Supplemental Materials TOC

Blood Sample Collection and Serum isolation.

Blood sample collection was performed once for each participant at the time of consent. Freshly-drawn peripheral venous blood samples were collected into serum separator tubes and allowed to coagulate for 2 hours at room temperature in a horizontal position. The tubes were then centrifuged at 800 RCF for 15 minutes at room temperature with full acceleration and brake setting 2. The supernatant (serum) was carefully removed from each tube using a 3mL Pasteur pipette and aliquoted into 2mL screw-top Eppendorf tubes which were stored at -80 °C until use.

Immunoassay Procedure for Serum Biomarker Determinations.

Calibrator standards and controls for both multiplex arrays were re-suspended in 1mL deionized water. Controls and calibrators were rolled for 30 minutes at room temperature. The biochips were prepared by first adding 200μL assay buffer to each well and then 100μL of either calibrators, controls or samples. The biochips were incubated for 1 hour at 37°C on a gentle shaker set to 370 RPM. The supernatant was removed using a gentle flicking motion and washed twice with wash buffer, with a further 4 wash cycles completed allowing a 2-minute incubation between each wash. The biochips were dried by gently tapping them on lint-free paper and 300μL specific conjugate was added to each well. The biochips were incubated for 1 hour at 37°C on a gentle shaker at 370 RPM. Supernatant was removed and the wash steps were carried out as described above. The biochips were dried by gently tapping them on lint-free paper and 250μL chemiluminescent solution was added to each well. The biochips were incubated for 2 minutes away from light prior to analysis using the Evidence Investigator® analyser (Randox Teoranta, Co. Donegal, Ireland). For the first 7-analyte array (lower abundance proteins), serum samples were undiluted. For the second 4-analyte array, serum samples were diluted at 1:200 in sample diluent. Each day, the first carrier from each kit was used for calibration (9 wells) and the second carrier was used for controls (3 controls in duplicate). Low, medium, and high concentration controls were included during each multiplex array plate run. The inter-assay coefficient of variation was calculated using the control samples across n=13 plate runs for the 7-analyte array and n=14 plate runs for the 4-analyte array. Samples and controls were tested in duplicate.

Biomarker Selection, Assay Performance, and Validation.

As detailed in the product patent (1), biomarker selection was based on a prior exploratory cross-sectional study wherein serum concentrations of 36 biomarkers were quantified in samples from 211 healthy controls and 376 patients with CKD (n=271 with CKD stages 1/2 and n=105 with CKD stage 3). Selection of the 36 biomarkers for quantification in the original exploratory study was based on an extensive review of the literature with respect to biomarker prognostication in CKD, and the 36 biomarkers originally selected largely reflect the state of the literature when this review was conducted during circa 2012-2013. Statistical analyses were performed to determine which individual biomarkers had the best predictive value for discriminating healthy controls and patients with CKD, as well as for accurately staging CKD. Additionally, analyses were performed to evaluate the complementary predictive value of all possible double, triple, and quadruple biomarker combinations. Based on these analyses, 11 biomarkers with the highest area under the receiver operating characteristic curve for diagnosis and staging of CKD, either alone or in combination with other biomarkers, were selected for inclusion on the two multiplex arrays which were prospectively evaluated in the current study cohort.

The multiplex arrays were extensively optimised by Randox Teoranta and provided to researchers at National University of Ireland, Galway at an advanced stage of development as part of a government-funded industry-academic collaboration (grant number IP-2013-0248). Performance of the multiplex arrays was validated within the study cohort itself by calculating inter-assay coefficient of variation (**Supplemental Table 1**). Further detailed assay performance metrics for each of the 11 biomarkers are available in the product instructions for use documents, including the quantification range, sensitivity, specificity, interference, intra- and inter-assay precision, and assay recovery.

Correlation analyses for individual biomarkers included on the multiplex arrays were performed at Randox Teoranta during the development of the assays, and not necessarily on the specific multiplex array platforms evaluated in the current study. Where available, quantification of biomarkers included on the multiplex arrays was correlated with values derived from enzyme-linked immunosorbent assays (ELISAs), Clinical Chemistry platforms, or other multiplexed platforms. Such correlations were performed for higher profile biomarkers for which comparator assays were available, including NGAL, sTNFR1, sTNFR2, C-reactive protein, cystatin C, epidermal growth factor, interleukin-8, and D-dimer. Correlation analysis was not performed for FABP1, C3a-desArg, and MIP-1-alpha due to more limited availability of comparator assays.

