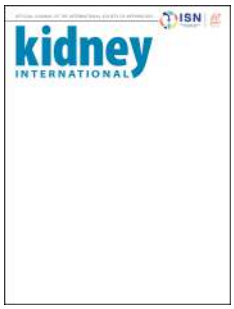

Altered vitamin K biodistribution and metabolism in experimental and human chronic kidney disease.

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PII: S0085-2538(21)01056-5

DOI: https://doi.org/10.1016/j.kint.2021.10.029

Reference: KINT 2828

To appear in: *Kidney International*

Received Date: 28 May 2021

Revised Date: 7 October 2021

Accepted Date: 18 October 2021

Please cite this article as: Kaesler N, Schreibing F, Speer T, Puente-Secades Sdl, Rapp N, Drechsler C, Kabgani N, Kuppe C, Boor P, Jankowski V, Schurgers L, Kramann R, Floege J, Altered vitamin K biodistribution and metabolism in experimental and human chronic kidney disease., *Kidney International* (2021), doi: https://doi.org/10.1016/j.kint.2021.10.029.

[QUERY TO AUTHOR: title and abstract rewritten by Editorial Office – not subject to change]

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

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Running Title:
Vitamin K metabolism in chronic kidney disease

Abstract

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.

Key words
Vitamin K, HDL particles, dialysis, 5/6 nephrectomy, statins, matrix gla protein

Translational statement

Understanding uremia-associated alterations in vitamin K pharmacokinetics is essential to conducting trials aimed at preventing progressive uremic vascular calcification. The results presented here suggest that supplementation strategies may have to be adapted in CKD patients.

INTRODUCTION

Cardiovascular morbidity and mortality markedly increase as chronic kidney disease (CKD) progresses[1]. Traditional risk factors and a number of non-traditional factors are implicated in this accelerated course of cardiovascular disease [2]. In particular, non-traditional 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 (cMGP) 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; CUA) that accompanies the therapy with vitamin K antagonists in patients with normal renal 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 non-phosphorylated, uncarboxylated fraction of MGP (dp-ucMGP), indicating subclinical vitamin K deficiency[7], has shown that this marker is strongly up-regulated in CKD. Dp-ucMGP is easily set free in the circulation as it has a very low affinity for calcification[5]. In these patients high circulating dp-ucMGP 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 (VKORC1), DT-diaphorase (NQO1) and γ-glutamyl-carboxylase (GGCX). Due to reduced GGCX 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 towards 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 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 chronic 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 above 18 years, chronic hemodialysis treatment for at least 6 months or for healthy subjects a documented GFR above 60 ml/min/1.73m², and written consent to the study. Key exclusion criteria were gastro-intestinal 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, Kerkrade, The Netherlands) containing 1000 µg of vitamin K1, 1000 µg of MK4 and 200 µg of 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 prior to hemodialysis after a long dialysis interval as well as at 1 and 3 h during dialysis and finally at 6 h. Blood samples were centrifuged at 2000g at 4°C and either immediately analyzed or stored at –80°C.

Serum Biochemistry

Serum HDL cholesterol, LDL cholesterol and triglycerides were determined with the HDL/LDL/VLDL Cholesterol Kit according to the manufacturer's instructions (Abcam, Cambridge, UK). To determine total triglyceride levels, serum was mixed with triglyceride reagent (Triglycerides FS, DiaSys, Holzheim, Germany). After incubation at room temperature

for 20 min, plasma triglycerides and cholesterol levels were measured by absorbance at 490 nm using a plate reader (FLUOstar OPTIMA, BMG Labtech GmbH, Ortenberg, Germany). C-reactive protein (CRP) was measured in serum by turbodimetric CRP4 Tina-quant®assay (Cobas 8000, Roche, Basel, Switzerland) 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 (ELISA; Biozol, Eching, Germany) or, in case of the 4D study samples, using a conformation-specific monoclonal antibody in an ELISA-based assay [15]. Results were expressed as arbitrary units per liter (AU/l) since 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 pre-dialysis plasma (from 10 dialysis patients and 9 healthy participants, both different from those above) by density gradient ultracentrifugation (HDL: density 1.063 to 1.21 g/cm³, LDL: density 1.006-1.063 g/cm³) 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

Four mg of HDL were diluted in PBS 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, Berlin, Germany) and incubated for 2 h at room temperature. After incubation, Atto-488-labeled HDL was purified using gel filtration.

Spiking of lipoproteins with vitamin K species

HDL or LDL were 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 HPLC setup (Dionex UltiMate, Thermofisher) with isocratic methanol at pH 5 as mobile phase and a MaxRPC12 column (Phenomenex, CA, USA) 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 were normalized to the total amount of triglycerides, LDL, and HDL, respectively.

Cell culture

Human osteoblast-like MG63 cells were cultivated in alpha-MEM, 10% FCS, and 1% penicillin-streptomycin in a humidified atmosphere with 5% CO_2 at 37°C. To determine cellular HDL attachment and internalization, Atto488 labeled HDL [17] was incubated 15, 30, or 60 min at 4°C with MG63 cells [17]. Images were taken with a Nikon A1 laser confocal microscope (Nikon, Tokyo, Japan).

