Nephrology Dialysis Transplantation (2023) 38: 70–79
https://doi.org/10.1093/ndt/gfac187
Advance Access publication date 24 May 2022

ORIGINAL ARTICLE

Uromodulin and its association with urinary metabolites: the German Chronic Kidney Disease Study

Helena Bächle[1], Peggy Sekula[1], Pascal Schlosser[1], Inga Steinbrenner[1], Yurong Cheng[1], Fruzsina Kotsis[1,2], Heike Meiselbach[3], Helena Stockmann[4], Sebastian Schönherr[5], Kai-Uwe Eckardt[3,4], Olivier Devuyst[6], Jürgen Scherberich[7], Anna Köttgen[1] and Ulla T. Schultheiss[1,2]; GCKD investigators

[1]Institute of Genetic Epidemiology, Faculty of Medicine and Medical Center—University of Freiburg, Freiburg, Germany, [2]Department of Medicine IV—Nephrology and Primary Care, Faculty of Medicine and Medical Center—University of Freiburg, Freiburg, Germany, [3]Department of Nephrology and Hypertension, University Hospital Erlangen, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany, [4]Department of Nephrology and Medical Intensive Care, Charité, University-Medicine, Berlin, Germany, [5]Institute of Genetic Epidemiology, Department of Genetics and Pharmacology, Medical University of Innsbruck, Innsbruck, Austria, [6]Institute of Physiology, University of Zurich, Zurich, Switzerland and [7]Klinikum München-Harlaching, Nephrology & Clinical Immunology, Teaching Hospital of the Ludwig-Maximilians-University München, Munich, Germany

Correspondence to: Ulla T. Schultheiss; E-mail: ulla.schultheiss@uniklinik-freiburg.de

ABSTRACT

Background. The progression of chronic kidney disease (CKD), a global public health burden, is accompanied by a declining number of functional nephrons. Estimation of remaining nephron mass may improve assessment of CKD progression. Uromodulin has been suggested as a marker of tubular mass. We aimed to identify metabolites associated with uromodulin concentrations in urine and serum to characterize pathophysiologic alterations of metabolic pathways to generate new hypotheses regarding CKD pathophysiology.

Methods. We measured urinary and serum uromodulin levels (uUMOD, sUMOD) and 607 urinary metabolites and performed cross-sectional analyses within the German Chronic Kidney Disease study (N = 4628), a prospective observational study. Urinary metabolites significantly associated with uUMOD and sUMOD were used to build weighted metabolite scores for urine (uMS) and serum uromodulin (sMS) and evaluated for time to adverse kidney events over 6.5 years.

Results. Metabolites cross-sectionally associated with uromodulin included amino acids of the tryptophan metabolism, lipids and nucleotides. Higher levels of the sMS [hazard ratio (HR) = 0.73 (95% confidence interval 0.64; 0.82), P = 7.45e-07] and sUMOD [HR = 0.74 (95% confidence interval 0.63; 0.87), P = 2.32e-04] were associated with a lower risk of adverse kidney events over time, whereas uUMOD and uMS showed the same direction of association but were not significant.

Conclusions. We identified urinary metabolites associated with urinary and serum uromodulin. The sUMOD and the sMS were associated with lower risk of adverse kidney events among CKD patients. Higher levels of sUMOD and sMS may reflect a higher number of functional nephrons and therefore a reduced risk of adverse kidney outcomes.

Keywords: CKD, metabolites, nephron mass, uromodulin

INTRODUCTION

Chronic kidney disease (CKD) is a major public health problem [1] with a prevalence of ∼10% in the general population [2]. Various etiological risk factors and diseases underlying CKD [3] are known, but its pathophysiology is incompletely understood. Reduced nephron mass induces hyperfiltration of the remaining nephrons and may lead to glomerulosclerosis as a secondary complication [4] with subsequent deterioration of glomerular filtration rate (GFR). A dedicated assessment of nephron mass and/or tubular function is missing in the current definition of CKD. To date, there is no valid method to evaluate nephron mass. The only indirect way is to estimate nephron mass via atrophy and fibrosis assessed semi-quantitatively from kidney biopsies [5]. New methods allowing a quantitative assessment of nephron mass of both kidneys and not only glomerular but also tubular function are warranted.

The kidney-specific protein uromodulin (UMOD) has been suggested as a potential marker for tubular functional mass in population-based studies [6, 7] and could potentially constitute a marker for structural integrity of the distal nephron [8]. Higher UMOD levels in urine or blood were reported to be associated with a lower risk of kidney disease progression and mortality [9–11]. In addition, metabolite profiling is an increasingly used tool to assess global metabolic disturbances in many diseases [12]. Numerous studies examined

