Altered vitamin K biodistribution and metabolism in experimental and human chronic kidney disease

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Chronic kidney disease (CKD) is accompanied with extensive cardiovascular calcification, in part correlating with functional vitamin K deficiency. Here, we sought to determine causes for vitamin K deficiency beyond reduced dietary intake. Initially, vitamin K uptake and distribution into circulating lipoproteins after a single administration of vitamin K1 plus K2 (menaquinone 4 and menaquinone 7, respectively) was determined in patients on dialysis therapy and healthy individuals. The patients incorporated very little menaquinone 7 but more menaquinone 4 into high density lipoprotein (HDL) and low-density lipoprotein particles than did healthy individuals. In contrast to healthy persons, HDL particles from the patients could not be spiked with menaquinone 7 *in vitro* and HDL uptake was diminished in osteoblasts. A reduced carboxylation activity (low vitamin K activity) of uremic HDL particles spiked with menaquinone 7 vs. that of controls was confirmed in a bioassay using human primary vascular smooth muscle cells. Kidney menaquinone 4 tissue levels were reduced in 5/6-nephrectomized versus sham-operated C57BL/6 mice after four weeks of a vitamin K rich diet. From the analyzed enzymes involved in vitamin K metabolism, kidney HMG-CoA reductase protein was reduced in both rats and patients with CKD. In a *trial* on the efficacy and safety of atorvastatin in 1051 patients with type 2 diabetes receiving dialysis therapy, no pronounced vitamin K deficiency was noted. However, the highest levels of PIVKA-II (biomarker of subclinical vitamin K deficiency) were noted when a statin was combined with a proton pump inhibitor. Thus, profound disturbances in lipoprotein mediated vitamin K transport and metabolism in uremia suggest that menaquinone 7 supplementation to patients on dialysis therapy has reduced efficacy.

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Cardiovascular morbidity and mortality markedly increase as chronic kidney disease (CKD) progresses.[1] Traditional risk factors and several nontraditional factors are implicated in this accelerated course of cardiovascular disease.[2] In particular, nontraditional risk factors appear to contribute to the pronounced cardiovascular calcification that characterizes advanced stages of CKD and are potent predictors of cardiovascular events and death.[3]

Cardiovascular calcification in CKD involves the arterial intima and media as well as heart valves. Apart from a disturbed mineral homeostasis, ample evidence indicates that inhibitor systems of extraosseous calcification are dysfunctional in CKD.[4] The most important inhibitor system in the arterial vascular wall and heart valves is matrix Gla protein (MGP). Inactive, uncarboxylated MGP (ucMGP) is produced by vascular smooth muscle cells and is then activated (i.e., carboxylated MGP) through a vitamin K–mediated carboxylation step.[5] The identification of this inhibitor system has provided a mechanistic explanation for accelerated vascular calcification and the increased risk for calciphylaxis (calcific uremic arteriolopathy) that accompanies the therapy with vitamin K antagonists in patients with normal kidney function, but particularly in CKD patients.[6] However, even without vitamin K antagonist therapy, many patients with advanced CKD exhibit markedly elevated plasma levels of uncarboxylated, inactive vitamin K–dependent proteins. Assessment of the

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nonphosphorylated, uncarboxylated fraction of MGP, indicating subclinical vitamin K deficiency,[7] has shown that this marker is strongly upregulated in CKD. The nonphosphorylated, uncarboxylated fraction of MGP is easily set free in the circulation as it has a low affinity for calcification.[5] In these patients, high circulating nonphosphorylated, uncarboxylated fraction of MGP levels are not only associated with more extensive vascular calcification, but also predict worse survival.[7]

The origin of the functional vitamin K deficiency in advanced CKD is likely multifactorial. One contributor is dietary restriction, given the high potassium content in most vitamin K–rich, green vegetables.[8] Beyond dietary intake, vitamin K is recycled via the vitamin K cycle, encompassing vitamin K epoxide reductase, DT-diaphorase, and γ-glutamyl-carboxylase. Because of reduced γ-glutamyl-carboxylase activity, over a mechanism similar to the action of coumarins,[9] impaired vitamin K recycling has been found in CKD rats. In the present study, the focus is directed toward uremia-related alterations of vitamin K pharmacokinetics, metabolism, and biodistribution in particular as it relates to altered lipid metabolism, another potential contributor to vitamin K deficiency in CKD. Vitamin K is fat soluble, and uptake and transport in the blood are mediated by lipoproteins.[10] Recognition of the pronounced alteration in lipoprotein-particle composition that occurs in advanced CKD[11,12] led us to hypothesize that such alterations also affect the uptake and biodistribution of vitamin K in dialysis patients and experimental models of CKD. In addition, the impact of lipid-lowering therapy on the vitamin K status in patients from the Die Deutsche Diabetes Dialyse Studie (4D) study[13] was evaluated, as was the expression of vitamin K–dependent enzymes in rodent and human kidney samples.[14]

METHODS

Human interventional study

Healthy volunteers from the general population and patients on long-term hemodialysis within the University Hospital Aachen were recruited (Table 1). This single-center, prospective, controlled, open, non-randomized, 2 armed parallel, interventional study was approved by the Aachen University Ethics Committee (EK 164-17) and registered in the German Trial Registry with the number DRKS00025281. Inclusion criteria were age >18 years, long-term hemodialysis treatment for at least 6 months or for healthy subjects a documented glomerular filtration rate >60 ml/min per 1.73 m^2, and written consent to the study. Key exclusion criteria were gastrointestinal disease likely to interfere with vitamin K uptake, anemia with a hemoglobin level <10 g/dl, therapy with vitamin K antagonists, pregnancy, and lactation.

Fasted subjects received a standardized breakfast and a single oral vitamin K supplement (Super K Komplex; Fairvital) containing 1000 μg of vitamin K1 (K1), 1000 μg of menaquinone 4 (MK4), and 200 μg of menaquinone 7 (MK7). Serum samples were obtained at baseline, and 1, 3, and 6 hours after ingestion of vitamin K. In hemodialysis patients, baseline samples were obtained before hemodialysis after a long dialysis interval as well as at 1 and 3 hours during dialysis and finally at 6 hours. Blood samples were centrifuged at 2000*g* at 4 °C and either immediately analyzed or stored at –80 °C.

