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Urine neutrophil gelatinase-associated lipocalin levels do not improve risk prediction of progressive chronic kidney disease

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Abstract

Novel biomarkers may improve our ability to predict which patients with chronic kidney disease (CKD) are at higher risk for progressive loss of renal function. Here we assessed the performance of urine neutrophil gelatinase-associated lipocalin (NGAL) for outcome prediction in a diverse cohort of 3386 patients with CKD in the CRIC study. In this cohort, the baseline mean estimated glomerular filtration rate (eGFR) was 42.4 ml/min/1.73m^2; the median 24-hour urine protein was 0.2 gm/day; and the median urine NGAL concentration was 17.2 ng/mL. Over an average follow-up of 3.2 years, there were 689 cases in which the eGFR was decreased by half or incident end-stage renal disease developed. Even after accounting for eGFR, proteinuria and other known CKD progression risk factors, urine NGAL remained a significant independent risk factor (Cox model hazard ratio 1.70 highest to lowest quartile). The association between baseline urine NGAL levels and risk of CKD progression was strongest in the first two years of biomarker measurement. Within this time frame, adding urine NGAL to a model which included eGFR, proteinuria and other CKD progression risk factors led to net reclassification improvement of 24.7%; but the C-statistic remained nearly identical. Thus, while urine NGAL was an independent risk factor of

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progression among patients with established CKD of diverse etiology, it did not substantially improve prediction of outcome events.

INTRODUCTION

There is great interest currently in defining novel biomarkers that will improve our ability to predict which patients with chronic kidney disease (CKD) are at higher risk for progressive loss of renal function, adding to currently available risk factors such as amount of total proteinuria and glomerular filtration rate (GFR).[1, 2] Better risk stratification may potentially improve clinical outcomes by facilitating the application or intensification of evidence-based therapies among higher-risk patients.

One such promising novel biomarker is urine neutrophil gelatinase-associated lipocalin (NGAL).[3] A recent comprehensive review identified urine NGAL as the most promising novel biomarker among those being evaluated as predictors of CKD progression.[2] NGAL was originally identified in animal models by microarray analysis to be one of the earliest induced genes and proteins in the kidney after ischemic or nephrotoxic injury.[4] It is a ubiquitous lipocalin iron-carrying protein, highly expressed in the tubular epithelium and released from tubular epithelial cells following damage.[2] Although initially studied in the context of acute kidney injury, urine NGAL levels are also abnormally elevated (albeit at a much lower level) in a large number of individuals with chronic kidney disease.

A number of cross-sectional studies have shown that urine NGAL levels correlate with level of GFR or severity of underlying renal parenchyma injury.[5-10] Several small studies in selected populations have provided conflicting reports about whether urine NGAL is an independent risk factor of more rapid loss of renal function after controlling for established CKD progression risk factors (such as proteinuria and blood pressure level).[11-18]

We undertook the present study to assess the performance of urine NGAL as a novel independent risk factor of CKD progression in a large prospective diverse cohort of individuals with CKD.

RESULTS

Of the 3939 enrolled Chronic Renal Insufficiency Cohort (CRIC) study participants, 229 did not have a specimen available for NGAL testing and 3386 had valid NGAL measurements. The characteristics of the 3386 study participants are shown in Table 1. Urine NGAL distribution was highly skewed with a median of 17.2 ng/mL and interquartile range (IQR) 8.1 to 39.2 ng/mL; 5% of the highest measurements were between 178.9 and 3069.6 ng/mL. The 24-hour urine protein distribution was also highly skewed with a median of 0.2 gm/day, and interquartile range of 0.1 to 1.0 gm/day; 5% of the highest measurements were between 5.3-30.1 gm/day. Mean estimated glomerular filtration rate (eGFR) was 42.4 ± 13.6 (SD) ml/min/1.73m^2. Mean serum creatinine was 1.9 ± 0.7 mg/dL, blood urea nitrogen 30 ± 14 mg/dL, total cholesterol 183 ± 46 mg/dL, hemoglobin 12.6 ± 1.8 gm/dL, parathyroid hormone 76 ± 71 ng/L, and C-reactive protein 5.7 ± 10.0 mg/L.