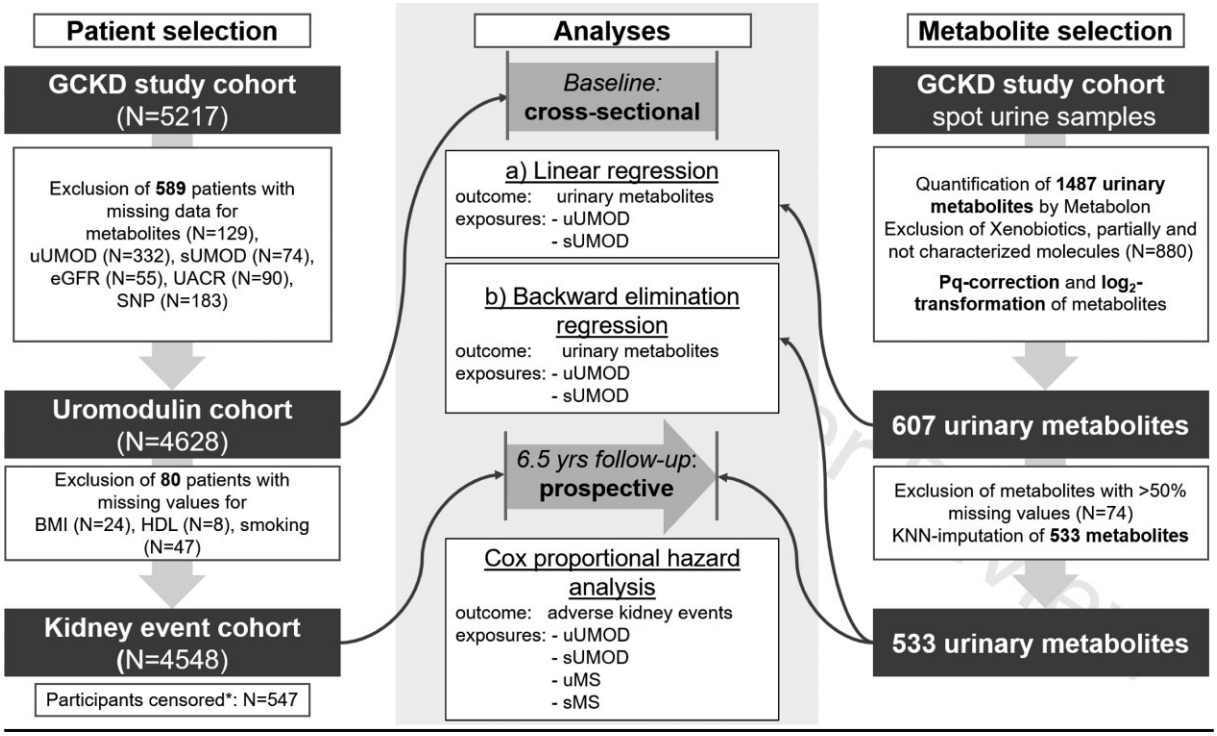


FIGURE 1: Selection of patients and metabolites for all carried out analyses. Workflow figure of metabolite and patient selection. GCKD: German Chronic Kidney Disease study; *N*: number; uUMOD: urinary uromodulin; sUMOD: serum uromodulin; eGFR: estimated glomerular filtration rate; UACR: urine albumin to creatinine ratio; SNP: single nucleotide polymorphism; BMI: body mass index; HDL: high-density lipoprotein cholesterol; yrs: years; uMS: metabolite score for urinary uromodulin; sMS: metabolite score for serum uromodulin; pq: probabilistic quotient; and KNN: k-nearest neighbor. *Censored: participants lost to follow-up or premature study end.

metabolomic alterations in patients with prevalent or new-onset CKD [13, 14], and several metabolites were identified to be associated with GFR and CKD [15] as well as CKD progression [16, 17]. In this hypothesis-generating study, we therefore investigated the association of urinary metabolites with urinary and serum UMOD levels in participants of the German Chronic Kidney Disease (GCKD) study using (i) cross-sectional linear regression and (ii) backward elimination regression analysis. We then generated weighted metabolite scores for urine and serum UMOD to (iii) consecutively study associations of urinary and serum UMOD as well as the metabolite scores with adverse kidney events. The detected associated metabolites may motivate further studies to provide insights into CKD pathophysiology.

MATERIALS AND METHODS

Study population

The GCKD study is an ongoing, multicentric, prospective observational study of 5217 CKD patients of European ancestry. Study procedures and baseline findings have been reported elsewhere [2, 18]. In short, 3132 male and 2085 female patients aged 18 to 74 years were included based on the following criteria: (i) eGFR 30–60 mL/min/1.73 m^2 or (ii) eGFR >60 mL/min/1.73 m^2 and "overt" albuminuria/proteinuria (albumin excretion of >300 mg/g creatinine or protein excretion of >500 mg/g creatinine or corresponding values of 24 h urinary excretion). Patients undergo annual, standardized study visits by trained personnel, including questionnaires and physical examinations, and bio-sampling every other year. We excluded 589 GCKD participants with missing measurements of metabolites, uromodulin, or any covariates from the overall population resulting in a sample size of 4628 for cross-sectional (uromodulin cohort) and 4548 for prospective analyses (kidney event cohort) (Fig. 1). Written informed consent and ethics approval were obtained for all participating institutions. The GCKD study is registered in the national registry for clinical studies (DRKS 00003971).

Data and measurements

Baseline biomarkers were measured in a central certified laboratory using standardized protocols as described before [2]. GFR was estimated using the CKD Epidemiology Collaboration (CKD-EPI) formula [19]. Urine albumin-to-creatinine ratio (UACR) was calculated and categorized according to the KDIGO (Kidney Disease: Improving Global Outcomes) classification into A1 (<30 mg/g), A2 (30–299 mg/g) and A3 (≥300 mg/g) [20]. High-density lipoprotein cholesterol (HDL) was quantified with an enzymatic colorimetric method (CHOD-PAP, MODULAR (P), Roche, Germany).

At baseline, age, sex, body mass index (BMI, corrected for amputations), smoking habits, and the presence of hypertension and diabetes were recorded. Current medication intake was obtained for all GCKD patients and coded using

the ATC (Anatomical Therapeutic Chemical Classification) system.

Spot-urine and serum samples collected during the baseline visit were directly processed and shipped to a central biobank frozen on dry ice, where samples were stored at −80°C. While time of measurement differed for metabolites and UMOD (metabolites: between February 2017 and February 2018; UMOD in urine: January 2017 to May 2017, in serum: February 2018), they were based on aliquots of the same biosamples collected at the baseline visit (March 2010 to March 2012).

Metabolite quantification from baseline spot urine samples was performed by Metabolon, Inc. (Durham, NC, USA) [21] using a non-targeted metabolomics gas and liquid chromatography coupled to mass spectrometry approach (HD4). Metabolite levels were $\log_2$-transformed prior to analyses. To develop a metabolite panel associated with UMOD, we limited our analyses to 607 endogenous metabolites, excluding partially or not characterized molecules and xenobiotics (Supplementary Table S1, Fig. 1), but did not restrict analyses to certain metabolic pathways in this untargeted metabolomics approach. The number of missing values for the metabolites ranged from 0 to 4576 (median: 47).