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 VSMC were thawed and cultured in DMEM media (31966 047 Gibco), 10% FCS, and 1% penicillin-streptomycin for three days and subsequently immortalized. Two retroviral particles SV40LT and HTERT were produced by transient transfection of HEK293T cells for 48h using TransIT-LT (Mirus), plasmids pBABE-puro-SV40-LT (Addgene #13970) or xlox-dNGFR-TERT (Addgene #69805) in combination with a packaging plasmid pUMVC (Addgene #8449) and a pseudotyping plasmid pMD2.G (Addgene #12259). Cell transduction was performed for 48h 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 h with native or vitamin K spiked HDL, isolated from healthy volunteers or dialysis patients. DMEM was supplemented with vitamin D (1,25 OH D3, 10nM, Sigma Aldrich) and did not contain FCS 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 % 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, Soest, Germany, **Suppl. Table 1**) was continued for 7 weeks, after which blood and organs were harvested. Blood pressure was measured by the tail-cuff method (Coda, Kent Scientific, CT, USA).

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, Lage, Germany), followed by 2 weeks on a standard diet, followed by one week of adenine.

Serum was obtained by centrifugation at 2,000*g for 10min 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 tissue 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 due to 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 20min. Blocking was performed with bovine or horse serum. UBIAD1 antibody (SC-271595, Santa Cruz Biotechnology, Dallas, Tex., USA) was incubated overnight at 4°C. HMGCR antibody (GTX32134, Genetex, Irvine, CA., USA) was enhanced by adding biotinylated anti-mouse IgG (BA-200 Vector labs, Burlingame, CA., USA). Secondary antibodies were streptavidin-FITC (SA-5001, Vector labs, Burlingame, CA., USA) and anti-rabbit Cy3 (711-165-152 Jackson ImmunoResearch, USA). Nuclei were counterstained with DAPI (D-1306, Molecular Probes, the Netherlands). Finally, the slides were mounted with VECTASHIELD® Mounting Media (Vector labs, Burlingame, CA., USA) and images were taken with a Nikon A1 laser confocal microscope (Nikon, Tokyo, Japan).

RT-PCR

RNA was extracted from rat kidneys by the Qiagen RNeasy kit (74104 Qiagen, Hilden, Germany), according to the manufacturer's instructions. The amount of RNA was quantified using a Nanovue Plus Spectrophotometer (GE Healthcare, Freiburg, Germany) at 260 and 280nm. cDNA was synthesized after incubating each sample with 100 µM oligo dT primer

(Eurofins Scientific, Luxembourg) at 70°C for 5 min. Afterwards, samples were incubated at 37°C for 1 h with M-MLV RT 5x Buffer, M-MLV RT Enzyme and dNTPs Mix (Promega, Madison, Wisconsin, USA) to achieve cDNA synthesis. In the final step, samples were diluted in a 1:8 ratio in RNase free water (Braun, Melsungen, Germany).

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

Primer sequences were HMGCR Fw CCTCCATTGAGATCCGGAGG, HMGCR Rev AAGTGTCACCGTTCCCACAA, UBIAD1 Fw AAGTGCGCCTCCTATGTGTT, UBIAD1 Rev CAGGAGTGAGTGAGGCACTG, Ppia Fw CAAATGCTGGACCAAACACAA, Ppia rev TTCACCTTCCCAAAGACCACAT.

Statistics

The D'Agoustino Pearson normality test and visual classification were utilized to check for a Gaussian distribution. Student's t-test with independent variance was applied to check for differences between two groups for parametric data. Non-parametric 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 non-parametric data was computed by the two-tailed Spearman analysis with a confidence interval of 95%. Softwares 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 chronic hemodialysis patients and 9 age-matched, healthy subjects were included. As shown in **Table 1**, age, body mass index (BMI), serum triglyceride or high-density lipoprotein (HDL) cholesterol levels did not differ significantly between the two groups. The hemodialysis group, as compared to controls, had more males and higher serum levels of low-density lipoprotein (LDL) cholesterol and C-reactive protein (CRP) 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, Suppl. Figure 1**). Incorporation of MK4 was low to absent in all measured lipoprotein fractions. In contrast, MK7 showed significant incorporation within 3 h after ingestion into HDL particles isolated from healthy persons.

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

Vitamin K incorporation into human lipoprotein particles *in vitro*

Since the most pronounced differences in vitamin K lipoprotein content between healthy subjects and dialysis patients occurred in HDL particles (**Figure 1, Suppl. Figure 1**), 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 (**Suppl. Table 2**).

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 chronic hemodialysis patients exhibited an almost absent MK7 incorporation (1.2-fold), while 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 6h incubation was used as a biological read-out 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 atto488 labeled HDL, isolated from healthy controls or dialysis patients (**Figures 2c, 2d**). These experiments demonstrated

reduced cellular HDL internalization for particles isolated from uremic patients compared to healthy controls, with significant differences after 15 and 30 min incubation. The amount of HDL attached to cells was not significantly different between the two groups (**Figures 2c, 2d**).

Longterm 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**), while serum phosphate, serum calcium, systolic and diastolic blood pressure remained unchanged (**Suppl. Table 3**).