Supplemental Table 1. Inter-Assay Coefficient of Variation of Multiplex Serum Biomarker Arrays.[a,b,c,d]

	Individual biomarkers										
	Multiplex array 1							Multiplex array 2			
	D-dimer	Epidermal growth factor	FABP1	Interleukin-8	MIP-1-alpha	sTNFR1	sTNFR2	C3a-desArg	C-reactive protein	Cystatin C	NGAL
Low concentration control	6.4	8.0	11.7	11.0	5.2	6.5	5.0	6.6	17.3	4.2	12.9
Medium concentration control	4.3	6.1	13.8	16.3	6.8	7.3	6.2	8.8	14.8	5.5	9.0
High concentration control	7.1	9.4	9.5	5.8	5.5	8.8	6.5	12.0	12.7	8.3	12.0
Mean values across all control concentrations	5.9	7.8	11.7	11.1	5.8	7.5	5.9	9.1	14.9	6.0	11.3
	Performance of each multiplex array										
	Multiplex array 1							**Multiplex array 2**			
Mean values across all biomarkers on each array	8.0							10.3			
	Overall array performance										
Mean values across both arrays	9.2										

[a]Values are given as percentage (%) inter-assay coefficient of variation.

[b]C3a-desArg = complement protein C3a (cleaved at C-terminal arginine); FABP1 = fatty acid-binding protein-1; MIP-1-alpha = macrophage inflammatory protein-1-alpha; NGAL = neutrophil gelatinase-associated lipocalin; sTNFR1 = soluble tumour necrosis factor receptor-1; sTNFR2 = soluble tumour necrosis factor receptor-2.

[c]Multiplex array 1: 7-analyte array to measure lower abundance proteins.

[d]Multiplex array 2: 4-analyte array to measure higher abundance proteins.

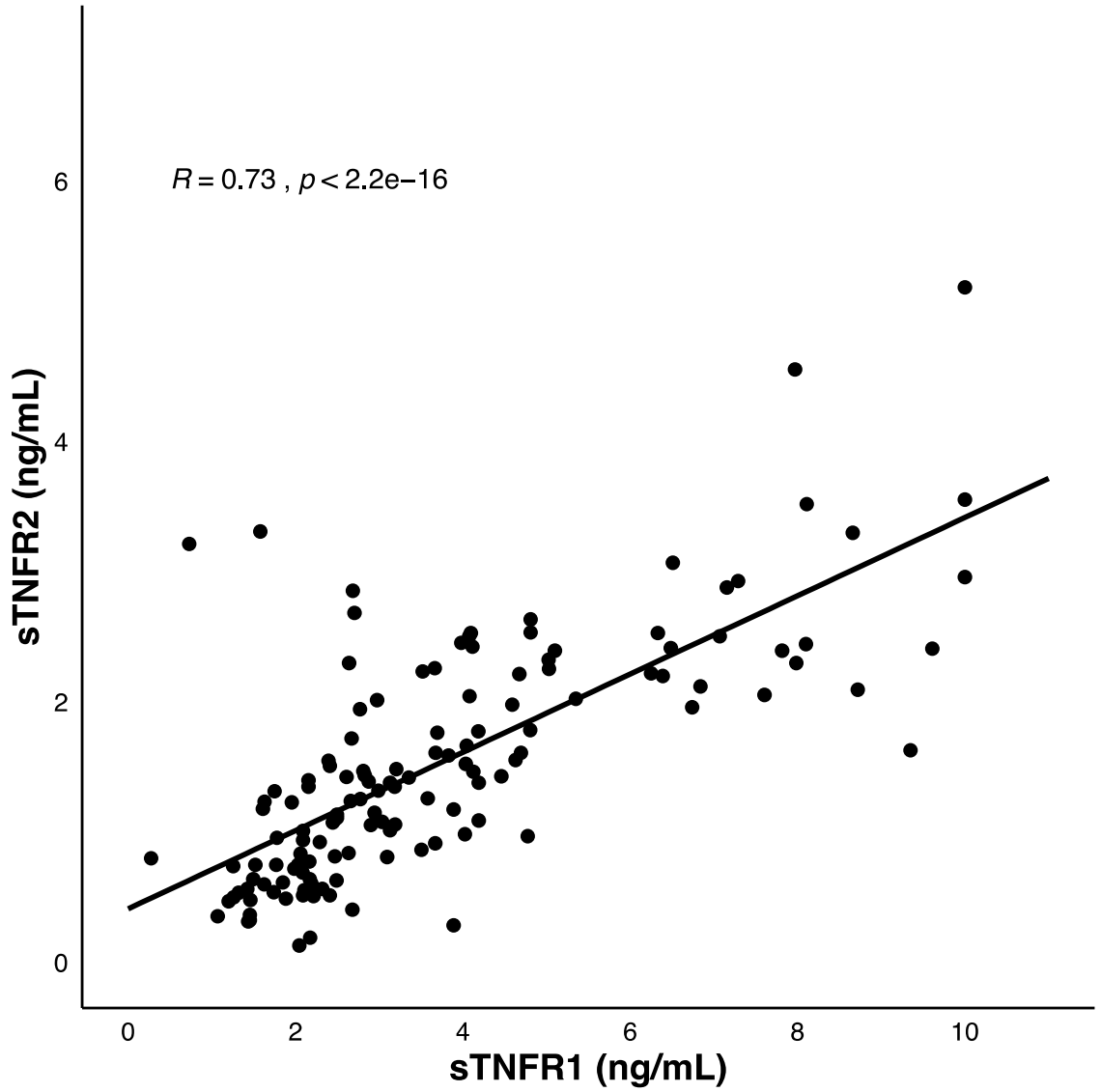
Supplemental Table 2. Characteristics of Study Participants with Available and Missing Baseline uACR Data.[a,b,c]