To account for urine dilution, metabolites were normalized using a probabilistic quotient (pq) normalization method, a robust median-based normalization approach suitable for the GCKD study samples [22]. All urinary creatinine values were within a plausible range and no samples had to be discarded.

K-nearest neighbor (KNN) imputation [23] was used to infer missing values for all metabolites with less than 50% missing observations to deal with incomplete values in multivariable analyses, resulting in a final number of 533 imputed metabolites (Fig. 1).

Urinary UMOD (uUMOD) concentrations were measured using an ELISA (Meridian Life Science, USA) as described previously [24]. Serum UMOD (sUMOD) concentrations of 5143 blood samples were obtained with a certified sensitive ELISA (EuroImmun) as reported by Scherberich *et al.* [8]. Both uUMOD and sUMOD were log-transformed, and uUMOD was pq-normalized [25] to account for urine dilution.

The adjudication of adverse events is continuously performed within the GCKD study based on hospital discharge letters, nephrologist outpatient letters and death certificates using a standardized catalogue. For this project, adjudicated events over a median follow-up of 6.5 years from baseline were available. Adverse kidney events included kidney failure requiring kidney-replacement therapy (KRT, i.e. dialysis or kidney transplantation) and death due to discontinuation of KRT. The time to event was calculated from study entry to first adverse kidney event [26]. Death due to other causes was treated as a competing event. Participants of the GCKD study were censored at the time of the first event, after 6.5 years of follow-up, or date of last contact when lost to follow-up.

Data were collected using Askimed, a Web-based eCRF (electronic case report form) system for data collection and management (https://www.askimed.com/).

Statistical analyses

Cross-sectional linear regression analysis. The correlation of uUMOD and sUMOD was assessed using Spearman's correlation coefficient. Linear regression models were fitted to evaluate the association of the two outcomes uUMOD and sUMOD with levels of each of the 607 unimputed urinary metabolites. Age, sex, eGFR, log-transformed UACR [log(UACR)], intake of loop diuretics (ATC code C03C) and genotype at single nucleotide polymorphism (SNP) rs77924615 at the *UMOD* locus [27] were included as adjustment variables based on biological plausibility and an extended literature search. Rs77924615 was genotyped as part of the Illumina HumanOmni2.5–8 v1.2 BeadChip [22].

To account for multiple testing, we applied a Bonferroni correction dividing the type I error level of 0.05 by the number of evaluated metabolites [(0.05/607) = 8.24e-05]. In sensitivity analyses, we restricted the uromodulin cohort to patients with UACR values <30 mg/g (KDIGO category A1) to assess whether high amounts of urinary protein altered the associations between uromodulin and metabolites. Linear regression analyses were repeated with KNN-imputed metabolites as sensitivity analysis.

Backward elimination regression and metabolite score generation. For both outcomes (uUMOD, sUMOD), backward elimination regression (selection criterion, $P = .05$) was performed on all 533 KNN-imputed metabolites, with adjustment covariates [age, sex, eGFR, log(UACR), loop diuretics intake and rs77924615] forced into the model. All metabolites remaining in the regression model after backward elimination were used to build the metabolite scores for uUMOD (uMS) and sUMOD (sMS) by calculating the model-based linear predictor for each individual, i.e. the sum over the individual metabolite levels multiplied by respective effect estimates. Prior to subsequent analyses, scores were normalized (mean = 0, SD = 1).

Prospective analysis. Cox proportional-hazard regression models were fitted for the time-to-adverse kidney event analysis, separately for the uMS, sMS, uUMOD and sUMOD as independent variables. Estimated risks are expressed as cause-specific hazard ratios (HRs), because death of other causes is a competing event [28]. The significance threshold was Bonferroni-corrected for four exposures [(0.05/4) = 1.25e-02]. Subdistribution hazard analyses were carried out for the adverse kidney event outcome on all four exposures to assess potential indirect effects of exposures on the outcome [29]. Effect estimations were adjusted for age, sex, eGFR, log(UACR), log(HDL), loop or thiazide diuretic intake, renin–angiotensin system (RAS) blocker intake, prevalent diabetes, smoking and log(BMI). Exclusion of patients ($N = 80$) with missing values for adjustment variables and missing follow-up time resulted in 4548 patients of the kidney event cohort for prospective analyses (Fig. 1). In sensitivity analyses, time to adverse kidney events additionally including acute kidney injury (AKI; applying the AKI network criteria) [30] was examined. The proportional-hazard assumption was visually inspected for every model by plotting Schoenfeld residuals. No major violations were identified (data not shown). Cumulative

Table 1. Study sample characteristics of the GCKD study cohort and the uromodulin cohort

	GCKD study cohort ($N = 5217$)	Uromodulin cohort ($N = 4628$)
Age, years, mean (±SD)	60.1 (±12.0)	60.0 (±12.0)
Sex, female, *N* (%)	2085 (40.0)	1821 (39.3)
eGFR[a], mL/min/1.73 m²	46.4 (37.1, 57.4)	46.5 (37.2, 57.5)
UACR[a], mg/g	50.9 (9.7, 391.7)	50.1 (9.4, 384.2)
UACR categories		
A1 (<30 mg/g), *N* (%)	2188 (41.9)	1991 (43.0)
A2 (30–299 mg/g), *N* (%)	1495 (28.7)	1339 (28.9)
A3 (≥300 mg/g), *N* (%)	1534 (29.4)	1298 (28.0)
uUMOD[a], µg/mL	7.0 (4.1, 11.4)	6.7 (4.0, 11.4)
sUMOD[a], ng/mL	83.9 (55.7, 125.7)	84.5 (55.8, 126.7)
Diuretics intake, *N* (%)	3184 (61.0)	2810 (60.7)
Loop diuretics, *N* (%)	2014 (38.6)	1772 (38.3)
Thiazide diuretics, *N* (%)	1386 (26.6)	1233 (26.6)
BMI[a], kg/m²	28.9 (25.7, 33.2)	28.8 (25.7, 33.1)
Diabetes, *N* (%)	1868 (35.8)	1610 (34.8)
Hypertension, *N* (%)	5021 (96.3)	4450 (96.2)
RAS blocker intake, *N* (%)	3802 (72.9)	3374 (72.9)
HDL cholesterol[a], mg/dL	48.4 (39.3, 61.4)	48.4 (39.3, 61.4)
Smoking, current[a], *N* (%)	828 (15.9)	739 (16.0)
rs77924615[a]		
GG, *N* (%)	3326 (66.0)	3132 (67.7)
GA, *N* (%)	1424 (28.2)	1342 (29.0)
AA, *N* (%)	338 (6.7)	154 (3.3)

GCKD: German Chronic Kidney Disease; uUMOD: urinary uromodulin (pq-corrected); sUMOD: serum uromodulin; *N*: number of observations; SD: standard deviation; UACR: urinary albumin to creatinine ratio; and RAS: renin–angiotensin system.