We divided the participants into quintiles of urine NGAL concentrations as shown in Table 1 (≤6.9, >6.9 to ≤12.9, >12.9 to ≤ 22.6, >22.6 to ≤49.5, >49.5 ng/mL). Patients with higher urine NGAL concentrations were more likely to have lower eGFR and have higher levels of proteinuria. They were also more likely to be female, non-white, have diabetes mellitus and higher blood pressure.

Over a mean follow-up period of 3.2 years, there were 689 cases of halving of eGFR or incident end-stage renal disease (ESRD)(incidence rate 6.37 per 100 person-years). In unadjusted analysis, there was a strong graded relationship between urine NGAL concentration and risk of CKD progression (Table 2). Compared with those with urine NGAL concentration ≤6.9 ng/mL, CRIC participants with urine NGAL concentration >49.5 were nearly 10 times more likely to experience ESRD or halving of eGFR (hazard ratio [HR] 9.34; 95% confidence interval [CI] 6.86-12.72; p <0.001). This relationship was similar after adjustment for demographic attributes (Table 2). The association between urine NGAL concentration and ESRD or halving of eGFR was substantially attenuated when eGFR and 24-urine protein were taken into account (HR 1.58 for urine NGAL concentration >49.5 vs. ≤6.9ng/mL; 95% CI 1.09-2.29; p=0.01). Additional adjustment for other risk factors for CKD progression gave similar results (HR 1.70 for urine NGAL concentration >49.5 vs. ≤6.9ng/mL; 95% CI 1.16-2.48; p=0.006)(Table 2).

Results were consistent when urine NGAL was analyzed as a linear term with a HR of 1.11 per one standard deviation increase in log urine NGAL in the fully adjusted model (HR 1.11; 95% CI 1.01-1.21; p = 0.02). Compared with urine protein and eGFR the effect size of urine NGAL is much more modest (Figure 1).

Similar results were seen when the exposure was changed to urine NGAL/urine creatinine ratio or when the outcome was defined using ESRD alone in sensitivity analyses (results not shown).

There were no statistically significant interactions between urine NGAL and proteinuria (p=0.27) or eGFR (p=0.42); nor with diabetes status (p= 0.78), age (p=0.13), sex (p=0.32) or race (p=0.18).

The C-statistic for the final model was 0.847. Removal of urine NGAL from the final model did not change the C-statistic to the third decimal place (0.847).

In exploratory analysis, we relaxed the assumption of a constant hazard ratio over time by modeling urine NGAL and renal progression within each of the four time intervals (<2, 2-<3, 3-<4 and >4 years). We noted that the effect of urine NGAL was stronger in the first two years after measurement of urine NGAL vs. more than 2 years of follow-up (Figure 2). The HR per SD of urine NGAL for outcomes occurring within 2 years of biomarker measurement was 1.27 (95% CI 1.14-1.42). The HRs were 0.97 (95% CI 0.83-1.12), 1.00 (95% CI 0.84-1.20) and 0.98 (95% CI 0.78-1.22) for the later three time intervals respectively.

The C-statistic for the model limited to two years of follow-up was 0.880. Removal of urine NGAL from the final model lowered the C-statistic minimally to 0.879.

To assess reclassification in arena of potentially most promising clinical use which would be prediction of outcome in the first two years following biomarker ascertainment, we examined category free as well as category-based Net Reclassification Improvement (NRI) within a two year horizon. We *a priori* defined clinically meaningful differences in risks of 0-5%, 5-10% and >10% event rate per year. The category free NRI for events was 4.4% (95% CI -5.9% to 16.8%) and for non-events was 20.3% (95% CI 6.5% to 26.0%). The overall category free NRI was thus 24.7% (95% CI 0.4% to 38.5%). The three-category NRI for events was -0.3% (95% CI -2.8% to 2.9%), and for non-events was 0.4% (95% CI -0.4% to 1.1%). The overall three-category NRI was thus 0.1% (95% CI -2.7% to 3.5%). In the entire cohort, predicted probability of CKD progression was similar in models with and without urine NGAL. Table 3 summarizes the different metrics used to assess incremental improvement in risk reclassification.