Table 1 | Patient characteristics and serum biochemistry, interventional trial

Variable	Healthy (n = 9)	Dialysis (n = 10)	*P* value
Sex, male, %	57	80	NS
Age, mean (SD), yr	61 (12)	70 (10)	NS
BMI, mean (SD), kg/m^2	25.5 (2.1)	25.8 (4.7)	NS
GFR, mean (SD), ml/min	>60	NA	
Diabetes mellitus, %	0	40	0.033
Triglycerides, mean (SD), mg/ml	1.48 (0.26)	1.52 (0.33)	NS
LDL, mean (SD), mg/ml	1.12 (0.13)	1.35 (0.21)	0.02
HDL, mean (SD), mg/ml	0.49 (0.17)	0.37 (0.10)	NS
CRP, median (IQR), mg/L	0.5 (1.5)	7.5 (18.6)	0.015
PIVKA-II, median (IQR), ng/ml	1.0 (0.9)	37.2 (21.9)	0.002
Statin therapy, %	44	30	NS
Dialysis vintage, mean (SD), mo	NA	47.6 (46.2)	

BMI, body mass index; CRP, C-reactive protein; GFR, glomerular filtration rate; HDL, high-density lipoprotein; IQR, interquartile range; LDL, low-density lipoprotein; NA, not applicable; NS, not significant; PIVKA, prothrombin induced by vitamin K absence.
Serum was collected at baseline.

Serum biochemistry

Serum high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and triglycerides were determined with the HDL/LDL/VLDL Cholesterol Kit, according to the manufacturer's instructions (Abcam). To determine total triglyceride levels, serum was mixed with triglyceride reagent (Triglycerides FS; DiaSys). After incubation at room temperature for 20 minutes, plasma triglycerides and cholesterol levels were measured by absorbance at 490 nm using a plate reader (FLUOstar OPTIMA; BMG Labtech GmbH). C-reactive protein was measured in serum by turbidimetric CRP4 Tina-quantassay (Cobas 8000; Roche) with a detection limit of >0.2 mg/L (Central Laboratory Diagnostics University Hospital Aachen, Aachen, Germany).

As ucMGP measurements require plasma, we also assessed a serum marker of vitamin K deficiency (namely, protein induced by vitamin K absence or antagonist II [PIVKA-II]). PIVKA-II serum levels were assessed by enzyme-linked immunosorbent assay (Biozol) or, in case of the 4D study samples, using a conformation-specific monoclonal antibody in an enzyme-linked immunosorbent assay–based assay.[15] Results were expressed as arbitrary units per liter (AU/L) because in states of vitamin K deficiency, circulating PIVKA-II may comprise multiple forms of partially carboxylated PIVKA-II and neither their relative abundance in serum nor their relative affinities for the antibody are known. Using electrophoretic techniques, 1 AU is equivalent to 1 mg of purified PIVKA-II.[15] The detection limit was 0.15 AU/ml PIVKA-II in serum. Patients with vitamin K antagonist therapy were excluded.

Lipoprotein isolation

Lipoproteins were isolated from blood samples of the human interventional study, mentioned above, in 9 healthy volunteers and 10 dialysis patients. In addition, lipoproteins were isolated from fresh, fasting predialysis plasma (from 10 dialysis patients and 9 healthy participants, both different from those above) by density gradient ultracentrifugation (HDL: density, 1.063–1.21 g/cm^3; LDL: density, 1.006–1.063 g/cm^3), as previously described.[16] Potassium bromide was used to adjust density. Lipoprotein concentrations used

in the present study were based on protein content, determined by the Bradford assay.

Fluorescent labeling of HDL using Atto-488

HDL (4 mg) was diluted in phosphate-buffered saline to achieve a final volume of 300 μl. Afterwards, the HDL solution together with 35 μl sodium bicarbonate buffer (1.0 M; pH 9.5) was added to a tube containing 25 μl Atto-488-NHS (AttoTec) and incubated for 2 hours at room temperature. After incubation, Atto-488–labeled HDL was purified using gel filtration.

Spiking of lipoproteins with vitamin K species

HDL or LDL was incubated at the concentrations observed in the *in vivo* study with K1 (15 ng/mg), MK4 (200 ng/mg), and MK7 (30 ng/mg) overnight at 4 °C on a shaker. Lipoproteins were then used for *in vitro* studies after extensive dialysis against Krebs-Henseleit buffer. MK7-spiked HDL was either extracted for MK7 quantification (healthy, n = 6; dialysis, n = 5 randomly selected in the lipoprotein isolation experiments) and/or incubated on vascular smooth muscle cells (VSMCs) for functional MGP carboxylation (healthy, n = 5; dialysis, n = 5).

Vitamin K quantification

Vitamin K was extracted from each lipoprotein fraction by hexane, and the lipid extract was cleaned on a Sep-Pak waters cartridge. Vitamin K was separated on a reversed phase high-performance liquid chromatography setup (Dionex UltiMate; Thermo Fisher) with isocratic methanol at pH 5 as mobile phase and a MaxRPC12 column (Phenomenex) as stationary phase. K1, MK4, and MK7 were quantified by internal and external standards. The intra-assay coefficient of variation was 5.4%, the inter-assay coefficient was 13.4%, and the assay linearity was 0.99.[9] Serum vitamin K content in chylomicrons, LDL particles, and HDL particles was normalized to the total amount of triglycerides, LDL, and HDL, respectively.

Cell culture

Human osteoblast-like MG63 cells were cultivated in α-Eagle minimal essential medium, 10% fetal calf serum, and 1% penicillin-streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C. To determine cellular HDL attachment and internalization, Atto-488–labeled HDL[17] was incubated for 15, 30, or 60 minutes at 4° C with MG63 cells.[17] Images were taken with a Nikon A1 laser confocal microscope (Nikon).

VSMCs were isolated from the aorta of a 53-year-old man undergoing vascular surgery. The use of this tissue was approved by the University of Maastricht (METC 2019-1235). Primary VSMCs were thawed and cultured in Dulbecco's modified Eagle's media (31966 047; Gibco), 10% fetal calf serum, and 1% penicillin-streptomycin for 3 days and subsequently immortalized. Two retroviral particles, SV40LT and HTERT, were produced by transient transfection of HEK293T cells for 48 hours using TransIT-LT (Mirus), plasmids pBABE-puro-SV40-LT (Addgene; number 13970) or xlox-dNGFR-TERT (Addgene; number 69805) in combination with a packaging plasmid pUMVC (Addgene; number 8449) and a pseudotyping plasmid pMD2.G (Addgene; number 12259). Cell transduction was performed for 48 hours by incubating the target cells with serial dilutions of produced retroviral supernatants (1:1 mix of concentrated particles containing SV40-LT or hTERT). After 3 days, transduced cells were selected with 2 μg/ml puromycin for 7 more days.

VSMCs were incubated *in vitro* for 6 hours with native or vitamin K–spiked HDL, isolated from healthy volunteers or dialysis patients. Dulbecco's modified Eagle's medium was supplemented with vitamin D (1,25 OH D3; 10 nM; Sigma Aldrich) and did not contain fetal calf serum during treatment. Cells were harvested, fixed with 4% paraformaldehyde, and stained with ucMGP antibody.[18] Staining intensity was quantified by gating on single cells against an unstained control, on a BD FACS Fortessa, and analyzed by FACS Diva software, and then calculated as percentage positively stained cells.