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 nephrectomised 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 levels in liver (**Suppl. Figure S2**). Pooled serum samples contained higher concentrations of MK4 and MK7 in 5/6 nephrectomised as compared to sham-operated mice (**Figure 3b**). In the kidney, the MK4 content was significantly lower in nephrectomised 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 (**Suppl. 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, renal vitamin K dependent enzymes were next investigated. Since the

amount of tissue available from mice is limited, a switch to the rat adenine CKD model was made **(Figure 4a and 4b**)[9, 21]. In this model, we previously described an altered vitamin K cycle associated with reduced GGCX activity [9]. To extend these data, the key enzyme regulating the conversion of vitamin K1 into K2 (MK4), UbiA prenyltransferase domain-containing protein 1 (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 renal UBIAD1 protein or gene expression (**Figure 4c, 4f, Suppl. Figure S3**) and no difference in gene expression of HMG-CoA reductase between CKD and control rats were detected (**Suppl. Figure S3)**. However, HMG-CoA reductase protein expression was markedly reduced in CKD rat kidney (**Figures 4c, 4e, 4f**), significantly correlating with the extent of calcification as evidenced by von Kossa staining ($p<0.001$) (**Figure 4d, Figure 4f-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, were podocytes. Of all enzymes, VKORC1 was expressed most prominently and VKORC1 mRNA expression in these cells decreased in samples from CKD patients (**Figure 5b**).

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**).

Pharmacological modifiers of vitamin K deficiency in chronic 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 VSMC [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 chronic hemodialysis patients had a PIVKA-II serum level above 0.2 AU/mL, which is the upper limit of normal for this assay (**Figure 6**)[7], yielding 98-119 data points per group (**Suppl. Table 4A**). While serum levels tended to increase in patients receiving atorvastatin and even more so if proton pump inhibitors were taken as well, ANOVA 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 (**Suppl. table 4B**).

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 as compared to healthy controls. Since correcting vitamin K deficiency would particularly benefit

CKD patients and since it is very safe[26], various randomized controlled trials have been performed or are ongoing to assess the effects of vitamin K supplementation on cardiovascular calcification and other morbidity in CKD patients [27-30]. The majority of these trials employ dietary supplements of MK7, since long-chain menaquinones are believed to also act in non-hepatic tissues. Additionally, 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 to 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 very 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 3-5 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 cardiovascular calcification, despite a 47% reduction in ucMGP levels with the 200 µg dose over one year [27, 28]. In the British K4Kidneys trial, patients with CKD stages 3-5 randomly received 400 µg MK7 or placebo daily and, again, pulse wave velocity at one year did not differ between the two groups [29]. An even higher MK7 dose (857 µg daily) administered to chronic hemodialysis patients did not lead to differences in vascular calcification progression, compared to the control group, despite a fall in ucMGP levels by approximately 50% in the group receiving MK7 [36]. It will be of interest to see the outcome of two additional trials in dialysis patients, where pharmacological doses of vitamin K1 (15 and 30 mg/week corresponding to 2143 and 4286 µg per day) are tested in chronic hemodialysis patients with extensive cardiovascular calcification (iPACK-HD, NCT 01528800; VitaVasK; 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 to 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, GGCX activity is reduced [9]. Here, 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 differences in renal 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 non-significant 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, are 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 small, especially compared to that of patients on vitamin K antagonists. These patients exhibit an approximately 15-fold higher PIVKA-ll level, compared to those not receiving a vitamin K antagonist (Suppl. Table 3). A limitation is that in the 4D trial all dialysis patients were diabetic and thus extrapolation to non-diabetic 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. Chronic 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/week of vitamin K1 given to chronic 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 (Suppl. Figure S1).

In summary, a marked vitamin K deficiency in chronic 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 pharmacological doses of vitamin K1 are eagerly awaited.

Disclosures

JF has received consultancy or speaker honoraria from Amgen, Astellas, AstraZeneca, Bayer, Boehringer, and Fresenius Vifor. LS has received consultancy fees from Immunodiagnostic systems (IDS), 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, Evotec AG, and research funding from Chugai, Travere Therapeutics and Galapagos.

Acknowledgements

We would like to thank Claudia Noll, Roya Soltan, Katrin Härthe, Cecile Maassen and Petra Lux for their excellent technical support. We also thank Javier Perales-Patón for technical support in visualizing single-cell gene expression data. This project was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – TRR 219 – Project-ID 322900939 and by the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska‐Curie grant agreement No 764474 (CaReSyAn). PB was funded by the DFG (Project-ID 454024652) and the European Research Council (ERC), under the European Union's Horizon 2020 research and innovation program (grant agreement No 101001791).

Figure legends

Figure 1: Vitamin K contents of lipoproteins isolated from healthy subjects and dialysis patients at 0, 1, 3, and 6h after a single administration of a vitamin K supplement; Row 1: vitamin K1, MK4, and MK7 content of chylomicrons, normalized to total triglycerides; Row 2 vitamin K1, MK4, and MK7 content of LDL, normalized to total LDL; Row 3:) vitamin K1, MK 4, MK7 content of HDL, normalized to total HDL. *: significant difference between healthy and dialysis $P<0.05$; #: significant versus time point t0 $P<0.05$ (red: hemodialysis, black: control); data are means +/- SD.CKD: Chronic kidney disease; h: Hours; HDL: High density lipoprotein; K1: Vitamin K1; LDL: Low density lipoprotein; MK4: Menaquinone 4; MK7: Menaquinone 7; TG: Triglycerides.