[a]Incomplete variables; see Fig. 1 for number of missing values in the GCKD study cohort. Continuous variables are reported as p50 (p25, p75), unless otherwise noted. UACR categories (A1–A3) are based on the KDIGO classification [20].

incidence function (CIF, parametric) of adverse kidney events overall and stratified by quartiles of the sMS, uMS, sUMOD and uUMOD were plotted for illustration.

All analyses were performed using Stata SE 15.1 (StataCorp, 2017, *Stata Statistical Software: Release 15*; StataCorp LLC, College Station, TX, USA) and *R* version 3.6.1.

RESULTS

Study population and baseline characteristics

Table 1 displays the summary of the baseline characteristics of the 4628 individuals in the uromodulin cohort and of all GCKD participants ($N = 5217$). Compared with the full GCKD study cohort, patient characteristics in the uromodulin cohort were similar. The mean age in the uromodulin cohort was 60 years, 39.3% were female. The median eGFR was 46.5 mL/min/1.73 m², the median UACR was 50.1 mg/g creatinine. In most of the participants (60.7%), intake of diuretics was found. Diabetes occurred in 34.8% of participants, current smoking in 16%. Both sUMOD and uUMOD showed near-normal distributions after log-transformation (Fig. 2) and were moderately correlated with each other ($\rho = 0.31$, Supplementary data, Fig. S1).

Cross-sectional association analyses

Metabolites significantly associated with uUMOD or sUMOD. Log-transformed uUMOD levels were significantly associated with levels of 149 metabolites as measured from urine after correction for multiple testing ($P < 8.24e\text{-}05$, Supplementary data, Table S2). Indoleacetate, an intermediate of the tryptophan metabolism, showed the lowest *P*-value {$\beta = 0.15$ [95% confidence interval (CI) 0.13; 0.16], $P = 6.28e\text{-}78$, $r^2 = 0.16$} (Table 2). Another strongly associated metabolite was the dicarboxylic fatty acid 3-carboxy-4-methyl-5-pentyl-2-furanpropionate (3-CMPFP). Cysteinylglycine disulfide, 5-methylthioadenosine and α-ketoglutaramate were amino acid metabolites of different sub-pathways belonging to the top five associations by *P*-value. The first two were among the metabolites with the largest effect estimates, in addition to dihydroxy-5-methylthio-4-pentenoate (DMTPA) and urate. The largest effect estimate was observed for hydroxyasparagine [$\beta = -0.29$ (95% CI −0.37; −0.22), $P = 3.07e\text{-}14$, $r^2 = 0.10$].

Significant associations with sUMOD were found for 235 metabolites measured from urine (Supplementary data, Table S2). The metabolite with the lowest *P*-value was N-acetylaspartate (NAA) [$\beta = 0.29$ (95% CI 0.26; 0.32), $P = 9.68e\text{-}68$, $r^2 = 0.42$] (Table 3). Other metabolites strongly associated with sUMOD were *C*-glycosyltryptophan, citrate, 3-(3-amino-3-carboxypropyl)uridine and 2-aminoadipate, with the same proportion of variance in sUMOD explained by those metabolites ($r^2 = 0.41$). Interestingly, the largest effect estimate for sUMOD, as well as for uUMOD, was detected for hydroxyasparagine [$\beta = -0.39$ (95% CI −0.44; −0.34), $P = 3.27e\text{-}54$, $r^2 = 0.41$] followed by *C*-glycosyltryptophan, 3-(3-amino-3-carboxypropyl)uridine, pseudouridine, and *N*-acetylneuraminate.

Cross-sectional regression analyses of uUMOD and sUMOD on each of the KNN-imputed metabolites did not show major differences compared with the unimputed metabolite levels (data not shown).