Animals

C57BL/6J mice underwent either 5/6 nephrectomy (n = 8) or sham surgery (n = 7). One week later, a vitamin K–rich diet (1 mg K1/kg, 500 μg MK4/kg, and 500 μg MK7/kg rodent chow; menadione-free S0382-E240; Ssniff; Supplementary Table S1) was continued for 7 weeks, after which blood and organs were harvested. Blood pressure was measured by the tail-cuff method (Coda; Kent Scientific).

In a second set of experiments, CKD was induced in rats (weighing 282–419 g) by 4 weeks of an adenine-containing diet (0.75%; Altromin), followed by 2 weeks on a standard diet, followed by 1 week of adenine.

Serum was obtained by centrifugation at 2000*g* for 10 minutes at 4 °C and stored at –80 °C until analysis. Serum parameters were measured by automatized, colorimetric, potentiometric chemistry (Vitros 350; Institute for Animal Science, University Hospital of the RWTH Aachen).

The animal protocols were approved by the local ministry (LANUV 84-02.04.2011.A144 and 84-02.04.2017.A324).

Human nephrectomy specimens

Normal human kidney and CKD tissues were obtained during nephrectomy for localized cancer; tissue was obtained as far distant as possible from the tumor and verified to be normal by histology. Kidneys were processed as previously described.[14] The local ethics committee of the University Hospital RWTH Aachen approved all human tissue protocols (EK-016/17). Kidney tissue was collected from the Urology Department of the Hospital Eschweiler from patients undergoing (partial) nephrectomy because of kidney cancer. All patients provided informed consent, and the study was performed in accordance with the Declaration of Helsinki.

Expression of vitamin K–related enzyme mRNAs was analyzed in human CD10-negative kidney single cells,[14] including a total number of 51,849 cells from 6 CKD and 9 non-CKD specimens. A dot plot was generated using Seurat (R package, v.3.2.2),[19] with the normalized gene expression by deconvolution method[20] implemented in scran (R package, v1.16.0; Bioconductor; open-source software).

Immunofluorescence

Immunohistochemical staining was performed using deparaffinized kidney sections from CKD and control rats and human kidneys. Antigen retrieval was performed by boiling in citrate buffer for 20 minutes. Blocking was performed with bovine or horse serum. UbiA prenyltransferase domain-containing protein 1 (UBIAD1) antibody (SC-271595; Santa Cruz Biotechnology) was incubated overnight at 4 °C. 3-Hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) antibody (GTX32134; Genetex) was enhanced by adding biotinylated anti-mouse IgG (BA-200; Vector Labs). Secondary antibodies were streptavidin–fluorescein isothiocyanate (SA-5001; Vector Labs) and anti-rabbit Cy3 (711-165-152; Jackson ImmunoResearch). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (D-1306; Molecular Probes). Finally, the slides were mounted with

VECTASHIELD Mounting Media (Vector Labs), and images were taken with a Nikon A1 laser confocal microscope (Nikon).

Real-time polymerase chain reaction

RNA was extracted from rat kidneys by the Qiagen RNeasy kit (74104; Qiagen), according to the manufacturer's instructions. The amount of RNA was quantified using a Nanovue Plus Spectrophotometer (GE Healthcare) at 260 and 280 nm. cDNA was synthesized after incubating each sample with 100 μM oligo dT primer (Eurofins Scientific) at 70 °C for 5 minutes. Afterwards, samples were incubated at 37 °C for 1 hour with M-MLV RT 5x Buffer, M-MLV RT Enzyme, and dNTPs Mix (Promega) to achieve cDNA synthesis. In the final step, samples were diluted in a 1:8 ratio in RNase free water (Braun).

To determine the gene expression levels, quantitative real-time polymerase chain reaction was performed using SYBR Green dye (Applied Biosystems). A total of 10 μl per reaction was used, comprising 5 μl SYBR green (Powerup; Thermo Fisher), 1 μl 2 mM forward primer, 1 μl 2 mM reverse primer, and 3 μl of the cDNA sample. All primers used for the real-time polymerase chain reaction were synthesized by Eurofins. The reactions were performed in triplicate to obtain accurate cycle threshold values. All data were analysed by the "2(–ΔΔCt)" (where CT is cycle threshold) method, comparing all the values with the housekeeping gene (PPIA).

Primer sequences were as follows: HMGCR, CCTCCATTGAGATCCGGAGG (forward) and AAGTGTCACCGTTCCCACAA (reverse); UBIAD1, AAGTGCGCCTCCTATGTGTT (forward) and CAGGAGTGAGTGAGGCACTG (reverse); and Ppia, CAAATGCTGGACCAAACACAA (forward) and TTCACCTTCCCAAAGACCACAT (reverse).

Statistical analysis

The D'Agoustino Pearson normality test and visual classification were utilized to check for a Gaussian distribution. The Student *t*-test with independent variance was applied to check for differences between 2 groups for parametric data. Nonparametric data were analyzed by the Mann-Whitney *U* test. Significance level was set to $P < 0.05$. Data are mean ± SD unless stated otherwise. The correlation of nonparametric data was computed by the 2-tailed Spearman analysis with a confidence interval of 95%. Software systems used were GraphPad Prism 8 and IBM statistics SPSS 26.

RESULTS

Incorporation of vitamin K into lipoproteins in uremic versus healthy participants

Study population. In our prospective clinical study, 10 long-term hemodialysis patients and 9 age-matched, healthy subjects were included. As shown in Table 1, age, body mass index, and serum triglyceride or HDL cholesterol levels did not differ significantly between the 2 groups. The hemodialysis group, compared with controls, had more males and higher serum levels of LDL cholesterol, and C-reactive protein was significantly higher (Table 1). Dialysis patients exhibited a state of functional vitamin K deficiency, evidenced by significantly higher serum levels of the vitamin K–dependent protein PIVKA-II (Table 1).

Vitamin K incorporation into lipoproteins in vivo. In healthy control subjects, uptake of vitamin K1 into chylomicrons or HDL particles was hardly detectable, and only minimal uptake into LDL particles was noted at 3 hours (Figure 1 and Supplementary Figure S1). Incorporation of MK4 was low to absent in all measured lipoprotein fractions. In contrast, MK7 showed significant incorporation within 3 hours after ingestion into HDL particles isolated from healthy persons.

In long-term hemodialysis patients, vitamin K1 uptake was low in all lipoprotein fractions (Figure 1 and Supplementary Figure S1). In contrast to healthy controls, MK4 was significantly incorporated into LDL and HDL cholesterol particles at 3 to 6 hours after ingestion. Uptake of MK7 was largely confined to HDL particles but was markedly depressed in hemodialysis patients, compared with controls.

Vitamin K incorporation into human lipoprotein particles *in vitro*

Because the most pronounced differences in vitamin K lipoprotein content between healthy subjects and dialysis patients occurred in HDL particles (Figure 1 and Supplementary Figure S1), HDL particles from each group were isolated and *in vitro* uptake of different vitamin K forms was investigated. Patients and control subject further were characterized regarding serum biochemistry and medication (Supplementary Table S2).