Figure 2: HDL in vitro assays. A: Vitamin K contents in HDL isolated from healthy subjects and dialysis patients, spiked with vitamin K1, MK4, or MK7, in vitro; normalized to unspiked HDL; **B**: uncarboxylated MGP (ucMGP) after treatment of VSMCs with spiked (+MK7) and unspiked HDL, isolated from healthy (ctrl) and dialysis patients (HD); **C**: Incorporation of atto4888 labeled HDL into MG63 cells after 15, 30, and 60min of exposure; Row 3 right: Attachment of atto4888 labeled HDL to MG63 cells after 15, 30, and 60min of incubation; (n=3) **D**: Confocal images of MG63 cells incubated with atto488 labeled HDL after 15, 30, and 60min. Scale bar = 50 µM; Red: Rhodamine-phalloidine, blue: DAPI, green: HDL-Atto488. *: P<0.05

CKD: Chronic kidney disease; ctrl: healthy control; HDL: High density lipoprotein; K1: Vitamin K1; MK4: Menaquinone 4; MK7: Menaquinone 7.

Figure 3: 5/6 nephrectomy in mice. Mice with either sham surgery or 5/6 nephrectomy, receiving a vitamin K rich diet; **A**: Animal protocol; vitamin K rich diet was initiated one 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, MK4, and MK7 concentrations in kidneys and pooled serum from sham operated and nephrectomised mice. *: P<0.05; **: P<0.001

K1: Vitamin K1; MK4: Menaquinone 4; MK7: Menaquinone 7; Nx: Nephrectomy

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 to control diet for 7 weeks; **B**: Serum urea concentration after 7 weeks of adenine diet, compared to healthy controls; **C**: Confocal images of co-staining for UBIAD1 (red) and HMGCR (green) in rat kidneys, healthy versus adenine. Scale bar = 50 µM;

D: Von Kossa staining of rat kidneys, healthy versus adenine. Scale bar = 200 µM; **D**: Planimetric quantification of UBIAD1 (red), HMGCR (green) and Co-stained area (yellow) of kidneys from healthy and adenine rats (n=9); **E**: Association of von Kossa positive staining with HMGCR positive area in kidneys from healthy and adenine rats. Data are means +/- SD **: P<0.001

Ctrl: Control; HMGCR: Hydroxymethyl-glutaryl-coenzyme A reductase; IF: Immunofluorescence; UBIAD1: UbiA prenyltransferase domain containing 1.

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 (non-zero 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 over-expressing the gene as compared to the others, coded by color. Cells in total 51749; **B:** Expression of GGCX, UBIAD1, HMGCR, VKORC1, NQO1 in podocytes, CKD: n=6, non-CKD: n=9; **C**: Confocal images of HMGCR (green) and UBIAD1 (red) co-stainings (yellow) of human kidney tissue from non-CKD and CKD patients. Scale bar = 50 µM **D**: Quantification of HMGCR (green), UBIAD1 (red) and co-staining (yellow) in human kidney from non-CKD and CKD patients (n=6-7).

CKD: Chronic kidney disease; GGCX: Gamma-glutamyl Carboxylase; HMGCR: Hydroxymethyl-glutaryl-coenzyme A reductase; NQO1: NAD(P)H quinone dehydrogenase 1; UBIAD1: UbiA prenyltransferase domain containing 1; VKORC1: Vitamin K epoxide reductase subunit C1

Figure 6: **Serum PIVKA-II levels in dialysis patients of the 4D trial**, either receiving placebo or atorvastatin, with or without a proton pump inhibitor; depicted are all values above detection limit (N=440, n= 469 below detection limit).

AU: Arbitrary units; PIVKA: Protein induced by vitamin K absence; PPI: Proton pump inhibitor.

Figure S1: Vitamin K contents of lipoproteins isolated from healthy subjects and dialysis patients at 0, 1, 3, and 6h after a single administration of a vitamin K supplement; Higher resolution Y-axis; Row 1: vitamin K1, MK4, and MK7 content of chylomicrons, normalized to total triglycerides; Row 2 vitamin K1, MK4, and MK7 content of LDL, normalized to total LDL; Row 3:) vitamin K1, MK 4, MK7 content of HDL, normalized to total HDL. *: significant difference between healthy and dialysis $P<0.05$; #: significant versus time point t0 $P<0.05$ (red: hemodialysis, black: control); data are means +/- SD.CKD: Chronic kidney disease; h: Hours; HDL: High density lipoprotein; K1: Vitamin K1; LDL: Low density lipoprotein; MK4: Menaquinone 4; MK7: Menaquinone 7; TG: Triglycerides.

Figure S2: Vitamin K contents in various tissues 5/6 nephrectomized mice

A) Serum protein was significantly increased in nephrectomized mice

B) Vitamin K1, MK4, and M7 contents in liver, heart, brain, pooled aorta, and lung in nephrectomized mice versus control

Ctrl: Control; CKD: nephrectomized mice

All units are ng/g tissue weight

Due to technical problems some samples were lost.

Figure S3:

Gene expression of UBIAD1 and HMG-CoA reductase (HMGCR) in adenine rats (CKD) versus healthy controls

Table 1: Patient characteristics and serum biochemistry, interventional trial

	Healthy (n=9)	Dialysis (n=10)	*P
Sex, male [%]	57	80	n.s.
Age, mean (SD) [years]	61 (12)	70 (10)	n.s.
BMI, mean (SD) [kg/m²]	25.5 (2.1)	25.8 (4.7)	n.s.
GFR, mean (SD) [mL/min]	>60	n/a	
Diabetes mellitus [%]	0	40	0.033
Triglycerides, mean (SD) [mg/mL]	1.48 (0.26)	1.52 (0.33)	n.s.
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)	n.s.
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	n.s.
Dialysis vintage [month]	n/a	47.6 (46.2)	