DISCUSSION

In this large and diverse cohort of individuals with CKD, urine NGAL level was elevated in a substantial fraction of the study population. Urine NGAL levels correlated with severity of CKD at baseline as patients with lower eGFR and greater 24-hour proteinuria had higher urine NGAL levels.

Baseline urine NGAL levels correlated strongly with risk of CKD progression, defined as halving of estimated GFR or development of ESRD. The strength of this association was substantially attenuated after adjustment for *a priori* defined “traditional” risk factors, but urine NGAL remained an independent risk factor for CKD progression (with those in the highest quintile at 70% increased risk compared with those in the lowest quintile).

Our results are consistent with some but not all of the prior literature. In a study of 63 patients with type 1 diabetes mellitus (DM) and mean GFR of 87 ml/min/1.73m^2, Nielsen et al showed that elevated NGAL was predictive of more rapid decline in ^{51}Cr-EDTA measured GFR; but it was no longer an independent risk factor after adjusting for known promoters of progression.[15] In a study of 65 patients with IgA nephropathy, Peters found that urine NGAL was a risk factor of ESRD risk in univariable analysis but not after adjusting for other factors such as serum creatinine.[14] Nielson showed in another cohort of 78 type 1 DM patients that elevated urine NGAL was not related to decline in GFR during a 4-year follow-up; it was associated with the development of ESRD, but not after adjustment (albeit confidence intervals were wide).[16] Nauta found in 606 kidney transplant recipients that urine NGAL did not predict graft failure after albuminuria was accounted for.[18]

Viau et al examined 87 subjects with polycystic kidney disease who had GFR 33±20 ml/min/1.73m^2 and reported that urine NGAL levels were higher in patients who progressed to ESRD. No attempts were made to adjust for other known risk factors for CKD progression.[11] In contrast, Parikh et al[17] found that that urine NGAL levels did not correlate with changes in total kidney volume or kidney function in 209 patients with polycystic kidney disease and preserved GFR (mean 89 ml/min/1.73m^2). In a study by Wu et al of 36 patients with drug induced chronic tubulointerstitial nephritis (GFR 37±20 ml/min/1.73m^2), urine NGAL was predictive of renal function decline and the only risk factor with a p-value

<0.05 in their multivariable models.[12] Bolignano et al reported that among 96 patients with CKD of various etiologies (GFR 42±19 ml/min/1.73m^2), urine NGAL (but not proteinuria) was an independent risk factor for CKD progression.[13]

We noted that the association between baseline urine NGAL levels and risk of CKD progression was strongest within the first two years of the biomarker measurement. Even within this time frame though, urine NGAL did not substantially improve risk classification.

The major strengths of this study include the large sample size, the detailed and rigorous phenotyping of study participants, the rigorous statistical analysis (encompassing both assessment of prediction and risk reclassification) and the diversity of the study population (in terms of both race-ethnicity as well as disease etiology).

Limitations of this study include the fact that NGAL measurements were made on urine samples which were not rapidly processed after voiding under conditions fully controlled by the research team. To the extent that suboptimal handling may have resulted in random error from protein degradation or other processes, this would bias our results towards the null. The CRIC study is composed of persons who volunteered for research and so our results may not be generalizable to all CKD patients. By design, some underlying causes of CKD, such as polycystic kidney disease, were not represented. Exact underlying kidney disease etiologies were not ascertained systematically or with certainty in CRIC through means such as kidney biopsies. Urine NGAL concentration was ascertained only at one point in time. Our subgroups and the 2-year time horizon were defined post-hoc and hence those results should be regarded as hypothesis generating findings which may be due to chance. With regard to possible explanations for the latter finding, we speculate that perhaps as an injury marker, urine NGAL levels may fluctuate over time due to (subtle) acute renal insults such as excessive lowering of blood pressure, for example. The impact of these transitory insults may be most evident in the short term.

To summarize, we determined that urine NGAL levels correlated with severity of CKD and were independently predictive of CKD progression, particularly in the first two years following measurement. However, this novel marker only very modestly improved prediction of outcome events. Thus it would be premature to consider introducing urine NGAL into clinical practice. Further studies, are needed to refine our understanding, in particular, ones in which urine collection and handling conditions were optimized.