Characteristic	Data available (n (%))	Total cohort (n=139)	uACR available (n=113)	uACR missing (n=26)
Clinical parameters				
Age (mean±SD; years)	139 (100)	63±17	62±17	68±14
Male (n (%))	139 (100)	78 (56)	64 (57)	14 (54)
Diabetes mellitus (n (%))	139 (100)	35 (25)	31 (27)	4 (15)
Hypertension (n (%))	139 (100)	115 (83)	95 (84)	20 (77)
CKD stage (n (%))	139 (100)			
Grade 1		5 (4)	5 (4)	0 (0)
Grade 2		17 (12)	13 (12)	4 (15)
Grade 3a		22 (16)	17 (15)	5 (19)
Grade 3b		42 (30)	34 (30)	8 (31)
Grade 4		46 (33)	38 (34)	8 (31)
Grade 5		7 (5)	6 (5)	1 (4)
CKD aetiology (n (%))	139 (100)			
Diabetes		23 (17)	20 (18)	3 (12)
Hypertension		12 (9)	11 (10)	1 (4)
Glomerulonephritis		30 (22)	27 (24)	3 (12)
Congenital		8 (6)	7 (6)	1 (4)
Polycystic kidney disease		6 (4)	6 (5)	0 (0)
Obstructive		6 (4)	3 (3)	3 (12)
Interstitial		7 (5)	5 (4)	2 (8)
Other/unknown		47 (34)	34 (30)	13 (50)
Laboratory data				
Serum creatinine (mean±SD; mg/dL)	139 (100)	2.0±1.0	2.0±1.0	1.8±0.8
CKD-EPI eGFR (median [IQR]; mL/min/BSA)	139 (100)	33 [24 - 51]	33 [23 - 51]	34 [26 - 49]
Haemoglobin (mean±SD; g/dL)	138 (99)	13.0±1.7	13.0±1.7	12.9±1.8

[a]BSA = body surface area; CKD-EPI = Chronic Kidney Disease-Epidemiology Collaboration; eGFR = estimated glomerular filtration rate; IQR = interquartile range; SD = standard deviation; uACR = urine albumin-to-creatinine ratio.