In vitro incubation of HDL particles from healthy blood donors with vitamin K1, MK4, or MK7 resulted in a mean 3.1-fold increase of vitamin K1 content, a 2.1-fold increase of MK4, and a 4.5-fold increase of MK7 (Figure 2a). In contrast, HDL particles isolated from long-term hemodialysis patients exhibited an almost absent MK7 incorporation (1.2-fold), whereas the incorporation of MK4 was not significantly different from healthy controls and vitamin K1 tended to be higher but was not significantly different (Figure 2a).

In additional experiments, MK7 incorporation into isolated LDL particles was also assessed. There was no difference between healthy persons and dialysis patients (data not shown).

Vitamin K bioactivity in human lipoprotein particles *in vitro*

Next, the potential biological relevance of the above findings was analyzed. For this, HDL particles from healthy controls were spiked with MK7 and carboxylation of MGP in human VSMCs after 6-hour incubation was used as a biological readout for vitamin K activity. Only treatment with spiked HDL, isolated from healthy subjects, but not that from dialysis patients significantly reduced ucMGP levels (Figure 2b).

To assess whether HDL particles from healthy controls and dialysis patients are comparably incorporated, osteoblastic MG63 cells were incubated with Atto-488–labeled HDL, isolated from healthy controls or dialysis patients (Figure 2c and d). These experiments demonstrated reduced cellular HDL internalization for particles isolated from uremic patients compared with healthy controls, with significant differences after 15- and 30-minute incubation. The amount of HDL attached to cells was not significantly different between the 2 groups (Figure 2c and d).

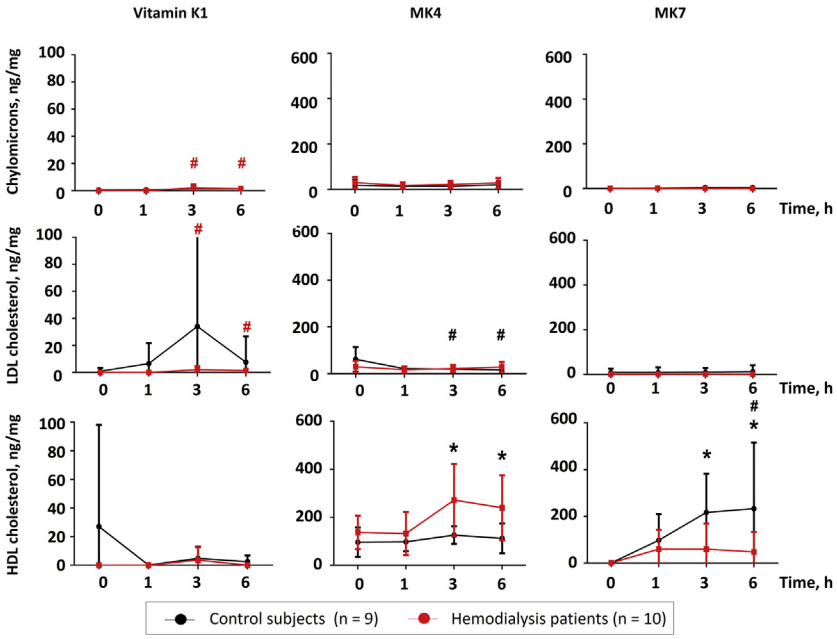

Figure 1 | Vitamin K contents of lipoproteins isolated from healthy subjects and dialysis patients at 0, 1, 3, and 6 hours after a single administration of a vitamin K supplement. Row 1: vitamin K1, menaquinone 4 (MK4), and menaquinone 7 (MK7) content of chylomicrons, normalized to total triglycerides. Row 2: vitamin K1, MK4, and MK7 content of low-density lipoprotein (LDL), normalized to total LDL. Row 3: vitamin K1, MK 4, and MK7 content of high-density lipoprotein (HDL), normalized to total HDL. *$P < 0.05$: significant difference between healthy and dialysis; #$P < 0.05$: significant versus time point 0 (red, hemodialysis; black, control); data are means ± SD.

Long-term incorporation of vitamin K into tissues of mice with CKD

To assess whether disturbances of vitamin K metabolism can experimentally be reproduced, CKD was induced in male C57BL6/J mice by 5/6 nephrectomy (Figure 3a). At week 1 after surgery, a 7-week high vitamin K diet (K1, MK4, and MK7) was initiated. Sham-operated mice served as controls. CKD was evidenced by significantly elevated serum urea at 8 weeks after 5/6 nephrectomy (Figure 3a), whereas serum phosphate, serum calcium, and systolic and diastolic blood pressure remained unchanged (Supplementary Table S3).

At week 8 after surgery, the vitamin K tissue contents of pooled serum, kidneys, livers, hearts, pooled aortas, brains, and lungs were analyzed. In both, 5/6 nephrectomized and sham-operated mice, the highest K1 level was found in the aorta, the highest MK4 level in the aorta and liver, and the highest MK7 level in liver (Supplementary Figure S2). Pooled serum samples contained higher concentrations of MK4 and MK7 in 5/6 nephrectomized compared with sham-operated mice (Figure 3b). In the kidney, the MK4 content was significantly lower in nephrectomized versus control mice, whereas vitamin K1 and MK7 levels were similar in both groups (Figure 3b). Vitamin K content (K1, MK4, and MK7) did not differ significantly between the groups in all other tissues (Supplementary Figure S2).

Vitamin K metabolizing enzymes in control and CKD rat kidney

Given that the most pronounced decrease in vitamin K (MK4) content was found in the kidney of CKD mice, kidney vitamin K–dependent enzymes were next investigated. Because the amount of tissue available from mice is limited, a switch to the rat adenine CKD model was made (Figure 4a and b).[9,21] In this model, we previously described an altered vitamin K cycle associated with reduced γ-glutamyl-carboxylase activity.[9] To extend these data, the key enzyme regulating the conversion of vitamin K1 into K2 (MK4), UBIAD1, was assessed. This enzyme, in turn, physically and functionally associates with 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase).[22] No differences in kidney UBIAD1 protein or gene expression (Figure 4c and f and Supplementary Figure S3) and no difference in gene expression of HMG-CoA reductase between CKD and control rats were detected (Supplementary Figure S3). However, HMG-CoA reductase protein expression was markedly reduced in CKD rat kidney (Figure 4c, e, and f), significantly correlating with the extent of calcification, as evidenced by von Kossa staining ($P < 0.001$; Figure 4d and f).

Vitamin K metabolizing enzymes in normal human kidneys and kidneys of CKD patients

To extend the rodent data and understand precisely which cells express vitamin K metabolizing enzymes, our recently published single-cell RNA sequencing data sets of human CKD and non-CKD kidneys were next analyzed[14] (Figure 5a). The most prominent cell type expressing all enzymes, except for HMG-CoA reductase, was podocytes. Of all enzymes, vitamin K epoxide reductase was expressed most prominently and vitamin K epoxide reductase mRNA expression in these cells decreased in samples from CKD patients (Figure 5b).