METHODS

Study population

The Chronic Renal Insufficiency Cohort (CRIC) study is a multi-center observational cohort which enrolled patients from seven clinical centers (consisting of 13 enrolling sites) located throughout the United States. Patients with reduced glomerular filtration rate (in the range of stages 2-4 CKD) between the ages of 21 and 74 were eligible for study participation. Those with polycystic kidney disease, multiple myeloma, or glomerulonephritis on active immunosuppression were excluded, as were those who had undergone kidney transplantation. Enrollment started June 2003 and ended August 2008. A total of 3939

participants were enrolled. The CRIC basic study design and baseline characteristics have been published.[19, 20]

Exposure

The CRIC protocol called for collection of a 24-hour urine specimen at baseline. Enrollees were allowed to start this timed collection at home up to a week prior to their in-person visit. They were instructed to keep the urine refrigerated or on ice prior to bringing it to the clinical center. Samples were rejected and re-collection attempted if total urine volumes were below 500 cc or collection times below 22 hours or more than 26 hours. After proper mixing, samples from the 24-hr urine were then transferred into airtight 10 mL cryovials (filled to 9 mL). The urine NGAL measurements for this analysis were made on urine aliquots stored at 4°C and then shipped overnight within 72 hours to the CRIC central lab with cold gel packs placed in the bottom of the mailing container.

Upon receipt at the central lab, the urine samples were further aliquoted into four 2mL aliquots and frozen at −80°C. The urine for the current studies therefore have undergone one freeze-thaw cycle. Urine NGAL concentration was measured using a two-step assay using Chemiluminescent Microparticle Immunoassay (CMIA) technology on an Abbott ARCHITECT i2000SR (Abbott Laboratories, Abbott Park, IL)(total imprecision was 3.8%).

All but 12 of the urine creatinines at baseline were measured on a BioTek Plate Reader ELX 808 using a Jaffe reaction with a colorimetric end point and reagents from Sigma (mean CV was 5.0%). A Roche Module P analyzer was used to quantify urine total protein using a turbidimetric reaction with benzothnium chloride (CV 5.2%).

Outcome

The primary outcome was progressive CKD, defined as a composite endpoint of incident end-stage renal disease (ESRD) or halving of estimated glomerular filtration rate (eGFR) (from baseline) using the Modification of Diet in Renal Disease (MDRD) study equation after calibration of serum creatinine.[21] ESRD was defined as receipt of chronic dialysis or kidney transplant. Ascertainment of ESRD in the CRIC study has been supplemented by cross-linkage with U.S. Renal Data System. Time to eGFR halving was imputed assuming a linear decline in kidney function between in-person annual visit measures. Follow-up for this analysis was through June 2009.

Analysis

Time-to-event analysis was conducted using Cox proportional hazards models. Recognizing that ESRD or halving of eGFR represent different degrees of loss of renal function depending on baseline renal function,[22] we adjusted for baseline eGFR in all analyses.

We also adjusted for 24-hr urine proteinuria and other potential confounders, based on the literature of known risk factors for CKD progression. For the latter, we *a prior* selected demographics (age, sex, race/ethnicity), presence or absence of diabetes mellitus status (defined by fasting blood glucose ≥126 mg/dl or random blood glucose ≥200 mg/dL or patient self-report of use of insulin or oral diabetes medication), systolic blood pressure

(SBP), body mass index (BMI), use of an angiotensin-converting enzyme inhibitor (ACE-I) or angiotensin receptor blocker (ARB) at baseline, history of cardiovascular disease (CVD), and education attainment (as a proxy for socio-economic status).[22, 23] (We also adjusted for CRIC clinical centers in final analyses).

We explored the non-linear relationship between urine NGAL and renal progression using splines[24] and discovered that by expressing urine NGAL on a natural logarithmic scale, a linear term of urine NGAL is sufficient to describe the relationship with CKD progression. For proteinuria and eGFR, the two strongest predictors for CKD progression,[25] we relaxed the linearity assumptions for their relations with progression by using quadratic splines (with one knot at the median) of eGFR and natural log transformed proteinuria. We calculated the C-statistic to measure the overall model fit.[26]

To explore whether there was effect modification, we entered multiplicative interaction terms into our statistical models for urine NGAL and proteinuria, eGFR, diabetes status, age, sex, or race.