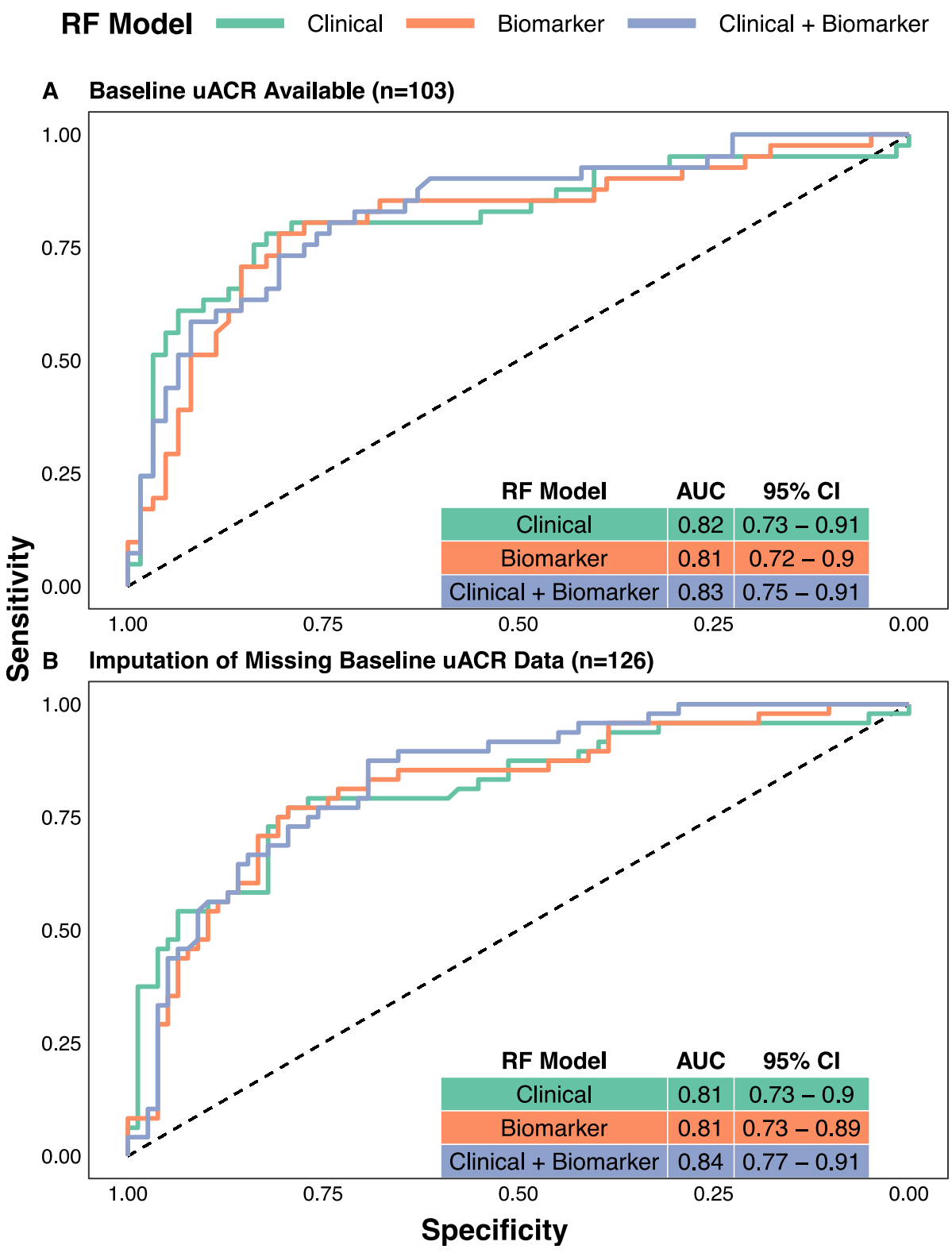
[b]Values are given as n (%) for categorical variables, or mean±SD for normally distributed continuous variables, unless otherwise indicated. Median [IQR] values are presented for continuous variables that are not normally distributed.

[c]uACR availability represents either measured uACR or calculated uACR from uPCR using the validated equation of Weaver et al. (2)



Supplemental Figure 1. Scatterplot of sTNFR1 and sTNFR2 reveals a strong positive correlation between both biomarkers.

The Pearson r correlation between sTNFR1 and sTNFR2 in the study cohort was 0.73.