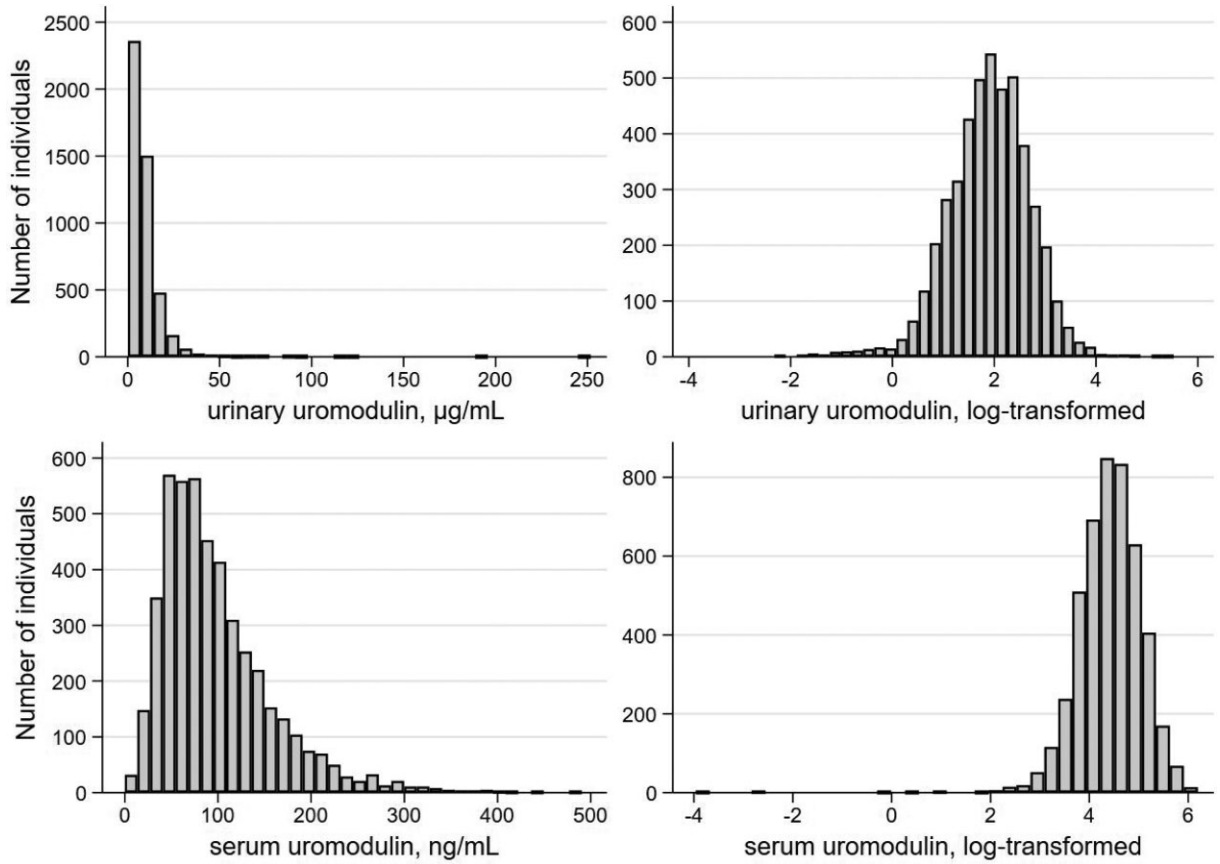


FIGURE 2: Distribution of urinary and serum uromodulin. Distribution of urinary uromodulin (pq-corrected) and serum uromodulin among 4628 persons in the uromodulin cohort.

Table 2. The five metabolites with the strongest associations with urinary uromodulin according to *P*-value (a) and effect size (b)

	Super-pathway	Sub-pathway	Effect on log(uUMOD)	SE	*P*-value	95% CI	r^2 adj.	*N*
(a) *P*-value-based selected associations:								
Indoleacetate	Amino acid	Tryptophan metabolism	0.15	0.01	6.28e-78	(0.13; 0.16)	0.16	4614
3-CMPFP**	Lipid	Fatty acid, dicarboxylate	0.15	0.01	4.16e-61	(0.13; 0.16)	0.14	4537
Cysteinylglycine disulfide*	Amino acid	Glutathione metabolism	−0.27	0.02	3.20e-46	(−0.31; −0.23)	0.13	4625
α-Ketoglutaramate*	Amino acid	Glutamate metabolism	0.19	0.01	2.11e-44	(0.16; 0.21)	0.13	4593
5-Methylthioadenosine	Amino acid	Polyamine metabolism	0.25	0.02	1.73e-41	(0.22; 0.29)	0.12	4627
(b) Effect-size-based selected associations:								
Hydroxyasparagine**	Amino acid	Alanine and aspartate metabolism	−0.29	0.04	3.07e-14	(−0.37; −0.22)	0.10	4628
Cysteinylglycine disulfide*	Amino acid	Glutathione metabolism	−0.27	0.02	3.20e-46	(−0.31; −0.23)	0.13	4625
5-Methylthioadenosine	Amino acid	Polyamine metabolism	0.25	0.02	1.73e-41	(0.22; 0.29)	0.12	4627
DMTPA	Amino acid	Methionine, cysteine, SAM and taurine metabolism	0.25	0.03	8.03e-22	(0.20; 0.30)	0.11	4627
Urate	Nucleotide	Purine metabolism	0.24	0.02	3.41e-30	(0.20; 0.29)	0.11	4628

SE: standard error; CI: confidence interval; *N*: number of observations; uUMOD: urinary uromodulin; r^2 adj.: adjusted r^2; 3-CMPFP**: 3-carboxy-4-methyl-5-pentyl-2-furanpropionate; DMTPA: dihydroxy-5-methylthio-4-pentenoate; and SAM: *S*-adenosyl methionine.

*The standard for this metabolite has not been run, but Metabolon, Inc., is highly confident in its identity.

**The standard for this metabolite has not been run, and Metabolon, Inc., is confident in its identity (but not as confident as with a *ID).

Linear regression model with log(uUMOD) as dependent variable fitted for each metabolite separately, adjusted for age, sex, eGFR, log(UACR), loop diuretics, and rs77924615. Metabolites were $\log_2$-transformed.

Table 3. The five metabolites with the strongest associations with serum uromodulin according to *P*-value (a) and effect size (b)

	Super-pathway	Sub-pathway	Effect on log(sUMOD)	SE	*P*-value	95% CI	r^2 adj.	*N*
(a) *P*-value based selected associations:								
N-acetylaspartate	Amino acid	Alanine and aspartate metabolism	0.29	0.02	9.68e-68	(0.26; 0.32)	0.42	4628
C-glycosyltryptophan	Amino acid	Tryptophan metabolism	−0.39	0.02	1.73e-62	(−0.43; −0.34)	0.41	4628
Citrate	Energy	TCA cycle	0.14	0.01	1.17e-61	(0.13; 0.16)	0.41	4628
3-(3-Amino-3-carboxypropyl)uridine*	Nucleotide	Pyrimidine metabolism	−0.36	0.02	5.98e-58	(−0.41; −0.32)	0.41	4628
2-Aminoadipate	Amino acid	Lysine metabolism	0.11	0.01	1.68e-57	(0.10; 0.12)	0.41	4451
(b) Effect-size based selected associations:								
Hydroxyasparagine**	Amino acid	Alanine and aspartate metabolism	−0.39	0.02	3.27e-54	(−0.44; −0.34)	0.41	4628
C-glycosyltryptophan	Amino acid	Tryptophan metabolism	−0.39	0.02	1.73e-62	(−0.43; −0.34)	0.41	4628
3-(3-Amino-3-carboxypropyl)uridine*	Nucleotide	Pyrimidine metabolism	−0.36	0.02	5.98e-58	(−0.41; −0.32)	0.41	4628
Pseudouridine	Nucleotide	Pyrimidine metabolism	−0.31	0.02	5.22e-37	(−0.36; −0.26)	0.40	4628
N-acetylneuraminate	Carbohydrate	Amino sugar metabolism	−0.29	0.02	1.06e-44	(−0.33; −0.25)	0.40	4628