To explore whether and how the association between urine NGAL and progression changed over time, we refit the Cox proportional hazard model assuming different hazard ratios for urine NGAL in the following four time intervals: <2, 2 to <3, 3 to <4 and >4 years. We found that the effect of NGAL was strongest over the first two years after measurement. Thus, improved model predictability due to NGAL was quantified using Net Reclassification Improvement (NRI) over a two year horizon. We calculated both category-free NRI and NRI based on three categories (*a priori* defined risks of <5%, 5-10% and >10% annual event rate)[27] for the risk of renal progression.

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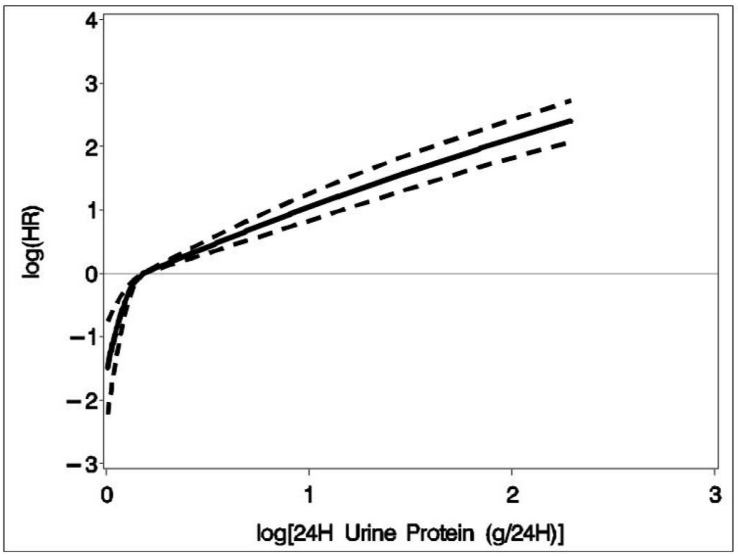

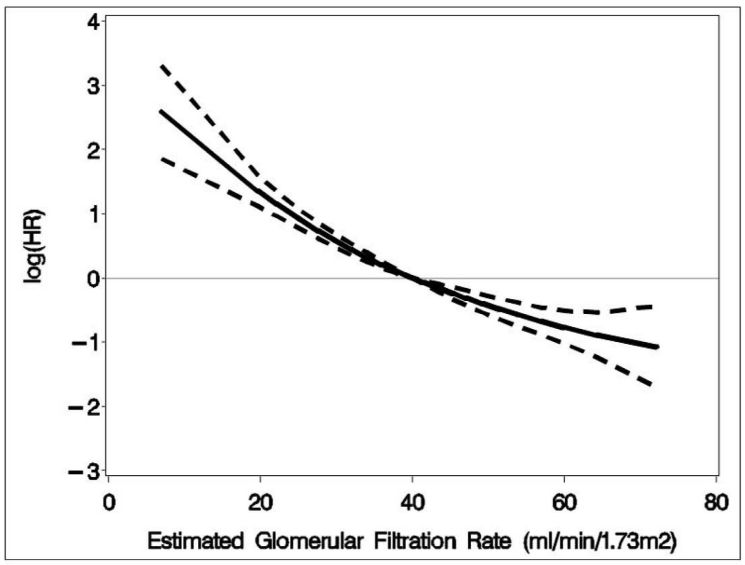

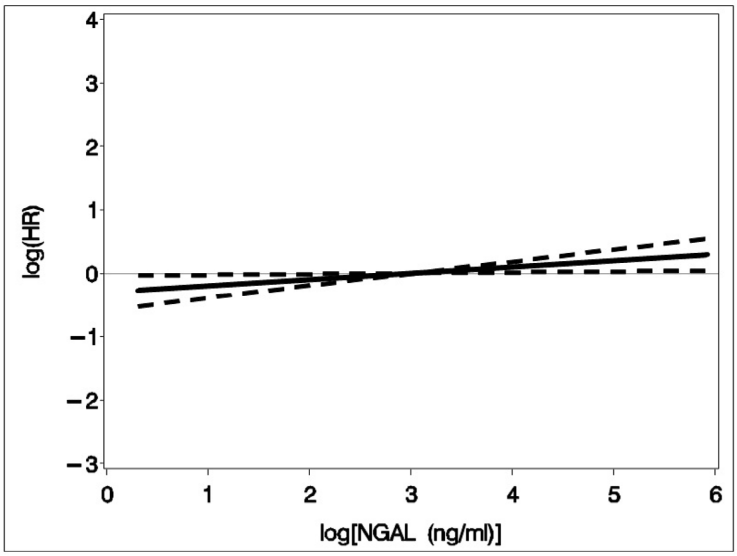

Figure 1.