Supplemental Figure 2. Sensitivity analyses of random forest classification models incorporating additional uACR adjustment illustrate added predictive value of serum biomarkers for a composite renal and mortality endpoint.

A: uACR-adjusted models were constructed for the subset of individuals with available uACR data and complete serum biomarker profiles (n=103).

Baseline uACR data was derived from both measured uACR values and from conversion of uPCR to uACR using the validated equation of Weaver et al. (2).

B: uACR-adjusted models were constructed for after imputation of missing baseline uACR data using a random forest proximity matrix (n=126).

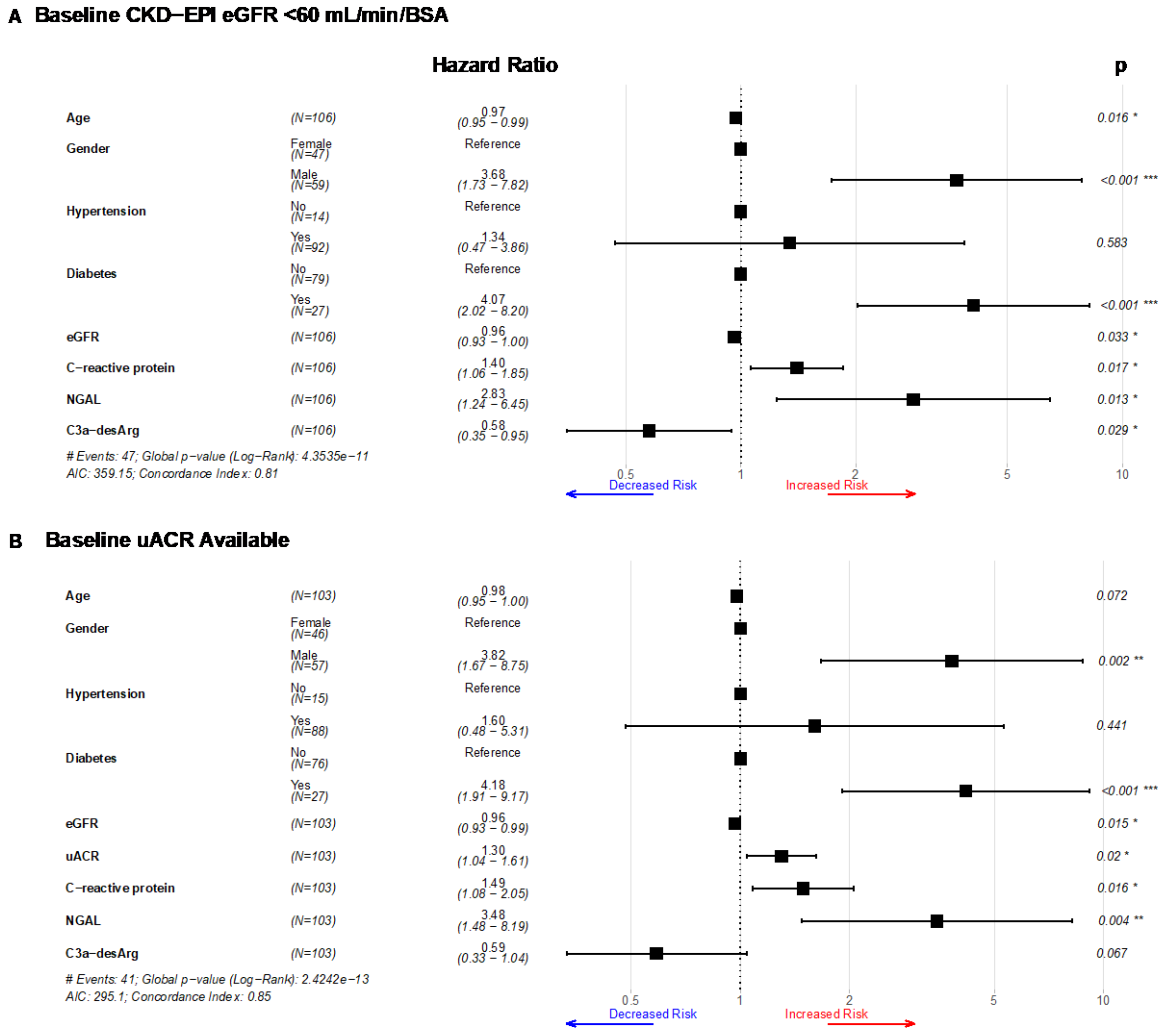
Missing baseline uACR data was imputed for 23 individuals using the function 'rfImpute' from the R package randomForest (3).