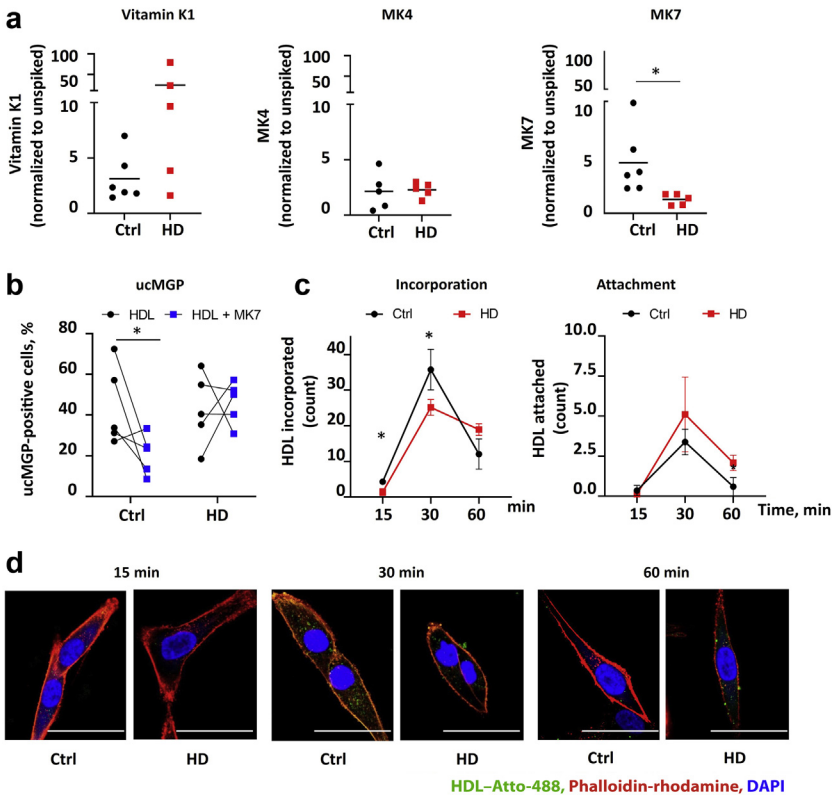

Figure 2 | High-density lipoprotein (HDL) *in vitro* assays. (**a**) Vitamin K contents in HDL isolated from healthy subjects and dialysis patients, spiked with vitamin K1, menaquinone 4 (MK4), or menaquinone 7 (MK7), *in vitro*; normalized to unspiked HDL. (**b**) Uncarboxylated matrix Gla protein (ucMGP) after treatment of vascular smooth muscle cells with spiked (+MK7) and unspiked HDL, isolated from healthy (control [Ctrl]) and dialysis patients (hemodialysis [HD]). (**c**) Incorporation of Atto-488–labeled HDL into MG63 cells after 15, 30, and 60 minutes of exposure. Right: attachment of Atto-488–labeled HDL to MG63 cells after 15, 30, and 60 minutes of incubation (n = 3). (**d**) Confocal images of MG63 cells incubated with Atto-488–labeled HDL after 15, 30, and 60 minutes. Bar = 50 μm. Red, rhodamine-phalloidin; blue, 4′,6-diamidino-2-phenylindole (DAPI); green, HDL–Atto-488. *$P < 0.05$. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

In non–tumor-bearing parts of kidneys derived from tumor nephrectomies, immunohistochemistry showed that HMG-CoA and UBIAD1 were mainly expressed in the distal and proximal tubules (Figure 5c). When evaluating the kidney areas stained positively for either enzyme in CKD versus non-CKD, there was no significant difference in UBIAD1 and HMG-CoA reductase protein expression (Figure 5d).

Pharmacologic modifiers of vitamin K deficiency in long-term hemodialysis patients

To assess the effect of HMG-CoA reductase inhibitors (statins) or proton pump inhibitor therapy on vitamin K status, 1051 stored serum samples from the 4D study[13] were analyzed. It has been shown that statins inhibit UBIAD1 activity,[23] reduce MK4 synthesis in VSMCs,[24] and independently predict coronary artery calcification in end-stage kidney disease.[24] Proton pump inhibitors alter the gut microbiome,[25] and thereby have an impact on a potential endogenous source of vitamin K2. Overall, 49% of the long-term hemodialysis patients had a PIVKA-II serum level >0.2 AU/ml, which is the upper limit of normal for this assay (Figure 6),[7] yielding 98 to 119 data points per group (Supplementary Table S4A). Although serum levels tended to increase in patients receiving atorvastatin and even more so if proton pump inhibitors were taken as well, analysis of variance failed to reveal significant PIVKA-II differences between patients receiving these medications or placebo (Figure 6).

In the above analyses, we had excluded 4D trial participants who were taking vitamin K antagonists. When we analyzed such patients separately, their PIVKA-II levels were markedly elevated (3.5–10.4 AU/ml), suggesting that statins and/or proton pump inhibitors exert a relatively minor effect on vitamin K uptake (Supplementary Table S4B).

DISCUSSION

In the present study, uremia-related alterations of vitamin K pharmacokinetics, metabolism, and biodistribution were investigated. CKD patients exhibit a functional vitamin K deficiency,[7] confirmed in the present study by a 37-fold higher PIVKA-II serum level in dialysis patients compared with healthy controls. Because correcting vitamin K deficiency would particularly benefit CKD patients and because it is safe,[26] various randomized controlled trials have been