A. Multivariable adjusted association between risk of progressive CKD and amount of 24-hour urine protein; log (HR) is log of the adjusted hazard ratio.

B. Multivariable adjusted association between risk of progressive CKD and estimated GFR; log (HR) is log of the adjusted hazard ratio.

C. Multivariable adjusted association between risk of progressive CKD and urine NGAL concentration; log (HR) is log of the adjusted hazard ratio.

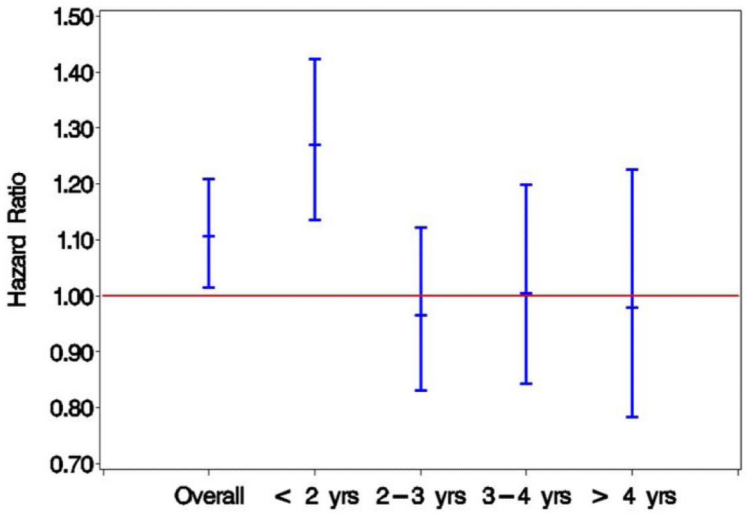

Figure 2.

Multivariable adjusted hazard ratio (HR)(per increase in log urine NGAL concentration) overall and by duration of follow-up.

Table 1

Baseline patient characteristics: overall and by quintiles of urine NGAL concentration