SE: standard error; CI: confidence interval; *N*: number of observations; sUMOD: serum uromodulin; and r^2 adj.: adjusted r^2.
*The standard for this metabolite has not been run, but Metabolon, Inc., is highly confident in its identity.
**The standard for this metabolite has not been run, and Metabolon, Inc., is confident in its identity (but not as confident as with a *ID). Linear regression model with log(sUMOD) as dependent variable fitted for each metabolite separately, adjusted for age, sex, eGFR, log(UACR), loop diuretics and rs77924615. Metabolites were $\log_2$-transformed.

Metabolite score composition

Using backward elimination, 104 and 102 of the 533 imputed metabolites remained in the regression model of uUMOD and sUMOD, respectively (Supplementary data, Table S3). The metabolites mostly belonged to the amino and fatty acid or nucleotide metabolism. Comparing the two panels of selected metabolites, 28 were associated with both uUMOD and sUMOD, e.g. *N*-acetylneuraminate, argininosuccinate, *N*1-methylguanosine, 1-methylguanidine and pyroglutamine. The metabolite scores were computed based on the selected metabolites and displayed an approximate normal distribution after standardization (Supplementary Fig. S2). The variance of the dependent variables (r^2), i.e. uUMOD or sUMOD, was explained with 36% and 58% by the uMS and sMS, respectively.

Prospective association analyses

Over a median follow-up of 6.5 years (range 0–6.5 years), 431 kidney events (KRT: 420, death due to discontinuation of KRT: 11) occurred. In cause-specific hazard regression analyses, neither uMS [HR = 0.98 (95% CI 0.88; 1.09), *P* = 7.33e-01] nor uUMOD [HR = 0.94 (95% CI 0.82; 1.07), *P* = 3.37e-01] showed a significant association with adverse kidney events (Table 4). In contrast, a higher sMS was significantly associated with a lower risk of adverse kidney events [HR = 0.73, per SD increase (95% CI 0.64; 0.82), *P* = 7.45e-07], as was sUMOD [HR = 0.74 (95% CI 0.63; 0.87), *P* = 2.32e-04, Table 4]. Figure 3 shows that the CIF of adverse kidney events was highest for persons in the lowest quartile (Q1) of the sMS quartiles, compared with Q4. Supplementary Fig. S3 displays the CIFs for quartiles of the uMS, sUMOD and uUMOD. The sub-distribution HRs of all exposures were similar in direction and magnitude to the cause-specific HRs, indicating absence of indirect effects on the outcome (Table 4). Sensitivity analysis examining cause-specific hazards of adverse kidney events including AKI (number of events: AKI: 357, KRT: 309, death due to discontinuation of KRT: 4, in total: 670) for all exposures were similar in direction and magnitude (Supplementary Table S4).

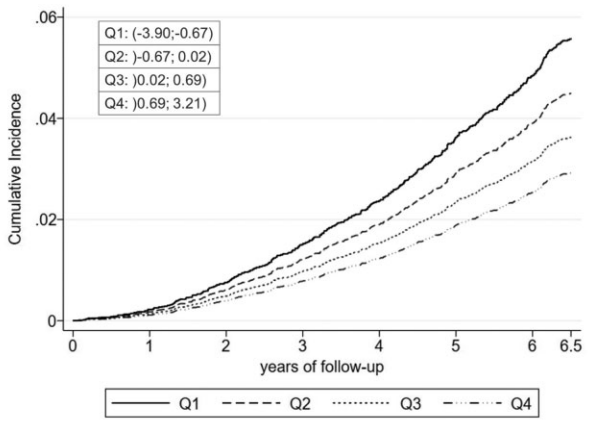
Table 4. Results of cause-specific and sub-distribution hazard regression analyses for adverse kidney events

	CSHR	95% CI	*P*-value	SDHR	95% CI	*P*-value
uMS	0.98	(0.88; 1.09)	7.33e-01	0.98	(0.88; 1.10)	7.78e-01
sMS	0.73	(0.64; 0.82)	7.45e-07	0.76	(0.66; 0.87)	7.88e-05
uUMOD	0.94	(0.82; 1.07)	3.37e-01	0.95	(0.83; 1.09)	4.63e-01
sUMOD	0.74	(0.63; 0.87)	2.32e-04	0.74	(0.64; 0.86)	1.24e-04

CSHR: cause-specific hazard ratio; CI: confidence interval; SDHR: sub-distribution hazard ratio; uUMOD: urinary uromodulin; uMS: metabolite score for uUMOD; sUMOD: serum uromodulin; and sMS: metabolite score for sUMOD.
Adjusted for eGFR, log(UACR), log(HDL), loop and thiazide diuretic intake, renin–angiotensin system blocker intake, diabetes, smoking and BMI. Number of patients = 4548. Number of events, total = 431, kidney-replacement therapy (KRT) = 420, death due to discontinuation of KRT = 11. Bonferroni-corrected significance threshold: 1.25e-02.