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

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Figure 1

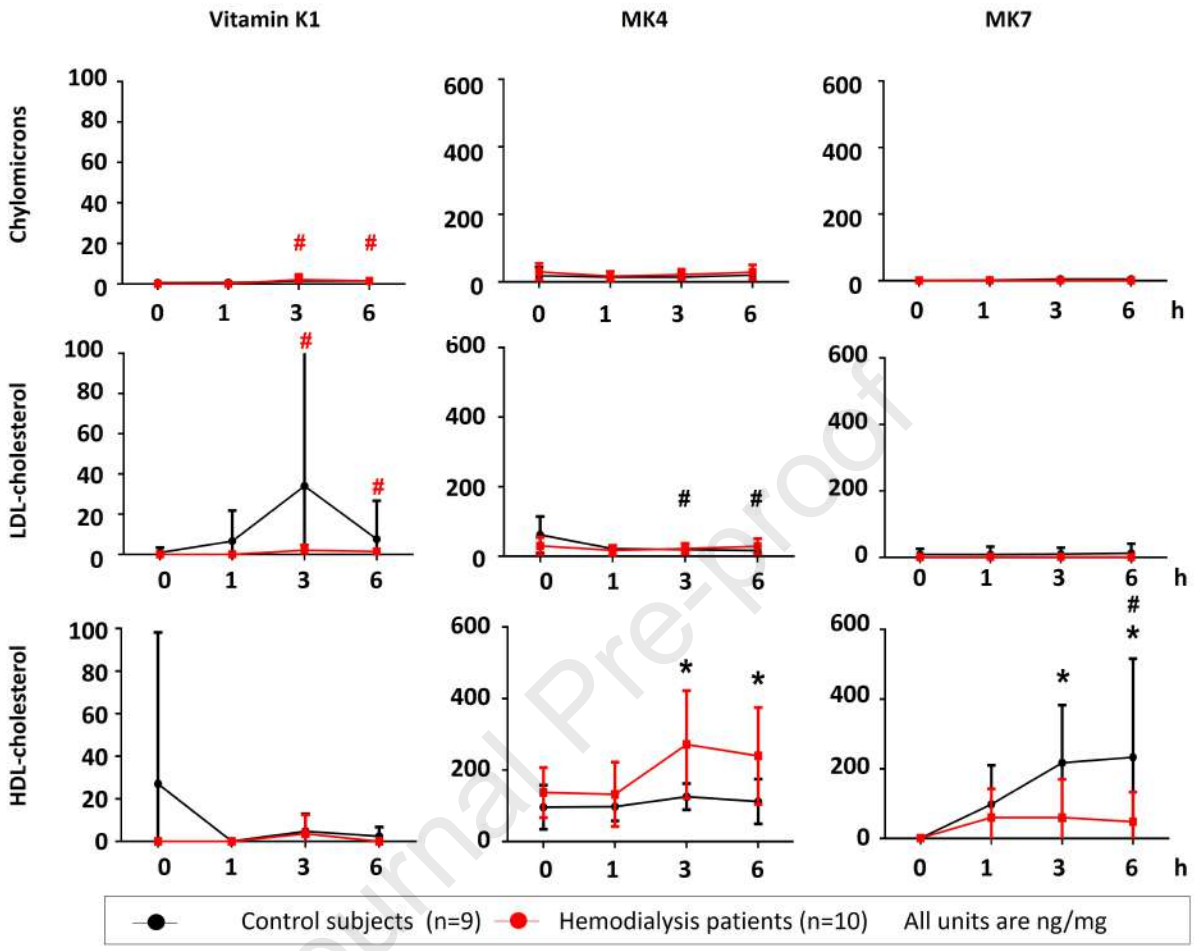


Figure 2

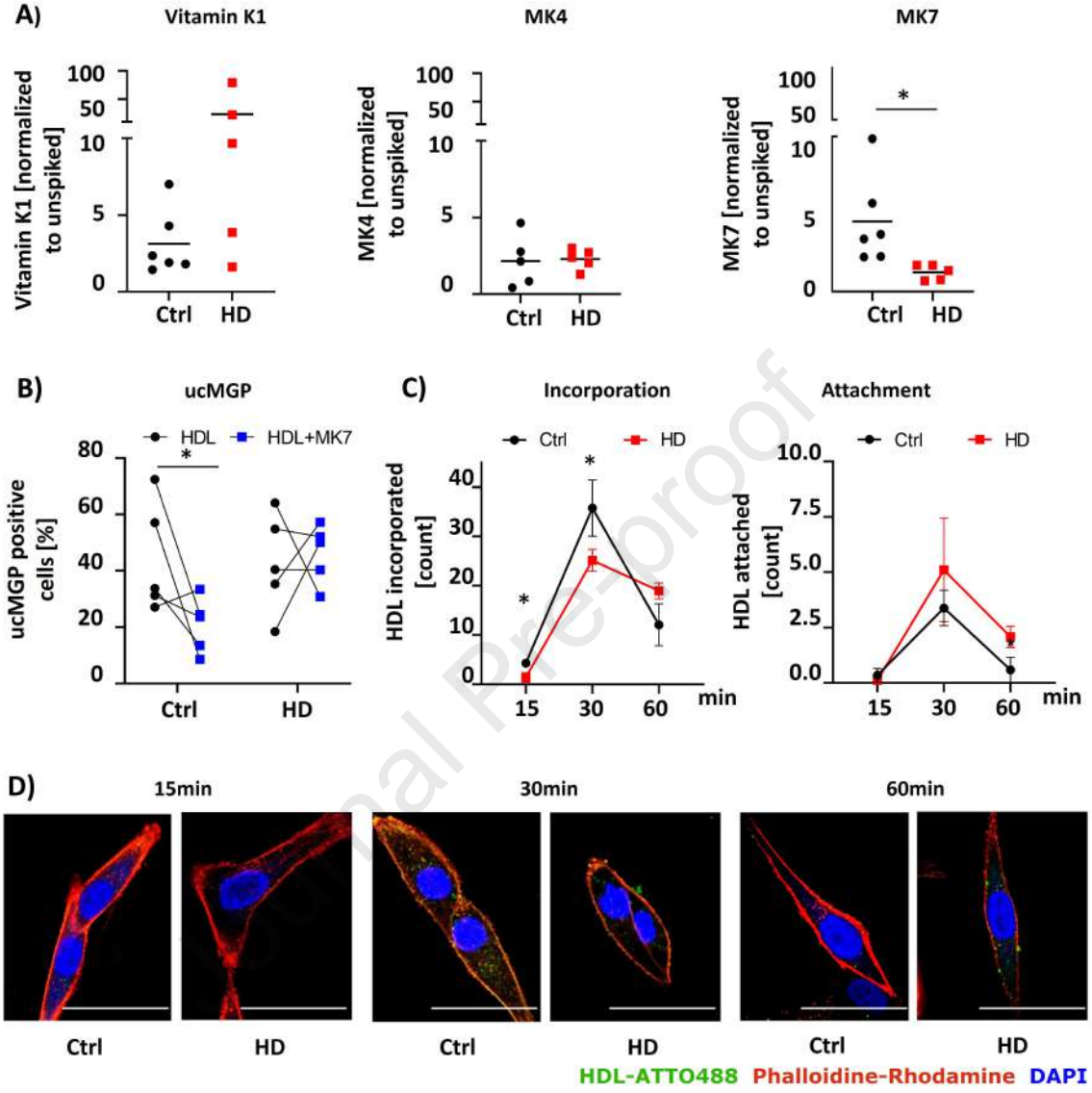
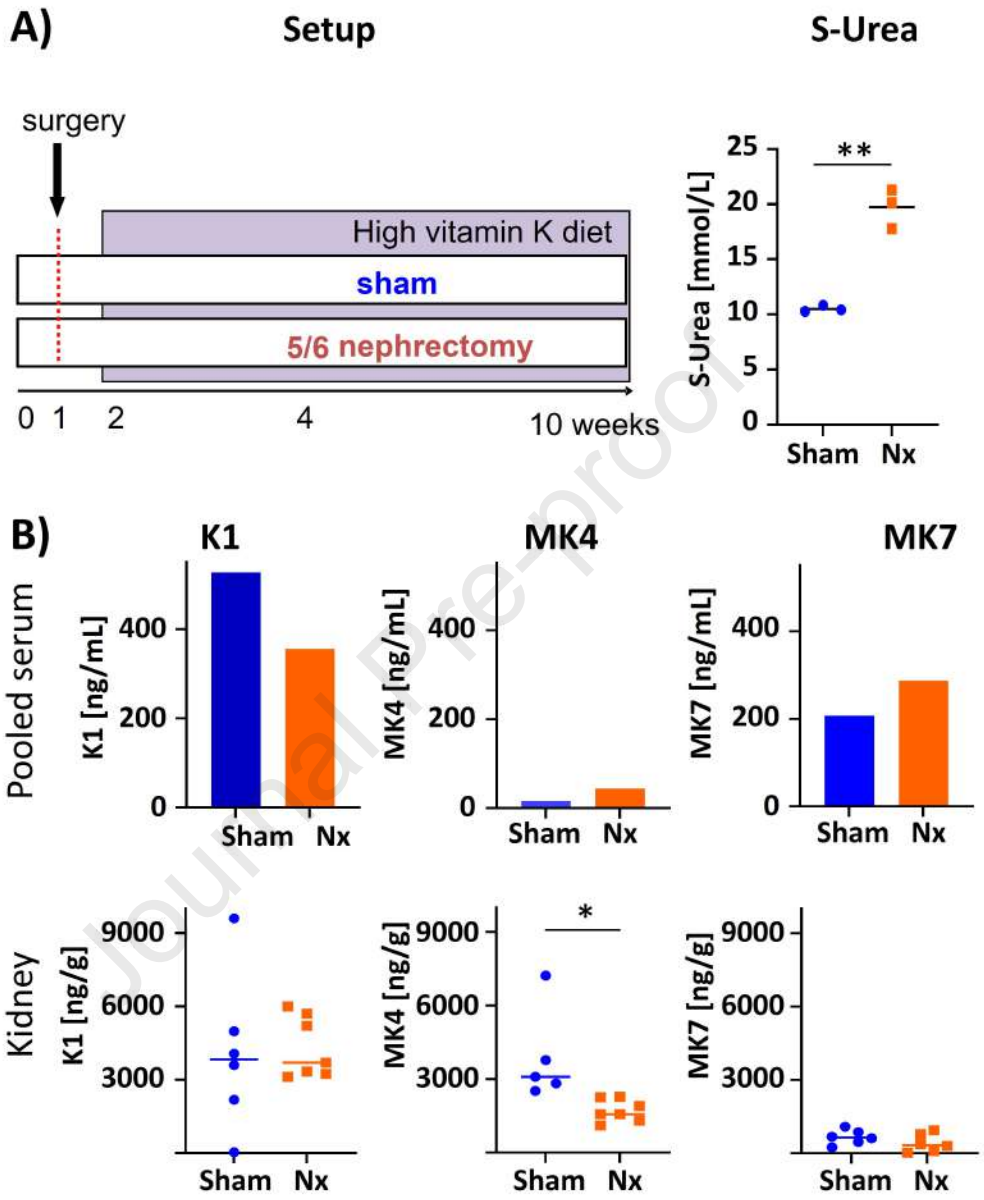