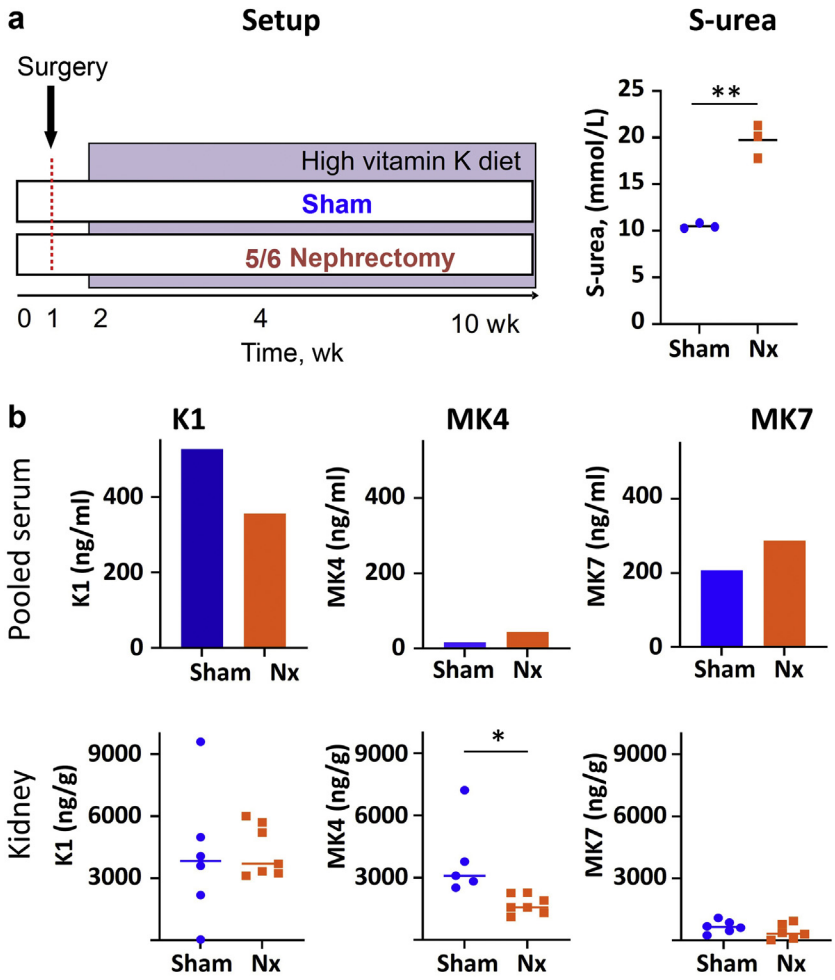

Figure 3 | 5/6 Nephrectomy (Nx) in mice. Mice with either sham surgery or 5/6 Nx, receiving a vitamin K–rich diet. (**a**) Animal protocol; vitamin K–rich diet was initiated 1 week after surgery and continued for 8 weeks. Right: serum urea concentration at the end of the experiment in sham and nephrectomized mice. (**b**) Vitamin K1 (K1), menaquinone 4 (MK4), and menaquinone 7 (MK7) concentrations in kidneys and pooled serum from sham operated and nephrectomized mice. *$P < 0.05$, **$P < 0.001$.

performed or are ongoing to assess the effects of vitamin K supplementation on cardiovascular calcification and other morbidity in CKD patients.[27–30] Most of these trials employ dietary supplements of MK7, because long-chain menaquinones are believed to also act in nonhepatic tissues. In addition, among the vitamin K2 species, MK7 is readily available in synthetic form (albeit not drug grade) and exhibits a long half-life in healthy subjects.[8,31] In addition, using the same dose, MK7 induces more carboxylation of osteocalcin than vitamin K1, indicating not only better absorption but also better bioactivity.[32] In healthy subjects of the present study, following a single oral dose, MK4 serum levels hardly changed, whereas MK7 levels rapidly increased in serum, confirming previously published data.[31]

The major finding of this study is that the pharmacokinetics of vitamin K1, MK4, and MK7 are profoundly altered in uremia. Following a single dose of vitamin K1, a significantly lower blood level of LDL-associated K1 in dialysis patients compared with healthy subjects was apparent. The most prominent alteration in MK4 and MK7 uptake was found in uremic HDL particles, whereby the uremic HDL particles took up more MK4 than did normal HDL and only little of the long-lived MK7. These observations support our data showing that the uremic HDL particle is highly altered, losing its protective vascular function[33] and turning into a proinflammatory particle that might contribute to the development of atherosclerosis.[34] Consistent with the low to negligible uptake of MK7 into uremic HDL, reduced cellular uptake and reduced bioactivity of uremic HDL particles spiked with MK7 were also apparent. This indicates that beyond dietary restriction[35] and reduced recycling,[9] altered transport and cellular uptake of vitamin K2 contributes to the vitamin K deficiency in the uremia characteristic of advanced CKD.

This major alteration in MK7 pharmacology connected with uremia may explain why several recent interventional trials in patients with CKD stages 3 to 5 have failed to demonstrate significant benefit of vitamin K2 supplementation.[27,30,36] Two studies investigating effects of oral MK7 administered at 90 or 200 μg daily noted no retardation of

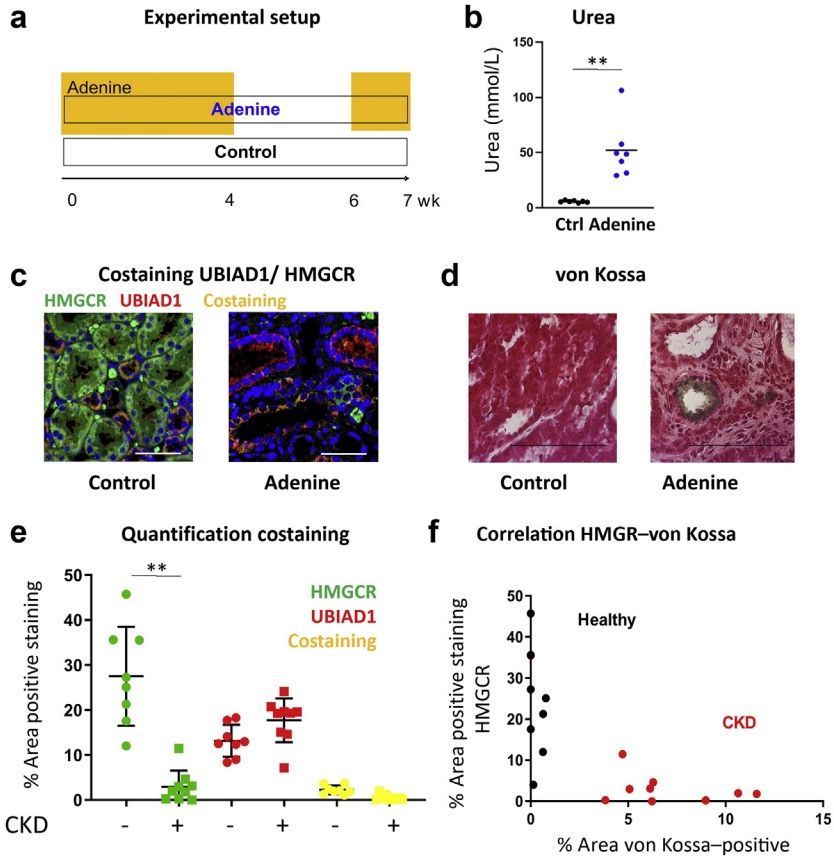

Figure 4 | Adenine nephropathy in rats. (**a**) Experimental setup; rats were supplemented with an adenine-enriched diet for 4 weeks, interrupted by 2 weeks of recovery, followed by a final week of adenine, compared with control (Ctrl) diet for 7 weeks. (**b**) Serum urea concentration after 7 weeks of adenine diet, compared with healthy controls. (**c**) Confocal images of costaining for UbiA prenyltransferase domain-containing 1 (UBIAD1; red) and hydroxymethyl-glutaryl-coenzyme A reductase (HMGCR; green) in rat kidneys, healthy versus adenine. Bar = 50 μm. (**d**) Von Kossa staining of rat kidneys, healthy versus adenine. Bar = 200 μM. (**e**) Planimetric quantification of UBIAD1 (red), HMGCR (green), and costained area (yellow) of kidneys from healthy and adenine rats (n = 9). (**f**) Association of von Kossa–positive staining with HMGCR-positive area in kidneys from healthy and adenine rats. Data are means ± SD. $^{**}P < 0.001$. CKD, chronic kidney disease. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