DISCUSSION

In the present study, we established associations between urinary metabolite profiles and urinary and serum uromodulin levels among 4628 persons with CKD. To our knowledge, this is the first metabolomic approach to identify urinary metabolites associated with a surrogate of tubular mass. Uromodulin is a Glycosylphosphatidylinositol-anchored, kidney-specific protein, which forms polymers in urine and is found as a monomer in much lower concentrations in blood. The basolateral excretion mechanisms and its function in blood



Number at risk

year	1	2	3	4	5	6	>6
Q1	1141	1089	1008	930	840	756	660
Q2	1141	1110	1063	1008	957	889	823
Q3	1141	1120	1090	1063	1026	975	913
Q4	1140	1129	1101	1081	1050	1014	970

FIGURE 3: Multivariable adjusted cumulative incidence function (CIF) for adverse kidney events, stratified by sMS quartiles. CIF for adverse kidney events. Adjusted for age, sex, eGFR, log(UACR), log(HDL), loop and thiazide diuretic intake, renin–angiotensin system blocker intake, diabetes, smoking and log(BMI). sMS: metabolite score for serum uromodulin and *Q1–Q4*: quartiles of sMS.

are still incompletely understood, despite uromodulin being the most abundant protein in urine. The pathophysiological mechanisms explaining levels of sUMOD and uUMOD are most likely different [7], further motivating this study to possibly generate hypotheses for both aspects by differing association patterns between metabolites and uUMOD and/or sUMOD. However, no causal inference can be drawn from the association of metabolites with uromodulin found in the cross-sectional analyses. The associated metabolites may represent a readout of the cell activity in Henle's loop, where uromodulin is uniquely synthesized. These metabolites and pathways might provide insights into cell activity and progression to kidney failure. The associations found in this study can therefore build a basis for further targeted experimental evaluation of implicated metabolites and their specific relationship to nephron mass and function.

Of all 359 metabolites associated with uUMOD and/or sUMOD, 109 were already known uremic solutes [31]. As it would be beyond the scope of this article to discuss all associated metabolites in detail, only those five metabolites with the strongest associations to uUMOD (Table 2) and sUMOD (Table 3) are discussed in the following paragraphs. In order to not introduce any bias to downstream analyses, metabolites were pq-normalized, which has been reported as a robust method of normalization [32].

Most of the metabolites associated with uromodulin belonged to pathways of amino or fatty acids and nucleotides. Many of them or their respective pathways have been described before in the context of kidney disease, e.g. intermediates of the tricarboxylic acid (TCA) cycle and tryptophan metabolism, as well as metabolites of nucleosides [14]. Several metabolites of the pyrimidine and purine metabolism are known uremic solutes and associated with CKD progression [15, 16]. The derivatives of nucleosides 3-(3-amino-3-carboxypropyl)uridine, also called nucleoside X, pseudouridine, and urate were strongly associated with sUMOD and uUMOD respectively. The excretion of urate is regulated by several transporters, e.g. ABCG2 [33], MRP4, SLC22A6 and SLC22A8, which enable tubular secretion of many other endogenous metabolites and uremic solutes and might therefore be pathophysiologically relevant in CKD [34]. Notably, *N*-acetylaspartate was suggested as a substrate for SLC22A6 [35] and was, like *N*-acetylneuraminate, an acetyl derivative of the amino sugar neuraminic acid, strongly associated with sUMOD. *N*-acetylneuraminate was detected as uremic solute and previously suggested as a predictive marker of kidney deterioration in CKD patients [31, 36].

Previous studies found amino acid profiles in plasma and urine, particularly those of the tryptophan metabolism, significantly altered in CKD [37]. Two of them, indoleacetate and *C*-glycosyltryptophan [31], were strongly associated with uUMOD or sUMOD in the present study and described as uremic toxins before. *C*-glycosyltryptophan was previously reported to be associated with CKD progression [15]. Cheng *et al.* recently suggested a causal relationship between lower eGFR and higher plasma levels of *C*-glycosyltryptophan and indoxyl sulfate [37], a uremic toxin associated with overall and cardiovascular mortality in CKD patients. It down-regulates the kidney-specific SLCO4C1 [38] and competes for transport capacity with other uremic toxins like CMPF due to a shared basolateral SLC22A8-mediated transport [39]. 3-CMPFP, a metabolite of furan fatty acids like CMPF, was strongly associated with uUMOD. Intracellularly accumulated CMPF was shown to induce cell damage by enhancing the production of reactive oxygen species (ROS) in proximal tubular cells *in vitro*, possibly contributing to cell damage and the progression of CKD [40].

As the processing of uromodulin itself causes oxidative stress, antioxidative mechanisms may be relevant for correct processing and its excretion [7]. One of the most important intrinsic antioxidants to eliminate ROS is glutathione [41]. A breakdown product of this, cysteinylglycine disulfide [42], displayed a strong negative association with uUMOD, indicating a potential relation between the metabolic reaction to oxidative stress in CKD and uromodulin levels and thus tubular function. Uromodulin was previously suggested to modify the response to oxidative stress in an anti-inflammatory manner [43, 44]. Since oxidative stress is known to be associated with CKD progression [41], this could partly explain the association of lower risk of adverse kidney events with higher levels of metabolites associated with sUMOD in our study. Antioxidant properties were further attributed to hydroxyasparagine, better known as L-aspartic acid β-hydroxamate [45], which exhibited strong association with both uUMOD and sUMOD in the present study. The 2-aminoadipate (L-α-aminoadipic acid), deriving from lysine degradation, was suggested as a marker of oxidative stress [46] and was associated with sUMOD. However, these metabolites were primarily discussed in the field of neurology [47] and

their association with CKD and tubular function needs further investigation.

Urinary uromodulin was strongly associated with 5-methylthioadenosine and DMTPA, intermediates of the methionine salvage pathway. Methionine and its related metabolites were reported to influence antioxidant mechanisms like glutathione synthesis to prevent oxidative stress [48]. The 5-methylthioadenosine, among other metabolites of this sub-pathway, was described as a uremic solute [31].