Figure 3



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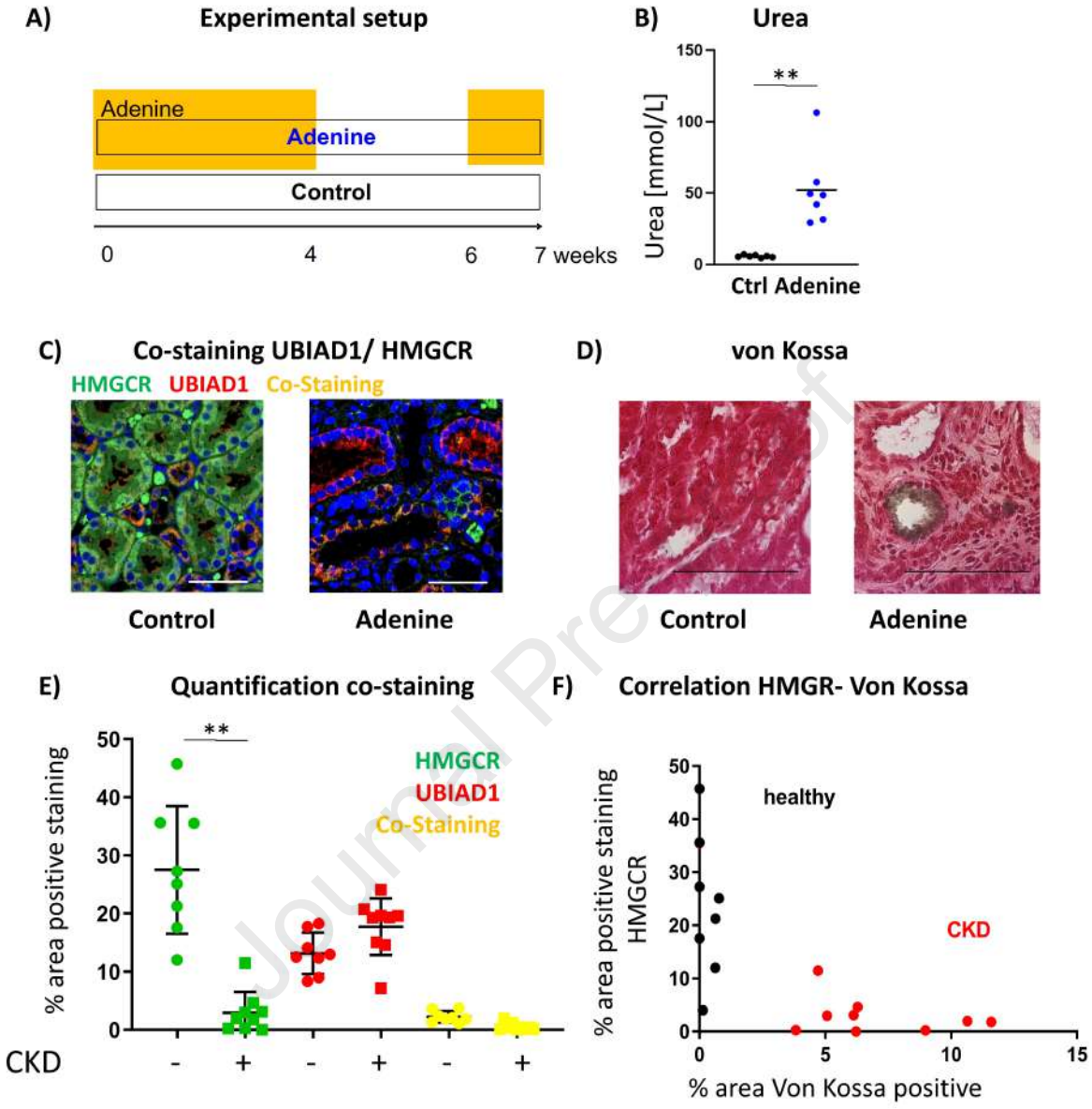