cardiovascular calcification, despite a 47% reduction in ucMGP levels with the 200-μg dose over 1 year.[27,28] In the British K4Kidneys trial, patients with CKD stages 3 to 5 randomly received 400 μg MK7 or placebo daily and, again, pulse wave velocity at 1 year did not differ between the 2 groups.[29] An even higher MK7 dose (857 μg daily) administered to long-term hemodialysis patients did not lead to differences in vascular calcification progression, compared with the control group, despite a decrease in ucMGP levels by ≈50% in the group receiving MK7.[36] It will be of interest to see the outcome of 2 additional trials in dialysis patients, where pharmacologic doses of vitamin K1 (15 and 30 mg/wk, corresponding to 2143 and 4286 μg/d) are tested in long-term hemodialysis patients with extensive cardiovascular calcification (Inhibit Progression of Coronary Artery Calcification with Vitamin K1 in Hemodialysis Patients [iPACK-HD], NCT 01528800; Vitamin K to Slow Vascular Calcification in Hemodialysis Patients [VitaVasK], European Union Drug Regulating Authorities Clinical Trials Database [EudraCT] No. 2010-021264-14).[37,38]

In contrast to humans, dietary vitamin K1 and K2 supplementation increased the serum levels of K1, MK4, and MK7 in CKD mice, compared with non-CKD mice. Only in certain tissues, such as the kidney, were MK4 levels low. Opposite observations have been reported in rats with adenine-induced CKD, where kidney levels of MK4 increased.[21] Lipoprotein distribution of supplemented vitamin K was not studied. Rodent HDL fundamentally differs from human HDL,[39] and cholesterol in mice is mostly transported in HDL rather than in LDL particles.[40] Even though preclinical rodent models of CKD indicated vitamin K benefits on cardiovascular calcification,[41,42] these rodent species differences render extrapolation to humans difficult. In addition, the induced degree of CKD in rodents never reaches that of human end-stage kidney failure.

Previous analysis regarding enzymes of the vitamin K cycle revealed that in rats with adenine-induced CKD, γ-glutamyl-carboxylase activity is reduced.[9] Herein, the focus was on UBIAD1 and HMG-CoA reductase, both involved in local tissue generation of MK4.[22] In contrast to previous data,[21] no

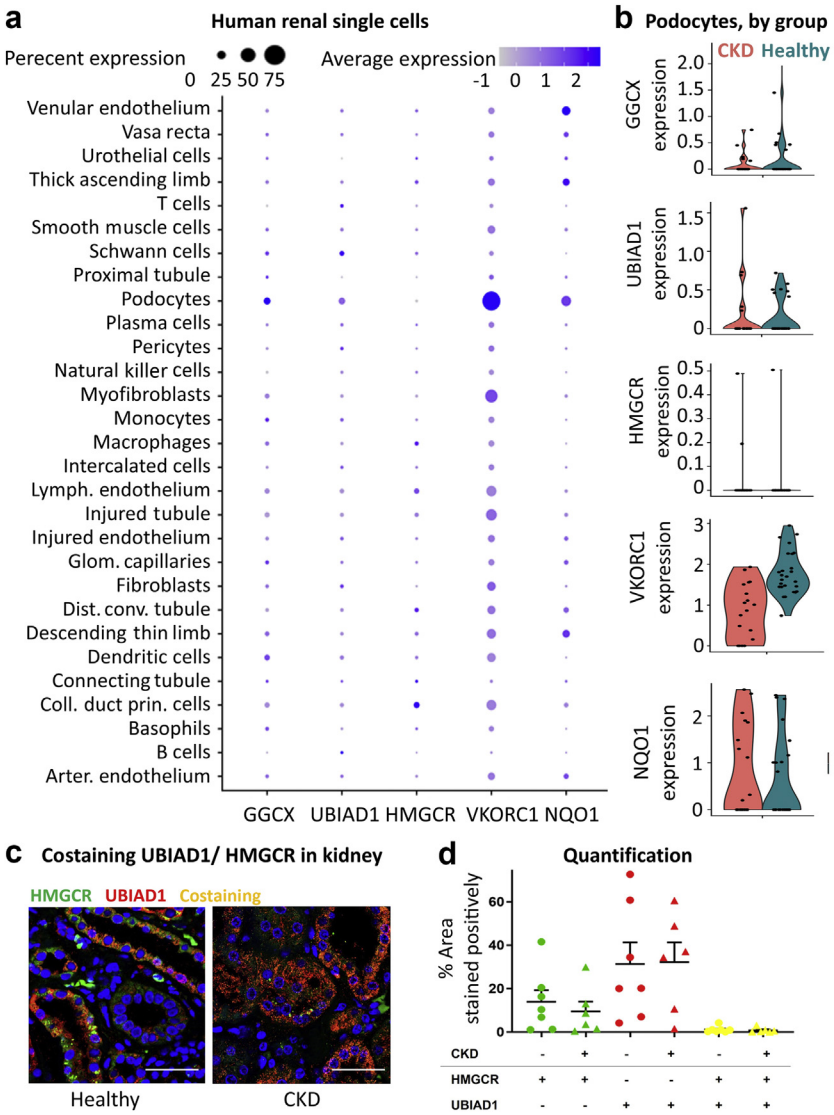

Figure 5 | Vitamin K enzymes in human kidneys. (**a**) Gene expression of vitamin K–related enzymes in CD10-negative single kidney cells. Percent expression: percentage of cells within a cluster that expresses the gene (nonzero expression), coded by sizes of the dot. Average expression: average expression of the cells within a cell cluster, scaled across all clusters to highlight the ones overexpressing the gene compared with the others, coded by color. Cells in total: 51,749. (**b**) Expression of γ-glutamyl-carboxylase (GGCX), UbiA prenyltransferase domain-containing 1 (UBIAD1), hydroxymethyl-glutaryl-coenzyme A reductase (HMGCR), vitamin K epoxide reductase subunit C1 (VKORC1), and NAD(P)H quinone dehydrogenase 1 (NQO1) in podocytes (chronic kidney disease [CKD], n = 6; non-CKD, n = 9). (**c**) Confocal images of HMGCR (green) and UBIAD1 (red) costainings (yellow) of human kidney tissue from non-CKD and CKD patients. Bar = 50 μm. (**d**) Quantification of HMGCR (green), UBIAD1 (red), and costaining (yellow) in human kidney from non-CKD and CKD patients (n = 6–7). To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

