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Urinary neutrophil gelatinase-associated lipocalin may aid prediction of renal decline in patients with non-proteinuric Stages 3 and 4 chronic kidney disease (CKD)

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ORIGINAL ARTICLE

ABSTRACT

Background. Chronic kidney disease (CKD) is an increasing public health issue. It is therefore potentially highly advantageous to identify patients at risk of accelerated renal progression and death. Neutrophil gelatinase-associated lipocalin (NGAL) is an established urinary biomarker for acute kidney injury, but it is not known whether adding urinary NGAL (uNGAL) measurements to conventional risk factors will improve risk assessment in the setting of chronic disease.

Methods. This is a prospective observational cohort study of 158 patients with Stage 3 or 4 CKD. The ability of baseline uNGAL to improve prediction of outcome was assessed by multivariate modelling and a number of metrics including net reclassification analysis. A primary composite endpoint of all-cause mortality or progression to end-stage renal disease (ESRD) requiring renal replacement therapy (RRT) at 2 years and a secondary endpoint of ≥5 mL/min/1.73 m^2 decline in the estimated glomerular filtration rate (eGFR) after 1 year were considered.

Results. Forty patients (25%) reached the primary composite endpoint, 20 of whom died. Twenty-seven patients (19%) reached the secondary endpoint of a ≥5 mL/min/1.73 m^2 decline in the eGFR. The baseline uNGAL-to-creatinine ratio (uNCR) was associated with the combined endpoint of death or initiation of RRT (HR per 5 μg/mmol increase 1.27, 95% CI: 1.01–1.60, P = 0.036) independent of conventional cardiovascular and renal risk factors, including proteinuria. In separate analysis of these two competing endpoints, however, uNCR only remained associated with increased risk of progression to ESRD requiring RRT. Higher baseline uNCR was also independently predictive of rapid renal decline over 1 year (HR per 5 μg/mmol increase 1.47, 95% CI: 1.06–2.06, P = 0.022). Addition of uNCR to the base model resulted in a significant increase in discrimination for the secondary (*C*-statistic 0.76–0.85, P = 0.001) but not the primary endpoint (P = 0.276). Reclassification analysis on the other hand, demonstrated an improvement in risk predication of both primary and secondary endpoints by incorporating uNCR into the base model, but only in those with low-level urine protein excretion (<28 mg/mmol), with category-free net reclassification improvement (NRI) scores of 64% (95% CI: 8–70; P = 0.019) and 79% (95% CI: 12–83; P = 0.009), respectively.

Conclusion. The utilization of uNCR in addition to conventional established cardiovascular and renal risk factors may improve the prediction of disease progression in elderly Caucasian pre-dialysis CKD patients with low-grade proteinuria.

INTRODUCTION

Chronic kidney disease (CKD) is a global public health concern with a steady increase in the prevalence of patients

reaching end-stage and requiring renal replacement therapy (RRT) [1, 2]. It is associated with an increased risk of cardiovascular events and all-cause mortality [3]. Much work has focused on identifying groups that are at a high risk of rapidly progressive kidney disease, and how to distinguish these patients from those with impaired but stable kidney function. Numerous risk factors for accelerated progression have been identified [4–7], with poorly controlled hypertension and heavy proteinuria being two of the best-recognized predictors [8]. The degree of albuminuria at a given level of kidney function is independently associated with progression to end-stage renal disease (ESRD) and all-cause mortality [9]. In addition, incorporation of the degree of albuminuria into the CKD staging system identifies patients with the reduced estimated glomerular filtration rate (eGFR) but minimal proteinuria, who may not develop a significant progression of kidney disease. Identification of such patients has been shown to reduce unnecessary nephrology referrals [10]. However, in clinical practice, if albuminuria is used as a urinary biomarker in combination with other established clinical predictors, it is still not possible to correctly identify a significant number of patients with accelerated deterioration of kidney function. Importantly, there appears to be a subset of patients in whom the renal decline may be rapid yet proteinuria remains relatively low [11]. Identifying these patients would be of particular clinical value.

Regardless of the primary cause of CKD, tubulointerstitial damage appears to be a final common pathway and a powerful predictor of progression to ESRD [12]. The detection of low-molecular weight (LMW) proteinuria of tubular origin in glomerular diseases has been associated with chronic tubulointerstitial injury on biopsies, poor response to treatment and long-term prognosis [13]. Some reported studies used sodium dodecyl sulphate–polyacrylamide gel electrophoresis to detect LMW protein species, which is labour intensive and requires considerable expertise [14, 15]. Others have measured β_2-microglobulin [16], retinol-binding protein [17] and α_1-microglobulin [18, 19]. These proteins are normally filtered at the glomeruli and reabsorbed by the proximal tubules. In the setting of glomerular disease, tubular markers may compete with albumin and other high-molecular weight proteins for tubular reabsorption and increased urinary excretion may result from the tubular reabsorptive capacity being exceeded, rather than tubular damage *per se*. The ideal urinary biomarker of tubular injury would be a non-invasive measurement of a urinary protein released directly from injured tubular cells before established tubulointerstitial damage results. This would identify at-risk patients for closer appraisal and more intensive intervention to limit this potentially reversible process.