Figure 5

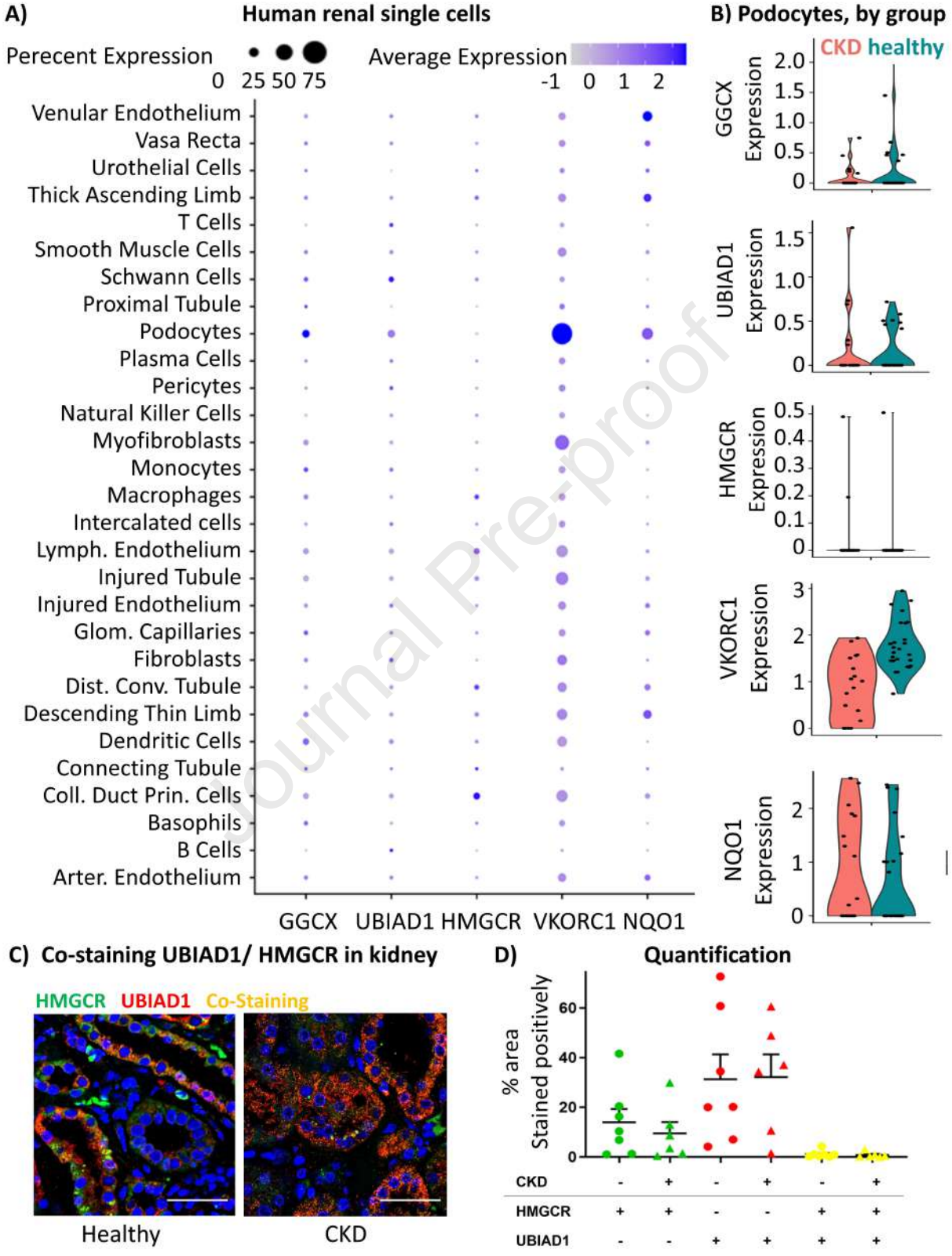
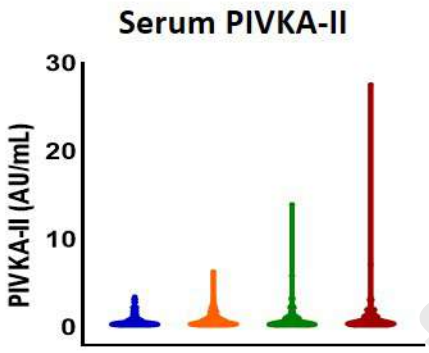


Figure 6



Placebo	+	-	+	-
Atorvastatin	-	+	-	+
PPI	-	-	+	+
n	119	119	104	98
Below detection limit of 0.15 AU/mL	148	142	104	112

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

kidney INTERNATIONAL

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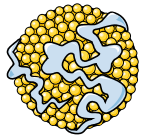
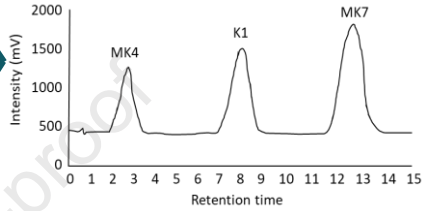
CKD vs non CKD

Vitamin K Uptake
- In vivo
- In vitro



Vit K Supplement Or lipoprotein spiking

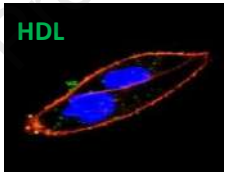
Quantification

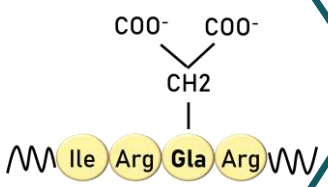


Reduced MK7 incorporation into HDL

Functionality & Cellular Uptake

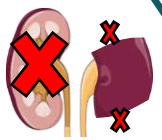
Treatment of VSMC → Carboxylation of MGP





HDL incorporation into cells is reduced; MGP carboxylation is reduced

Vitamin K Uptake & Vitamin K Enzymes



Reduced MK4 and HMGCR in kidneys

HMGCR: HMG-CoA reductase MK: Menaquinone
MGP: matrix gla protein
VSMC: Vascular smooth muscle cell

Kaesler, 2021

CONCLUSION

Altered vitamin K2 (MK7) handling via HDL contributes to uremia associated vitamin K deficiency in man