Receiver operating characteristic (ROC) curves for 3 types of random forest classification models of the composite renal and mortality endpoint: clinical variables alone (age, gender, hypertension, diabetes, baseline eGFR, and baseline uACR) (green), serum biomarkers alone (orange), and clinical variables plus serum biomarkers (purple). Panels A and B differ in the uACR data used to adjust random forest models as detailed above.

A leave-one-out cross-validation approach was implemented for the random forest models.

Optimal prediction of the composite renal and mortality endpoint was achieved in models incorporating both clinical variables and serum biomarkers.

AUC values and associated 95% confidence intervals for the 3 model types are presented in the inset tables.



Supplemental Figure 3. Sensitivity analyses examining the predictive value of serum biomarkers with respect to time to development of a composite renal and mortality endpoint (Cox proportional hazards regression).

A: Forest plot of Cox proportional hazards regression model illustrating the predictive value of serum biomarkers with respect to a composite renal and mortality endpoint in the subgroup of patients with CKD stages 3-5/eGFR <60 mL/min/BSA (n=106).

C-reactive protein (adjusted hazard ratio [aHR], 1.4; 95% confidence interval [95% CI], 1.1 to 1.9) and NGAL (aHR, 2.8; 95% CI, 1.2 to 6.5) were positively associated with time to the composite renal and mortality endpoint. C3a-desArg values were inversely associated with time to the composite renal and mortality endpoint (aHR, 0.6; 95% CI, 0.4 to 0.95).
B: Forest plot of Cox proportional hazards regression model incorporating log-transformed uACR alongside other clinical variables and serum biomarkers with respect to a composite renal and mortality endpoint (n=103).

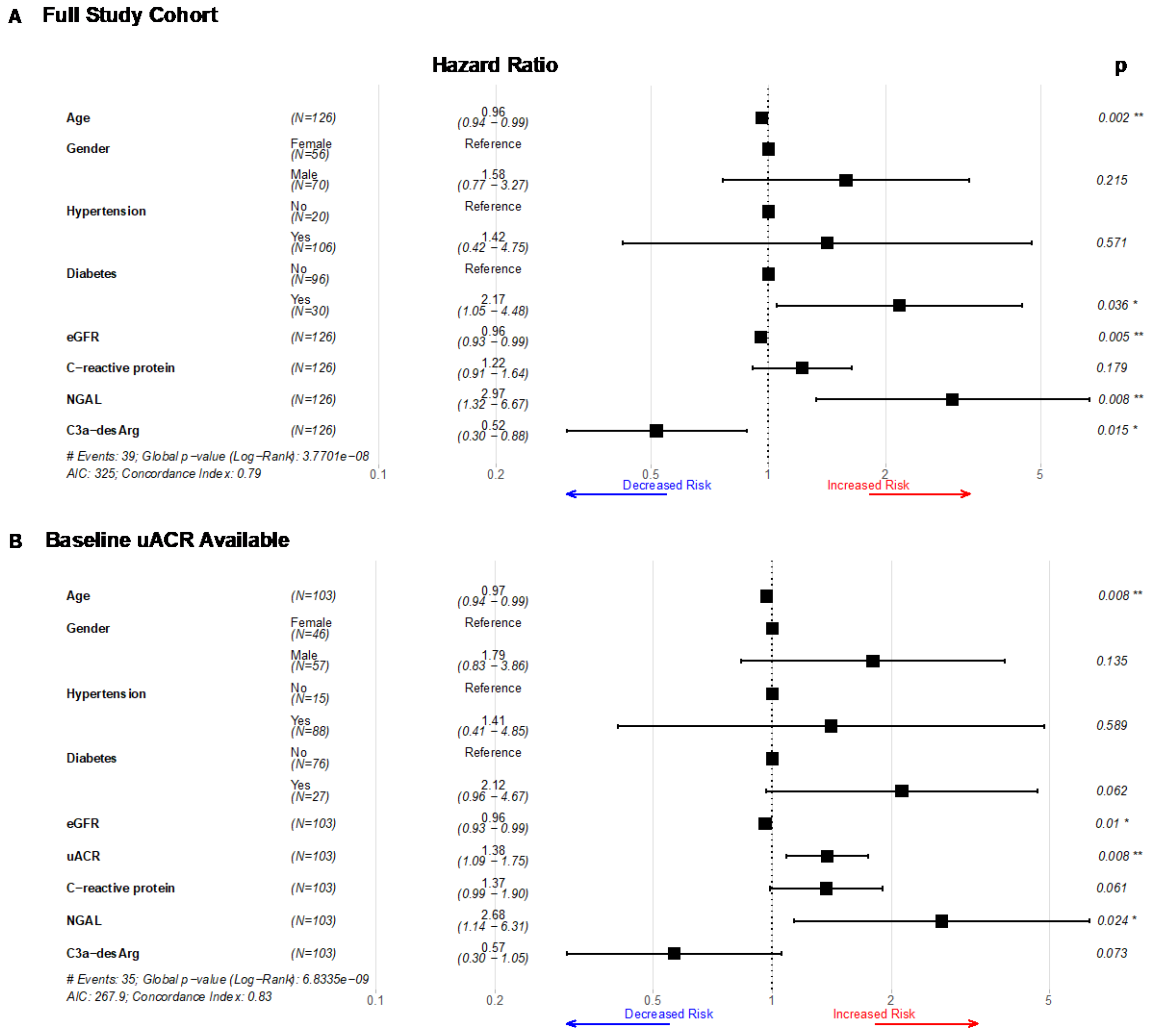
C-reactive protein (aHR, 1.5; 95% CI, 1.1 to 2.1) and NGAL (aHR, 3.5; 95% CI, 1.5 to 8.2) were positively associated with time to the composite renal and mortality endpoint. The inverse