Neutrophil gelatinase-associated lipocalin (NGAL), a 25-kD protein, is known to be released from injured renal tubular cells in acute kidney injury, before a decrease in the glomerular filtration rate can be detected [20–22]. Higher baseline levels of urinary NGAL (uNGAL) has also been shown to predict an increased risk of worsening kidney function in membranous nephropathy [23]. More recently, in patients with moderate kidney disease, serum and uNGAL concentrations were identified to be independent predictors of CKD progression [24]. However, the value of incorporating uNGAL measurements into a model of established risk factors to predict the risk of hard endpoints such as death or requirement for RRT, or rapid progression of kidney disease has not yet been evaluated.

In the present prospective study, we examined the value of incorporating uNGAL measurements into a model with established clinical risk factors in predicting the hard endpoints of death or initiation of RRT as well as renal progression, in a cohort of elderly patients with Stage 3 or 4 CKD of various aetiologies.

MATERIALS AND METHODS

Study participants

This is a substudy of an ongoing prospective study of cardiovascular risk in patients with Stage 3 and 4 CKD (the Academic Study) [25]. One hundred and fifty-eight of the 200 patients recruited to the study reached the primary composite endpoint of the substudy or completed at least 2 years of follow-up (median: 1477 days, IQR: 850–1691) and were included in subsequent analysis. Patients were predominantly recruited from outpatient nephrology clinics at Brighton and Sussex University Hospitals NHS Trust from March 2006 to July 2009. A full history covering renal disease, cardiovascular disease (CVD) and risk factors was obtained at study entry. Pre-existing cardiovascular comorbidity was defined by a history of transient ischaemic attack, stroke, myocardial infarction, angina or if the patient had undergone treatment for CVD (e.g. coronary artery bypass grafting or angioplasty). Anthropometric measurements were also made. This study was approved by the local regional ethics committee and conducted in accordance with the Declaration of Helsinki. All the participants provided written informed consent.

Laboratory measurements

Random, non-fasting, plasma, serum and plain urine samples were collected at entry to the study. Urine samples were immediately centrifuged at 15 min at 2000×*g* and 4°C, before storage at −70°C. Samples were only subjected to a single thaw at 4°C prior to analysis.

Standard biochemical analysis was performed using a routine automated analyser (Roche Modular, Haywards Heath, UK). The eGFR was calculated using the Chronic Kidney Disease-Epidemiology Collaboration (CKD-EPI) equation [26]. Urinary albumin concentration was measured by immunoturbidimetry, and urinary total protein concentration was measured by the turbidimetric assay after precipitation with benzethonium chloride as previously described [27]. uNGAL was measured using the particle-enhanced turbidimetric immunoassay (The NGAL Test™) from BioPorto Diagnostics (Gentofte, Denmark) on a Roche Modular *P* autoanalyser. Between-batch imprecision was 3.4% at 197.3 μg/L and the limit of analytical detection was 12 μg/L. Urine albumin and total protein concentration were expressed as ratios to urinary creatinine concentration, uACR and uPCR,

respectively. Urine creatinine concentration was measured by enzyme-linked spectrophotometry. uNGAL was expressed in terms of mass concentration (uNGAL, μg/L) or normalized to creatinine [uNGAL-to-creatinine ratio (uNCR) μg/mmol].

Outcomes assessment

The primary composite endpoint was defined as either death from any cause or initiation of RRT within 2 years of the recruitment to the study. A secondary endpoint of a ≥5 mL/min/1.73 m^2 decline in the eGFR in the first year of follow-up was also considered. The eGFR decline was estimated by ordinary least squares linear regression of all available eGFR measurements after expurgation of acute kidney disease (AKD) episodes (mean sample number per patient 8, range 3–24). AKD episodes were identified in 21 patients (14%) using current KDIGO Clinical Practice Guideline criteria (≥35 decrease in the eGFR for <3 months) [28]. Patients were dichotomized into groups with an eGFR decline of < and ≥5 mL/min/1.73 m^2. Since only decline after 1 year was considered, patients who died within the first 12 months of enrolment ($n = 12$) were excluded from the analysis of this secondary endpoint. An alternative secondary endpoint of a ≥3 mL/min/1.73 m^2 decline in the eGFR in 1 year was also considered for purposes of comparison with other studies using this cut-off.