differences in kidney UBIAD1 protein or gene expression between CKD and control rats were detected. However, HMG-CoA reductase protein expression was considerably reduced in kidneys from CKD rats and was related to the extent of kidney calcification. A nonsignificant reduction in HMG-CoA reductase expression was noted in human CKD tissue. These observations indicate a link between vitamin K deficiency and lipid-lowering therapy in humans. Indeed, lipophilic statins, such as atorvastatin, have been shown to directly inhibit the MK4 synthesizing enzyme UBIAD1,[23,43] and mice treated with atorvastatin had lower MK4 kidney levels.[44] However, PIVKA-II serum levels in the 4D study dialysis patients treated with atorvastatin were not significantly higher and thus vitamin K deficiency was not aggravated. Another type of medication, common in CKD, is proton pump inhibitors, which potentiate the effect of vitamin K antagonists, depending on the genotype of vitamin K epoxide reductase.[45,46] The highest PIVKA-II values were observed in patients receiving atorvastatin in combination with a proton pump inhibitor. However, the overall impact of statins and or proton pump inhibitors on PIVKA-II serum levels, and thus vitamin K deficiency, appear

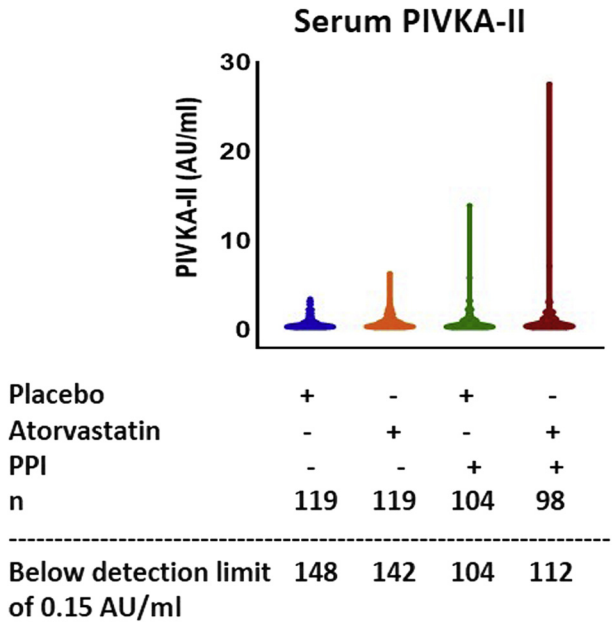

Figure 6 | Serum protein induced by vitamin K absence (PIVKA)–II levels in dialysis patients of the Die Deutsche Diabetes Dialyse Studie (4D) trial, receiving either placebo or atorvastatin, with or without a proton pump inhibitor (PPI); depicted are all values above detection limit (N = 440; n = 469 below detection limit). AU, arbitrary unit.

small, especially compared with that of patients on vitamin K antagonists. These patients exhibit an ≈15-fold higher PIVKA-II level, compared with those not receiving a vitamin K antagonist (Supplementary Table S3). A limitation is that in the 4D trial all dialysis patients were diabetic and thus extrapolation to nondiabetic dialysis patients or even patients with non–dialysis-dependent CKD is difficult.

A limitation of the present study is that we only investigated vitamin K1 and K2 uptake in a short-term experiment in dialysis patients and controls. Long-term administration of MK7 in high doses clearly led to a partial correction of the vitamin K deficiency in dialysis patients.[27,30,36,47] However, unpublished data of the VitaVasK trial[38] show that 15 mg/wk of vitamin K1 given to long-term dialysis patients is markedly more potent than MK7 in correcting the deficiency. With respect to vitamin K1, we only administered 1 mg in the single-dose study, which is lower than the amount used in VitaVasK and which may explain why we discovered only low amounts of K1 in chylomicrons (Supplementary Figure S1).

In summary, a marked vitamin K deficiency in long-term hemodialysis patients is confirmed and next to dietary restriction and reduced recycling, a novel pathomechanism is identified (namely, profound alteration in transport and tissue uptake of vitamin K2 in advanced CKD). These alterations will likely affect the outcome of clinical trials aiming to supplement vitamin K2 in dialysis patients and may provide an explanation why, thus far, no convincing cardiovascular benefits have been reported. The outcome of clinical trials assessing pharmacologic doses of vitamin K1 is eagerly awaited.

DISCLOSURE

JF has received consultancy or speaker honoraria from Amgen, Astellas, AstraZeneca, Bayer, Boehringer, and Fresenius Vifor. LS has received consultancy fees from Immunodiagnostic Systems, not related to the submitted work, and grants from NattoPharma, Boehringer Ingelheim, and Bayer, also not related to the submitted work. RK has received consultancy or speaker honoraria from Bayer and Evotec AG, and research funding from Chugai, Travere Therapeutics, and Galapagos. All the other authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary File (PowerPoint)

Figure S1. Vitamin K contents of lipoproteins isolated from healthy subjects and dialysis patients at 0, 1, 3, and 6 hours after a single administration of a vitamin K supplement; higher resolution, *y*-axis. Row 1: vitamin K1, menaquinone 4 (MK4), and menaquinone 7 (MK7) content of chylomicrons, normalized to total triglycerides. Row 2: vitamin K1, MK4, and MK7 content of low-density lipoprotein (LDL), normalized to total LDL. Row 3: vitamin K1, MK4, and MK7 content of high-density lipoprotein (HDL), normalized to total HDL. $^{*}P < 0.05$: significant difference between healthy and dialysis; $^{\#}P < 0.05$: significant versus time point 0 (red, hemodialysis; black, control); data are means ± SD.

Figure S2. Vitamin K contents in various tissues of 5/6 nephrectomized mice. (**A**) Serum protein was significantly increased in nephrectomized mice. (**B**) Vitamin K1, menaquinone 4 (MK4), and menaquinone 7 (MK7) contents in liver, heart, brain, pooled aorta, and lung in nephrectomized mice versus control (Ctrl). All units are ng/g tissue weight. Because of technical problems, some samples were lost. CKD, chronic kidney disease (nephrectomized mice).

Figure S3. Gene expression of UbiA prenyltransferase domain-containing 1 (UBIAD1) and hydroxymethyl-glutaryl-coenzyme A reductase (HMGCR) in adenine rats (chronic kidney disease [CKD]) versus healthy controls.

Table S1. Rodent diet.

Table S2. *In vitro* spiking experiment.

Table S3. Serum biochemistry and systolic blood pressure at the end of the experiment.

Table S4. Serum protein induced by vitamin K absence (PIVKA)–II levels in patients participating in the Die Deutsche Diabetes Dialyse Studie (4D) trial. (**A**) Main cohort (shown in Figure 6), excluding patients receiving vitamin K antagonists. (**B**) Subset, vitamin K antagonist users only (excluded in Figure 6).

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