNP from each locus with *P* value <1E–05 from urinary UMOD (uUMOD) meta-analysis.

Supplemental Table 8. List of *KRT40* variants, in high LD with rs8067385, and with *P* value <1E–05 in association with uUMOD.

Supplemental Table 9. Association of rs9672398 (*WDR72* locus) with urinary UMOD levels indexed to creatinine.

Supplemental Table 10. Association of rs13335818 (*UMOD-PDILT* locus) with urinary UMOD levels indexed to creatinine.

Supplemental Table 11. Most significant SNP from each locus with *P* value <1E–05 from urinary UMOD indexed to creatinine (uUCR) meta-analysis.

Supplemental Table 12. Encoded protein, expression, and disease association for candidate genes (*P* value <1E-05) at urinary UMOD (uUMOD)-associated and indexed urinary UMOD (uUCR)-associated variants.

Supplemental Table 13. Effect size and *P* values of rs8067385 from meta-analysis of uUMOD concentration and uUMOD_eGFR using seven cohorts.

Supplemental Table 14. Effect size and *P* values of rs9672398 from meta-analysis of uUMOD concentration and uUMOD_eGFR using seven cohorts.

Supplemental Table 15. VEGAS2 results for uUMOD and uUCR meta-analysis.

Supplemental Table 16. Candidate gene analysis for uUMOD and uUCR levels.

Supplemental Table 17. Look-up analyses of genes associated with specific segments of the kidney and candidate genes previously published by Olden *et al.* (2014).

Supplemental Table 18. Effect sizes of the most significant SNP in *UMOD* and *PDILT* in association with uUMOD and uUCR.

Supplemental Table 19. Effect sizes of the most significant SNP in *UMOD, KRT40* and *WDR72* in association with uUMOD and uUCR in 12 cohorts (FHS excluded).

Supplemental Appendix 1. Summary characteristics of the study cohorts.

Supplemental Figure 1. Genetic loci associated with uUMOD and uUCR.

Supplemental Figure 2. Effect size of rs12934455 and regional association plot of UMOD-PDILT locus from raw UMOD levels.

Supplemental Figure 3. Effect size of rs9672398 and regional association plot of *WDR72* locus for uUCR meta-analysis.

Supplemental Figure 4. Effect size of rs13335818 and regional association plot of *UMOD-PDILT* locus from uUCR meta-analysis.

Supplemental Figure 5. Manhattan plot of meta-GWAS of uUMOD and uUCR using sample size and *P* values for analysis of the 13 study cohorts.

Supplemental Figure 6. Forest plot showing effect sizes of rs8067385 (*KRT40* locus) on uUMOD and uUCR meta-analysis in the 13 cohorts.

Supplemental Figure 7. Effect of *UMOD* genotype on urinary UMOD (uUMOD and uUCR) levels.

Supplemental Figure 8. Manhattan plot showing GWAS results in association with uUMOD and uUMOD conditioned for rs12934455 or for rs11864909 using GS:SFHS.

Supplemental Figure 9. Candidate genes influencing the urinary excretion of UMOD.

Supplemental Figure 10. *In situ* hybridization for *Umod*, *Krt40* and *Krt39* on mouse kidney.

Supplemental Figure 11. Immunofluorescence staining for AQP2 and KRT40 or WDR72 on mouse kidney.

Supplemental Figure 12. eQTL data for the *KRT40* variant rs8067385.

Supplemental Figure 13. UMOD (Z-stack) and ROMK distribution in mTAL cells following KRT40 knockdown.

Supplemental Methods.

Supplemental References.

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