Statistical analysis

Data are presented as mean (± SD) or median (25–75th centile) as appropriate. uPCR, uACR, uNCR, parathyroid hormone (PTH) and high-sensitivity C-reactive protein (hsCRP) showed strongly skewed distributions and were natural log-transformed before further analysis. Comparisons of baseline parameters between those that reached endpoint (event) and those that did not (non-event) were performed using the unpaired Student's *t*-test with a Welch correction or the Mann–Whitney *U*-test for continuous variables and the χ^2 test for categorical variables. Pearson's or Spearman's correlation coefficients were calculated to assess the association between uNGAL/uNCR and other baseline variables. Cox proportional hazards models were used to estimate the risk associated with each variable. The proportional hazards assumption was tested by creating time-by-covariate interactions for each variable. Variables not associated with the outcome ($P > 0.10$) in univariate regression were excluded from multivariate modelling. Base models were defined by entering all baseline variables significantly associated with the outcome, except uNGAL/uNCR. Models were tested for colinearity using variance inflation factors and stability of the regression coefficients. Incremental improvement in model performance on the addition of uNGAL/uNCR was assessed using a number of metrics as suggested by Steyerberg *et al.* [29]. Discrimination, the capacity of models to correctly distinguish those with and those without the outcome, was evaluated by a comparison of concordance statistics (*C*-statistics) using the Delong–Delong test. Discrimination slopes were calculated as the difference between the mean predicted risk of those patients reaching endpoint and those not. The integrated discrimination index (IDI) was calculated as the difference between discrimination slopes of models without and with uNCR. Category-free NRI scores were calculated to assess reclassification as described by Pencina *et al.* [30]. NRI scores quantify changes in risk estimates when comparing the classification of risk by two models (e.g. base model versus base model plus uNCR). Event and non-event NRI were considered separately. Overall NRI scores were calculated as follows: P(up|event)−P(down|event)+P(down|non-event)−P(up|non-event). An increase in risk estimates for individuals who reached endpoint (event) indicates improved classification, whereas a reduction indicates worse reclassification. On the other hand, a reduction in NRI for event-free individuals denotes improved reclassification, while an increase suggests worse reclassification. Overall model fit was compared using the Akaike Information Criterion (AIC) and Nagelkerke's R^2 statistic. uNGAL and uNCR were considered as continuous variables (per 10 μg/L and 5 μg/mmol increase, respectively) and after dichotomization by the median value (high versus low). The potential interaction between uNGAL/uNCR and proteinuria was considered after dichotomization by the median uPCR (< and ≥28 mg/mmol) and entering a two-way cross-product term in the multivariable model. Interactions between uNGAL/uNCR and diabetes or pre-existing CVD were also assessed and evaluated using the likelihood ratio test. uACR and uPCR were modelled in separate regression analyses as markers of proteinuria. uPCR was more strongly associated with both primary and secondary endpoints than uACR, and yielded models with better overall fit compared with modelling with uACR, hence only uPCR was used in subsequent risk analysis.

A two-sided P-value of <0.05 was considered significant. Analysis was performed using SPSS version 20 (IBM Corporation, USA), Stata 12.1 (Stata Corp., College Station, TX, USA) and Analyse-It (Analyse-It, UK).

RESULTS

Baseline cohort characteristics and cross-sectional analysis

The baseline characteristics of the study cohort ($n = 158$) are summarized in Table 1. Subjects were predominantly elderly (mean age: 69 ± 12 years) and male (75%). The mean eGFR was 32 ± 11 mL/min/1.73 m^2. The mean systolic blood pressure (SBP) was 153 ± 21 mmHg. Most patients were overweight (median BMI: 28.4 kg/m^2). Twenty-five percent were diabetic and forty-seven percent had a documented history of pre-existing CVD. The primary aetiology of CKD included hypertension (30%), chronic glomerulonephritis (19%), obstructive or congenital disease (6%), diabetic nephropathy (6%), interstitial nephritis (7%), cystic kidney disease (5%), vasculitis (8%) and was unknown in 19% of the participants. Importantly, only 30% of patients had clinically significant proteinuria (uPCR >50 mg/mmol).

The median uNGAL concentration (uNGAL) was 70.2 (20.6–210.8) μg/L and uNCR was 10.8 (7.9–45.0) μg/mmol. uNGAL and uNCR were highly correlated ($r = 0.93$, $P < 0.001$). In baseline analysis, uNCR was significantly correlated with plasma PTH ($r = 0.28$, $P = 0.001$), uPCR ($r = 0.55$,

Table 1. Baseline characteristics of study participants according to primary outcome status

Parameter	All patients ($n = 158$)	Events[a] ($n = 40$)	No events[a] ($n = 118$)	P-value (events versus no events)
Gender (% male)	75	83	72	0.191
Age (years)	69 ± 12	72 ± 10	68 ± 12	0.031
BMI (kg/m^2)	28.4 (25.3–33.7)	28.1 (25.5–31.8)	28.4 (25.3–33.8)	0.793
SBP (mmHg)	153 ± 21	158 ± 21	151 ± 21	0.085
DBP (mmHg)	82 ± 11	83 ± 11	82 ± 11	0.388
Alcohol intake (units/week)	8 ± 9	8 ± 10	8 ± 9	0.946
Smoking (pack/year)	19 ± 29	31 ± 36	15 ± 24	0.002
Diabetes (%)	25	40	19	0.009
CVD (%)	47	78	38	<0.001
Statin use (%)	59	50	62	0.190
Beta blocker use (%)	30	30	30	0.968
ACEI/ARB use (%)	65	55	69	0.119
Diuretic use (%)	59	68	57	0.235
CBB use (%)	50	58	47	0.275
Blood haemoglobin (g/dL)	12.7 ± 1.7	12.2 ± 2.0	12.9 ± 1.6	0.039
Plasma total cholesterol (mmol/L)	4.40 ± 0.93	4.42 ± 0.98	4.37 ± 0.93	0.885
Plasma triglyceride (mmol/L)	1.50 ± 0.44	1.63 ± 0.50	1.46 ± 0.41	0.028
Plasma hsCRP (mg/L)	2.33 (0.94–6.08)	4.64 (1.50–10.68)	1.99 (0.86–4.54)	0.002
Plasma albumin (g/L)	43 ± 3	42 ± 3	43 ± 3	0.184
Plasma adjusted calcium (mmol/L)[b]	2.29 ± 0.11	2.28 ± 0.11	2.29 ± 0.12	0.515
Plasma phosphorus (mmol/L)	1.08 ± 0.20	1.12 ± 0.24	1.07 ± 0.19	0.186
Plasma PTH (ng/L)	77 (50–123)	109 (72–208)	70 (49–104)	<0.001
eGFR (mL/min/1.73 m^2)[c]	32 ± 11	26 ± 8	35 ± 11	<0.001
uCreat (mmol/l)	5.47 ± 3.33	5.46 ± 3.34	5.47 ± 3.34	0.994
uACR (mg/mmol)	7 (2–45)	43 (18–95)	4 (1–18)	<0.001
uPCR (mg/mmol)	28 (25–71)	50 (30–142)	22 (12–47)	<0.001
uNGAL (µg/L)	72 (26–218)	187 (67–689)	50 (21–177)	<0.001
uNCR (µg/mmol)	18 (8–45)	30 (15–18)	16 (6–30)	<0.001