Citrate and α-ketoglutaramate (a precursor of α-ketoglutarate, the key molecule of the TCA cycle), are both involved in mitochondrial energy production and were strongly associated with sUMOD and uUMOD, respectively. Citrate is freely filtered by the glomerulus and increasingly reabsorbed by proximal tubular cells in metabolic acidosis [49]. Decreased urinary citrate excretion may therefore reflect tubular dysfunction due to disturbances in acid–base homeostasis. In this study, urinary citrate was positively associated with sUMOD. It needs further examination is required to determine whether altered urinary concentrations of citrate or α-ketoglutaramate may reflect impaired mitochondrial energy production in dysfunctional tubular cells due to CKD.

Both sUMOD and the metabolite score based on metabolites associated with sUMOD (sMS) were significantly associated with adverse kidney events, with adjustment for prognostic factors including eGFR. In accordance with our results, it was previously shown that uUMOD and sUMOD were only moderately correlated with each other [43]. In this study, only lower sMS and sUMOD levels were significantly associated with a higher risk of adverse kidney events, and therefore might reflect decreased kidney function accompanied by impaired tubular function better than uUMOD and uMS. The detection of metabolites associated with UMOD might reveal molecular mechanisms of metabolic alterations in CKD. In the past, opposite effect directions were observed in genetic and prospective studies of UMOD; since UMOD is on one hand only produced in kidney tubular cells, uUMOD levels positively correlate with eGFR, tubular function, kidney length and volume, and uUMOD is considered a proxy of nephron mass. This is supported by findings where higher urinary uromodulin levels are associated with lower risk of kidney function decline in prospective at-risk cohorts [9]. On the other hand, common variants in the UMOD locus are associated with lower kidney function and risk of CKD [27], where the risk allele is the common allele with high allele frequencies [50]. Overall, the observational effects of uromodulin on endpoints and the *UMOD* genetics mediated effects on endpoints are therefore of opposite direction. However, the SNP effects on serum and urine uromodulin are the same, with the common risk allele at the UMOD locus associated with higher sUMOD levels [51]. Similarly, the effects of sUMOD and uUMOD go into the same direction, higher levels are associated with less CKD progression [7, 8, 43]. These observations motivated our study of both serum and urine uromodulin with metabolites under the same assumptions.

Our study's strengths include a large sample size, a long observation time, the measurement of uromodulin in both serum and urine, and the non-targeted metabolomic approach via MS-based quantification with a broad spectrum of metabolic pathways. This untargeted approach maximizes the hypothesis-generating potential of the study, including the study of metabolites that have not been examined in the context of CKD in metabolomics studies so far. While experimental generation of mechanistic insights is beyond the scope of this study, the detected associations motivate their further study. Some limitations deserve mention: first, baseline 24 h urine collections were not performed within the GCKD study, so that urinary sodium excretion could not be included as adjustment variable for uUMOD concentrations. Second, individual causes of CKD could not be evaluated separately because of small sample sizes. Third, despite the large study sample, the number of patients with adverse kidney events was still moderate, and our observations need to be extended to larger studies in the future. Fourth, kidney size was not evaluated within the study and could therefore not be used for validation of results. Finally, metabolite measurements were only conducted at baseline, which precludes longitudinal investigations of metabolic changes.

CONCLUSION

We identified urinary metabolites associated with urinary and serum uromodulin. Our findings suggest that lower levels of specific metabolites associated with sUMOD and of sUMOD itself may identify individuals at higher risk for adverse kidney events. Further investigation is needed to evaluate the application of these metabolites as potential biomarkers of tubular mass and CKD progression.

SUPPLEMENTARY DATA

Supplementary data are available at *ndt* online.

ACKNOWLEDGEMENTS

We are very grateful for the willingness and time of all study participants of the GCKD study. The enormous effort of the study personnel at the regional centers is highly appreciated. We also thank the large number of nephrologists for their support of the GCKD study (http://www.gckd.org). Current GCKD investigators and collaborators are listed in Supplementary Table S5.

FUNDING

The work of F.K. was supported by a scholarship from the Else Kroener Fresenius Foundation (NAKSYS). The work of U.T.S. and F.K. was supported by the German Ministry of Education and Research (BMBF) within the framework of the e:Med research and funding concept (grant 01ZX1912B). The work of P. Schlosser was supported by the EQUIP Program for Medical Scientists, Faculty of Medicine, University of Freiburg. The GCKD study was supported by the German Ministry of Education and Research (BMBF, FKZ 01ER 0804, 01ER 0818, 01ER 0819, 01ER 0820, and 01ER 0821) and the KfH Foundation for Preventive Medicine (Kuratorium

für Heimdialyse und Nierentransplantation e.V.—Stiftung Präventivmedizin) and corporate sponsors (www.gckd.org). O.D. is supported by the NCCR Kidney.CH program (Swiss National Science Foundation) and the Swiss National Science Foundation 310030_189044.

AUTHORS' CONTRIBUTIONS

The individual contributions of each co-author in the manuscript are: study conception: U.T.S., P.Sekula and A.K.; study design: U.T.S., P.Sekula and P.Schlosser; data acquisition: U.T.S., F.K., I.S., K.-U.E., H.M., A.K., J.S., O.D., P.Schlosser, S.S. and Y.C.; data analysis: H.B., U.T.S., P.Sekula, P.Schlosser and I.S.; data interpretation: H.B., U.T.S., P.Sekula and A.K.; critical intellectual input: U.T.S., P.Sekula, P.Schlosser, F.K., A.K., O.D. and J.S.; drafting the article: H.B., U.T.S., F.K., A.K., P.Sekula and P.Schlosser; and critical revision of article: U.T.S., P.Sekula, P.Schlosser, F.K., H.S., S.S., Y.C., O.D., K.-U.E., J.S. and A.K.

CONFLICT OF INTEREST STATEMENT

The authors have nothing to declare. The results presented in this paper have not been published previously in whole or part, except in abstract format.

DATA AVAILABILITY STATEMENT

Public posting of individual level participant data is not covered by the informed patient consent form. As stated in the patient consent form and approved by the ethics committees, a dataset containing pseudonyms can be obtained by collaborating scientists upon approval of a scientific project proposal by the steering committee of the GCKD study: www.gckd.org.

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Received: 6.10.2021; Editorial decision: 16.5.2022