Urine NGAL concentration (ng/mL)								
Characteristic		***<= 6.9 (N=681)***	***>6.9 - <=12.9 (N=678)***	***>12.9 - <=22.6 (N=673)***	***>22.6 - <=49.5 (N=677)***	***> 49.5 (N=677)***	***All (N=3386)***	***P value***
Age	Mean (SD)	58.7 (10.3)	59.4 (10.0)	59.5 (10.9)	57.5 (11.3)	56.1 (11.9)	58.2 (11.0)	<.0001
Sex	Female	103 (15.1%)	258 (38.1%)	381 (56.6%)	444 (65.6%)	406 (60.0%)	1592 (47.0%)	<.0001
Race/ethnicity	Non-Hispanic White	396 (58.1%)	331 (48.8%)	263 (39.1%)	240 (35.5%)	180 (26.6%)	1410 (41.6%)	<.0001
	Non-Hispanic Black	206 (30.2%)	277 (40.9%)	314 (46.7%)	332 (49.0%)	311 (45.9%)	1440 (42.5%)	
	Hispanic	40 (5.9%)	43 (6.3%)	76 (11.3%)	83 (12.3%)	162 (23.9%)	404 (11.9%)	
Diabetes		303 (44.5%)	270 (39.8%)	304 (45.2%)	357 (52.7%)	400 (59.1%)	1634 (48.3%)	<.0001
24-hr proteinuria (gm/day)	Mean (SD)	0.3 (0.4)	0.5 (0.8)	0.7 (1.1)	1.2 (1.7)	3.1 (4.3)	1.1 (2.4)	<.0001
	Median (IQR)	0.1 (0.1-0.3)	0.1 (0.1-0.5)	0.2 (0.1-0.7)	0.3 (0.1-1.7)	1.1 (0.2-4.4)	0.2 (0.1-1.0)	
Estimated GFR (ml/min/1.73m^2)	Mean (SD)	47.9 (12.0)	45.9 (12.4)	42.3 (12.7)	40.2 (13.6)	35.6 (13.5)	42.4 (13.6)	<.0001
	Median (IQR)	48.0 (39.5-56.1)	44.7 (37.1-54.1)	41.2 (33.1-50.8)	38.0 (29.2-49.9)	33.8 (25.1-43.6)	41.6 (32.1-51.5)	
Systolic BP (mmHg)	Mean (SD)	123 (18)	126 (20)	127 (22)	131 (23)	137 (25)	129 (22)	<.0001
Diastolic BP (mmHg)	Mean (SD)	71 (12)	71 (12)	71 (13)	72 (13)	73 (14)	72 (13)	0.0002
Body Mass Index (kg/m^2)	Mean (SD)	31.2 (6.7)	31.6 (6.9)	32.3 (7.8)	33.5 (8.8)	32.5 (9.0)	32.2 (7.9)	<.0001
Characteristic		*<= 6.9 (N=681)*	*>6.9 - <=12.9 (N=678)*	*>12.9 - <=22.6 (N=673)*	*>22.6 - <=49.5 (N=677)*	*> 49.5 (N=677)*	*All (N=3386)*	*P value*
History of cardiovascular disease		233 (34.2%)	201 (29.6%)	228 (33.9%)	212 (31.3%)	242 (35.7%)	1116 (33.0%)	0.12
Use of ACE-inhibitor or ARB		499 (73.7%)	468 (69.5%)	451 (67.3%)	437 (64.9%)	429 (64.0%)	2284 (67.9%)	0.0008

Table 2

Association between quintiles of urine NGAL concentration and risk of progressive CKD (halving of eGFR or ESRD)

Quintiles of baseline urine NGAL concentration (ng/mL)	Events	Rate (per 100 person-years)	Unadjusted hazard ratio (HR) (95% confidence intervals)	Age, sex, race/ethnicity adjusted HR	Additionally adjusted for baseline eGFR and 24-hr urine	Additionally adjusted for other baseline covariates*
<= 6.9	47	1.9	Ref	Ref	Ref	Ref
> 6.9 - <= 12.9	77	3.3	1.75 (1.22-2.51)	2.01 (1.40-2.89)	1.26 (0.87-1.82)	1.37 (0.94-1.98)
> 12.9 - <= 22.6	105	4.8	2.52 (1.79-3.56)	3.15 (2.22-4.47)	1.21 (0.84-1.74)	1.24 (0.86-1.79)
> 22.6 - <= 49.5	173	8.1	4.30 (3.11-5.93)	5.72 (4.10-7.97)	1.31 (0.92-1.88)	1.39 (0.97-2.00)
> 49.5	287	16.9	9.34 (6.86-12.72)	11.65 (8.45-16.05)	1.58 (1.09-2.29)	1.70 (1.16-2.48)
HR per 1 unit increase in log (urine NGAL)			1.75 (1.66-1.85) p-value: <.0001	1.78 (1.69-1.88) p-value: <.0001	1.09 (1.001-1.18) p-value: 0.0471	1.11 (1.01-1.21) p-value: 0.0230

* diabetes mellitus status, systolic blood pressure, body mass index, use of angiotensin-converting enzyme inhibitor or angiotensin receptor blocker, history of cardiovascular disease, and education attainment

Table 3

Summary of measures of risk reclassification

		C-statistic	Category free Net Reclassification Improvement (NRI) (95% confidence interval)	3-category (<5%, 5-10% and >10% annual event rate) NRI (95% confidence interval)
Overall	Without NGAL*	0.847		
	With NGAL	0.847		
2-year time horizon	Without NGAL*	0.879	24.5% (0.4% to 38.5%)	0.1% (−2.7% to 3.5%)
	With NGAL	0.880		

*with base model already including age, sex, race, eGFR, 24-hr urine, diabetes mellitus status, systolic blood pressure, body mass index, use of angiotensin-converting enzyme inhibitor or angiotensin receptor blocker, history of cardiovascular disease, and education attainment