Data are mean ± SD or median (25–75th percentile).
ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; BMI, body mass index; BP, blood pressure; CCB, calcium channel blocker; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; hsCRP, high-sensitivity C-reactive protein; PTH, parathyroid hormone; uACR, urine albumin-to-creatinine ratio; uCreat, urine creatinine concentration; uPCR, urine protein-to-creatinine ratio; uNGAL, urine neutrophil gelatinase-associated lipocalin; uNCR, urine NGAL-to-creatinine ratio.
[a]'event' denotes those patients who reached primary composite endpoint (all-cause death or initiation of RRT) in 2 years.
[b]Adjusted calcium concentration (mmol/L) = total calcium concentration (mmol/L) + 0.02 [(40—albumin concentration (g/L)].
[c]eGFR calculated using the CKD-EPI equation [26].

P < 0.001), SBP ($r = 0.163$, P = 0.041) and inversely with eGFR (−0.28, P < 0.001). uNGAL concentration was also associated with plasma PTH ($r = 0.18$, P = 0.001), uPCR ($r = 0.47$, P < 0.001) and inversely with the eGFR (−0.23, P < 0.001). There was no significant association between uNGAL concentration and SBP ($r = 0.138$, P = 0.083). Both uNGAL and uNCR were significantly higher in those patients with pre-existing CVD (P = 0.019 and P = 0.022, respectively), but were

not associated with antihypertensive or lipid-lowering therapy. Urinary creatinine concentration was strongly correlated with uNGAL concentration ($r = 0.241$, $P = 0.002$), but not with uNCR ($r = -0.091$, $P = 0.254$).

uNCR is associated with the primary composite endpoint of all-cause mortality or initiation of RRT

After 2 years, 40 patients (25%) reached the primary composite endpoint, 20 (12.5%) of whom died and the remainder requiring RRT. Two patients died after initiating RRT. Patients who reached the primary composite endpoint were more likely to be older, have known CVD or diabetes, have smoked, had lower baseline eGFR, haemoglobin, plasma albumin concentration, higher plasma PTH, serum hsCRP and triglyceride concentrations and higher SBP. uACR, uPCR, uNGAL and uNCR were all significantly higher in those with events than those without (Table 1).

In univariate Cox regression analysis, age, male gender, lower eGFR and haemoglobin concentrations, pre-existing CVD, higher triglyceride and PTH concentrations and uPCR were associated with the primary composite endpoint (Table 2). uNCR was more strongly associated with the outcome than uNGAL concentration both on a continuous scale (HR per 5 µg/mmol increase: 1.59; 95% CI: 1.32–1.91, $P < 0.001$ versus HR per 10 µg/L increase 1.12; 95% CI: 1.01–1.53, $P = 0.018$) and when comparing high and low groups dichotomized by the median value (data not shown). After multivariate adjustment, only eGFR, triglyceride concentration, CVD history, uPCR and uNCR remained independently associated with the primary composite endpoint (Table 2). Multivariate modelling with uACR instead of uPCR is shown in Supplementary Table S1. In separate analysis, uNGAL concentration was also retained in the adjusted model but with a smaller effect size compared with uNCR and borderline significance (HR: per 10 µg/L increase 1.08 95% CI: 1.01–1.19, $P = 0.049$) (Supplementary Table S2).

Although the addition of uNCR to the base model (containing age, gender, eGFR, haemoglobin, serum triglyceride, PTH concentrations, urine creatinine concentration, CVD and uPCR) resulted in an increase in Nagelkerke's R^2 (48.7–55.8%) and reduction in AIC statistic (434–420), the *C*-statistic showed only a modest increase (0.81–0.82, $P = 0.279$) (Supplementary Figure S1A). The discrimination slope increased from 35 to 49% yielding an IDI of 14% (Supplementary Figure S2). In reclassification analysis (Table 3), the addition of uNCR to the base model gave an overall NRI score of 36% ($P = 0.015$) (Figure 1A). Improvement in reclassification was only observed in the event group (NRI: 40%, $P < 0.001$) and not in those who remained event-free (NRI: −4%, $P = 0.515$). Incorporation of uNGAL into the same base model yielded a marginal improvement in overall fit (Nagelkerke's R^2 increased from 48.2 to 49.7% and AIC statistic decreased from 435 to 432), but no significant change in discrimination (*C*-statistic remained at 0.81).

To better understand the nature of the observed improvement in risk prediction due to the addition of uNCR to the base model, we next considered the association of uNCR with each component of the composite primary endpoint, separately. As shown in Supplementary Table S3, while uNCR remained strongly associated with an increased risk of progression to ESRD requiring RRT (HR: 1.25, 95% CI: 1.08–2.55; $P = 0.009$), significance in predicting all-cause mortality was lost after full multivariate adjustment (HR: 1.08, 95% CI:

Table 2. Cox proportional regression analysis of uNCR and other baseline factors associated with the primary composite endpoint (all-cause mortality or initiation of RRT) (*n* = 158)

Variable	Unit increase	Univariate			Multivariate		
		HR	95% CI	P-value	HR	95% CI	P-value
Age	10 year	1.32	0.98–1.78	0.069			
Gender	male = 1	1.56	0.79–3.53	0.094			
CVD	yes = 1	4.68	2.22–9.86	<0.001	3.52	1.62–7.65	0.001
eGFR	5 mL/min/1.73 m^2	0.66	0.54–0.79	<0.001	0.78	0.64–0.96	0.018
SBP	5 mmHg	1.05	0.98–1.13	0.089			
Haemoglobin	1 g/dL	0.82	0.68–0.98	0.029			
Triglyceride	1 mmol/L	2.49	1.19–5.19	0.015	1.64	1.15–5.64	0.022
PTH	10 ng/L	2.71	1.62–4.52	<0.001			
uCreat	1 mmol/L	1.04	0.93–1.16	0.524			
uPCR	10 mg/mmol	1.86	1.44–2.41	<0.001	1.85	1.29–2.64	0.001
uNCR	5 µg/mmol	1.59	1.32–1.91	<0.001	1.27	1.01–1.60	0.036

CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; HR, hazard ratio; PTH, parathyroid hormone; SBP, systolic blood pressure; uCreat, urine creatinine concentration; uPCR, urine protein-to-creatinine ratio; uNCR, urine NGAL-to-creatinine ratio.

Table 3. Reclassification of 2-year risk of death or initiation of RRT for patients with Stages 3 and 4 CKD according to uPCR

	Reclassified upwards(%)	Reclassified downwards(%)	Total	NRI (%)
All				
Event	28 (70)	12 (30)	40	40
Non-event	61 (52)	57 (48)	118	−4
		Overall	158	36 (95% CI: 9–52)
				P = 0.015
uPCR ≤28 mg/mmol				
Event	12 (80)	3 (20)	15	60
Non-event	30 (48)	33 (52)	63	4
		Overall	73	64 (95% CI: 8–70)
				P = 0.019
uPCR >28 mg/mmol				
Event	16 (64)	9 (36)	25	28
Non-event	31 (56)	24 (44)	55	−8
		Overall	73	20 (95% CI: 2–35)
				P = 0.047

CI, confidence interval; NRI, net reclassification improvement.

1.00–1.20; P = 0.068). Similar results were obtained using uNGAL concentration as covariate (data not shown).

Interaction between uNCR and uPCR and prediction of the primary composite endpoint

We next tested the possibility that the improvement of model performance was due to the identification of patients with relatively low proteinuria, but in whom the outcome was still poor. In support of this hypothesis, we found a strong interaction between uNCR and uPCR (dichotomized by the median value) and the primary endpoint (P = 0.003). Re-evaluation of model performance in these two groups showed a greater improvement in event reclassification (i.e. those reaching endpoint) in those with an uPCR ≤28 mg/mmol (event NRI: 60%, P = 0.009) compared with those patients with an uPCR above this level (event NRI 28%, P = 0.024). As before, no improvement in non-event reclassification was observed in either subgroup (Table 3). Overall, net reclassification was only substantially improved in those with lower urinary protein excretion.

uNCR is associated with secondary endpoint of≥5 mL/min/1.73 m^2 decline in eGFR over 1 year

Within the first year of the follow-up, 12 patients (8%) died, leaving 146 patients available for the analysis of the secondary endpoint. These individuals had a lower prevalence of pre-existing CVD (44 versus 47%) but otherwise did not differ significantly in terms of their baseline characteristics (data not shown). A ≥5 mL/min/1.73 m^2 decline in eGFR was observed in 27 patients (19%) and a ≥3 mL/min/1.73 m^2 decline in eGFR in 40 patients (27%). Of these, two patients were commenced on RRT.

In univariate analysis, age, baseline eGFR, plasma PTH concentration, SBP, uPCR and uNCR were associated with a ≥5 mL/min/1.73 m^2 decline in the eGFR (Table 4). Urinary creatinine concentration was not associated with the decline in the eGFR (P = 0.839). In multivariate regression, only eGFR, SBP, uPCR and uNCR remained independently associated with the progression of kidney disease after 1 year (Table 4). Multivariate modelling with uACR instead of uPCR is shown in Supplementary Table S4. In separate analyses, uNGAL concentration also remained significantly associated with renal decline but again showed borderline significance after multivariate adjustment (Supplementary Table S5). Similar results were obtained using the alternative cut-off of ≥3 mL/min/1.73 m^2 decline in the eGFR over 1 year, with the same covariates remaining independently associated with the progression of disease after multivariate analysis (uNCR; HR: 1.50, 95% CI: 1.12–2.02, P = 0.018).

In contrast to the primary endpoint, incorporation of uNCR into the base model (including age, eGFR, SBP and uPCR) resulted in a significant increase in *C*-statistic from 0.76 (95% CI: 0.65–0.86) to 0.85 (95% CI: 0.76–0.94) (P = 0.001) (Supplementary Figure S1B) and gave an IDI of 9.0% (Supplementary Figure S2), indicating improved discrimination of those who declined and those who maintained relatively stable renal function. The addition of uNCR to the base model improved overall net reclassification with an NRI

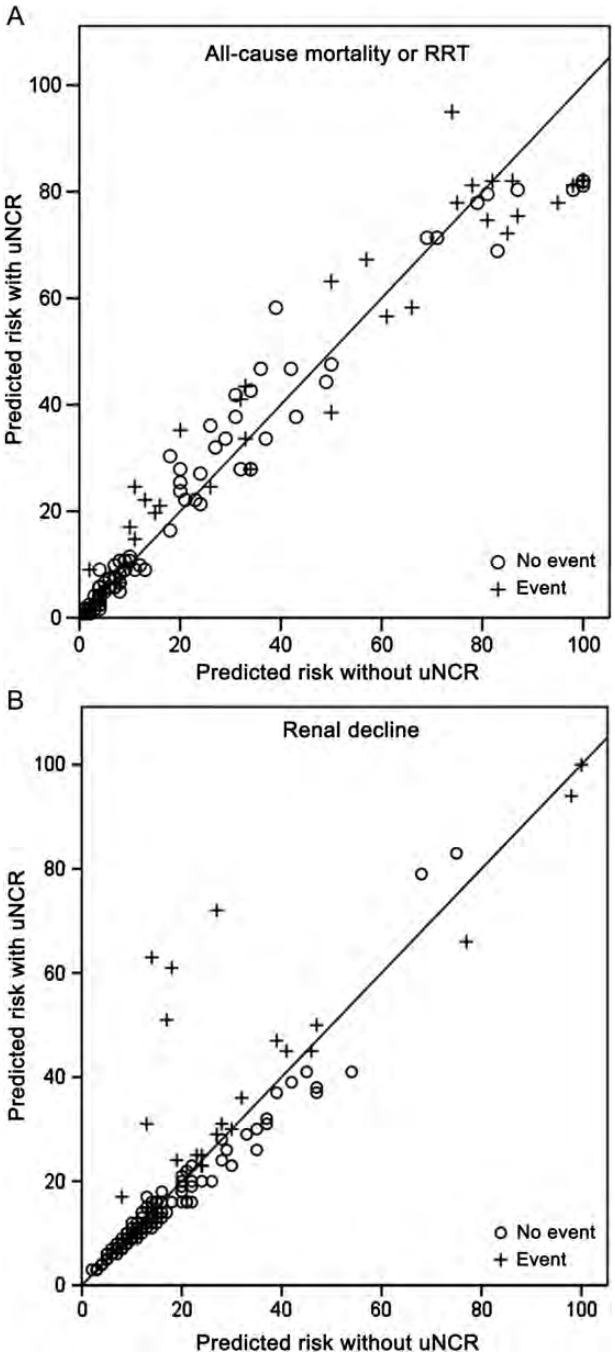

FIGURE 1: Reclassification plot showing difference in predicted risk of base model (*x*-axis) and model incorporating uNCR (*y*-axis) of study participants reaching endpoint (cross) and those remaining event-free (open circle) for (**A**) 2-year risk of all-cause mortality or initiation of RRT ($n = 158$) and (**B**) 1-year risk of renal decline ($n = 146$) in patients with Stages 3 and 4 CKD. Diagonal line indicates no change in predicted risk.

score of 26% (P = 0.018) (Table 5), with a significant increase in event NRI (18%, P = 0.010) but only a borderline improvement in non-reclassification (8%, P = 0.050). Overall model fit was improved as indicated by the increase in Nagelkerke's R^2 (52.0–68.3%) and a reduction in AIC statistic (502–489). Modest improvement in model performance was also observed with uNGAL, with a slight increase in overall model fit (Nagelkerke's R^2 increased 52.0–56.7%) and discriminative power (increase in *C*-statistic 0.76–0.80, P = 0.023).

We again noted a significant interaction between uNCR and uPCR in the prediction of eGFR decline (P = 0.015), assessment of risk reclassification after dichotomization by the median uPCR (≤26 mg/mmol) revealed an improvement in event and non-event risk estimates of disease progression in the lower proteinuric group (uPCR ≤ 26 mg/mmol), but not in those with higher urinary protein excretion (Table 5).

DISCUSSION

This is the first study to demonstrate that the ascertainment of uNCR improves the prediction of risk of kidney disease progression in patients with established pre-dialysis CKD. The addition of uNCR to a base model including conventional cardiovascular and renal risk factors significantly improved the prediction of a composite endpoint of all-cause mortality or initiation of RRT within 2 years. Separate analysis of these two competing endpoints, however, suggested that uNCR was only significantly associated with an increased risk of progression to ESRD and not with death, after multivariate adjustment. Perhaps more significantly, reclassification analysis dichotomized by median uPCR demonstrated that the greatest benefit was gained in identifying those patients who experienced progression to ESRD requiring RRT or a rapid decline in the eGFR over 1 year (≥5 mL/min/1.73 m^2), but who had relatively low-level proteinuria (uPCR < 28 mg/mmol). Conversely, in individuals with higher urine total protein excretion, the addition of uNCR conferred little enhancement in the reclassification of progressors from non-progressors.

The findings of the present study are consistent with that of a previous study by Bolignano *et al.* [24], which reported that uNGAL concentration was a strong and independent predictor of renal disease progression (defined by a doubling of serum creatinine concentration or initiation of RRT) in patients with pre-dialysis CKD (mean eGFR 41.8 ± 19.1 mL/min/1.73 m^2). Despite substantial differences in cohort demographics (patients in the present study were generally older, had lower baseline eGFR, higher SBP and were less proteinuric), a shorter follow-up and use of a different definition of renal disease progression, our study appears to validate these findings.

Although it appears from our data that uNCR is more strongly related to declining renal function rather than mortality (HR: 1.08 95% CI: 1.00–1.20, P = 0.068), we cannot rule out the possibility that uNGAL excretion may have prognostic significance with respect to this endpoint. It should be acknowledged that the *post hoc* analysis of individual components of the composite primary endpoint might be relatively underpowered. A cohort study of patients with heart failure and relatively preserved kidney function identified an association between death or heart failure-related hospitalizations and two other potential urinary biomarkers for tubular damage: urinary kidney injury molecule-1 (uKIM-1) and *N*-acetyl-beta-D-glucosaminidase, independent of the GFR [31]. uNGAL was not identified as a potential biomarker in that

Table 4. Cox proportional regression analysis of baseline factors associated the ≥5 mL/min/1.73 m^2 decline in eGFR over 1 year (n = 146)

Variable	Unit increase	Univariate			Multivariate		
		HR	95% CI	P-value	HR	95% CI	P-value
eGFR	5 mL/min/1.73 m^2	0.79	0.64–0.96	0.020			
SBP	5 mmHg	1.09	1.00–1.18	0.046	1.08	0.99–1.19	0.076
PTH	10 ng/L	1.95	1.10–3.48	0.023			
uPCR	10 mg/mmol	2.00	1.53–2.62	<0.001	1.85	1.29–2.64	0.001
uNCR	5 µg/mmol	1.82	1.41–2.35	<0.001	1.47	1.06–2.06	0.022

eGFR, estimated glomerular filtration rate; HR, hazard ratio; PTH, parathyroid hormone; SBP, systolic blood pressure; uPCR, urine protein-to-creatinine ratio; uNCR, urine NGAL-to-creatinine ratio.

Table 5. Reclassification of 1-year risk of renal decline (eGFR ≥5 mL/min/1.73 m^2) for patients with Stage 3 and 4 CKD according to uPCR

	Reclassified upwards (%)	Reclassified downwards (%)	Total	NRI (%)
All				
Event	16 (59)	11 (41)	27	18
Non-event	52 (44)	67 (56)	119	8
		Overall	146	26 (95%CI: 6–29)
				P = 0.018
uPCR ≤ 28 mg/mmol				
Event	7 (70)	3 (30)	10	50
Non-event	24 (37)	40 (63)	64	26
		Overall	74	76 (95%CI: 12–83)
				P = 0.009
uPCR > 28 mg/mmol				
Event	9 (53)	8 (47)	17	6
Non-event	28 (51)	27 (49)	55	−3
		Overall	72	3 (95%CI: −5–10)
				P = 0.339

CI, confidence interval; NRI, net reclassification improvement.

study; however, it has been shown to be predictive of death or RRT in adults undergoing cardiac surgery and in elderly patients with chronic heart failure [32, 33].

In terms of its clinical utility, however, the usefulness of a biomarker depends not so much on whether it is independently associated with an outcome, but rather whether such an ascertainment adds to established risk parameters. In the present study, we have used a number of performance metrics to demonstrate that the addition of uNCR to a multivariate model of conventional risk factors, improved risk estimates of renal disease progression, particularly in those with relatively low-level proteinuria. This latter point is especially important as, although proteinuria has long been recognized as a predictor for renal progression [9, 34], several epidemiological studies have identified a subset of patients that experience a rapid decline in the eGFR, but in whom total urine protein excretion remains relatively normal [11]. Our data suggest that uNCR measurement may greatly aid identification of these patients. In this context, the relatively high uNCR (not captured by total proteinuria measurement) presumably reflects ongoing tubular injury and explains why these individuals have a lower predicted risk in the base model but are, in fact, at

a higher risk of actual progression. In this regard, it would, therefore, appear to be a strength of the present study that relatively few of the patients recruited to the study had clinically significant proteinuria at recruitment (<30%), allowing us to specifically assess performance of uNGAL in this group.

It is likely that increased urinary excretion of albumin and NGAL reflects a different underlying pathophysiology in CKD. As opposed to most markers of tubular injury, overt albuminuria most commonly arises from increased glomerular leakage exceeding the tubular reabsorptive capacity [13], while microalbuminuria might be due to endothelial dysfunction [35]. In contrast, the urinary excretion of NGAL monomer, produced specifically from injured renal tubular cells, and unlike the dimeric form originating from neutrophils [36], has been significantly correlated with the severity of tubular atrophy and interstitial fibrosis on renal biopsies [37]. Tubulointerstitial injury is a powerful predictor of CKD progression regardless of primary cause of kidney disease [12]. Therefore, uNCR, in addition to total proteinuria or albuminuria, could potentially provide important prognostic information of tubular competence thereby avoiding the risk of invasive procedures (e.g. biopsy). Another potential explanation for increased uNGAL excretion is from glomerular filtration of systemically produced NGAL from neutrophils other inflammatory cells [36], or possibly even the cardiovascular system [38]. The patients who died or commenced RRT in our study displayed significantly higher hsCRP and lower plasma albumin concentrations, suggesting greater systemic inflammation. However, forced entry of serum hsCRP and albumin concentration into the multivariate model did not attenuate the strength of the association between uNCR and primary endpoint.

Interestingly, creatinine-normalized uNGAL concentrations consistently out-performed uNGAL concentrations alone in multivariate analysis and risk prediction models. The weaker signal from uNGAL concentration presumably reflects variability in urinary flow rates, which is partly 'corrected' by normalization to urinary creatinine concentration. However, given the well-recognized variability of urinary creatinine excretion, timed urine collections may provide a more accurate quantitation of NGAL excretion and thus enhance risk estimates further. Of note, urine creatinine concentration, although significantly correlated with uNGAL, was not itself associated with the primary or secondary endpoint, even in simple univariate analysis.

The utility of uNCR in predicting renal decline in patients with relatively preserved kidney function is less certain. Analysis of data from the Atherosclerosis Risk in Communities (ARIC) study showed that the association between a higher baseline uNGAL concentration and incident Stage 3 CKD was lost after adjustment for urinary creatinine and albumin concentration in a small cohort of individuals from the general population [39]. While data from the larger Multi-Ethnic Study of Atherosclerosis (MESA) failed to find any significant association between uNGAL concentration (or uNCR) and incident Stage 3 CKD or rapid decline in renal function [40]. Reconciling these findings with those of the present study and that of Bolignano *et al.* [24] is challenging, but might reflect the greater significance of tubular injury in patients with more advanced kidney disease. Marked differences in cohort demographics may also contribute to the apparent disparity with our results; both ARIC and MESA cohorts had a higher proportion of females and non-white Caucasian races and less hypertensive comorbidity. Moreover, differences in urine NGAL and creatinine methodology, in addition to the quite high analytical variability reported for NGAL measurement are also likely to contribute. Furthermore, the findings of Bhavsar *et al.* [39] and Peralta *et al.* [40] are not themselves consistent with the former reporting no significant association between renal progression and uKIM1 concentration, while the latter showed a strong association of this marker with a rapid renal decline and incident Stage 3 CKD, independent of albuminuria.

This study has several limitations. First, the follow-up, particularly for renal events, was relatively short and the confidence intervals derived for NRI estimates were quite wide. However, event rates were sufficiently high enough to achieve adequate statistical power for the analysis of primary and secondary endpoints. Analysis of the individual components of the composite primary endpoint may, however, have been relatively underpowered, and in particular, the relationship between uNCR and all-cause mortality may have been underestimated. Further adequately powered studies are needed to assess the potential association between uNCR and death in this setting. Secondly, the study cohort was predominantly Caucasian and from a single European centre, which limits the generalizability of these results to populations from other ethnic backgrounds such as African Americans, Asians and Hispanics. Validation of these findings is required in external cohorts.

In conclusion, we have demonstrated that uNCR ascertainment, in combination with established clinical risk factors, improves the prediction of renal disease progression in elderly Caucasian CKD patients with low-grade proteinuria. Although the measurement of uNCR appears to add value to risk prediction, further work is needed to replicate these findings in larger, more racially diverse cohorts and to understand the pathophysiological significance of increased uNGAL excretion in the context of CKD.

SUPPLEMENTARY DATA

Supplementary data are available online at http://ndt.oxfordjournals.org.

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CONFLICT OF INTEREST STATEMENT

None declared. The results presented in this paper have not been published previously in whole or part, except in abstract form.

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ORIGINAL ARTICLE

Effects of angiotensin receptor blockade (ARB) on mortality and cardiovascular outcomes in patients with long-term haemodialysis: a randomized controlled trial

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