trend between C3a-desArg and the composite renal and mortality endpoint was no longer statistically significant (aHR, 0.6; 95% CI, 0.3 to 1.04).

Biomarker values were log-transformed for modelling.

Hazard ratios from Cox models are expressed per one unit change in natural logarithm biomarker concentrations.

Composite renal and mortality endpoint: ≥40% decrease in CKD-EPI eGFR, doubling of serum creatinine, renal replacement therapy, or mortality.



Supplemental Figure 4. Sensitivity analyses examining the predictive value of serum biomarkers with respect to time to development of a renal-specific composite endpoint (Cox proportional hazards regression).

A: Forest plot of Cox proportional hazards regression model illustrating the predictive value of serum biomarkers with respect to a renal-specific composite endpoint in the full study cohort (n=126).

NGAL (adjusted hazard ratio [aHR], 3.0; 95% confidence interval [95% CI], 1.3 to 6.7) was positively associated with time to the renal-specific composite endpoint. C3a-desArg values were inversely associated with time to the renal-specific composite endpoint (aHR, 0.5; 95% CI, 0.3 to 0.88).

B: Forest plot of Cox proportional hazards regression model incorporating log-transformed uACR alongside other clinical variables and serum biomarkers with respect to a renal-specific composite endpoint (n=103).

NGAL (aHR, 2.7; 95% CI, 1.1 to 6.3) was positively associated with time to the renal-specific composite endpoint. The inverse trend between C3a-desArg and the renal-specific composite endpoint was no longer statistically significant (aHR, 0.6; 95% CI, 0.3 to 1.05).

C-reactive protein did not predict time to the renal-specific composite endpoint in the full study cohort or in the subgroup of patients with baseline uACR available.

Biomarker values were log-transformed for modelling.

Hazard ratios from Cox models are expressed per one unit change in natural logarithm biomarker concentrations.

Renal-specific composite endpoint: ≥40% decrease in CKD-EPI eGFR, doubling of serum creatinine, or renal replacement therapy.

References

1. McConnell I, Richardson C, Lamont J, FitzGerald S.P. Kidney Disease Biomarker. European Patent Office Number EP3052939B1. Filed on October 6th, 2014; granted on January 3rd, 2018. Available at: https://patents.google.com/patent/EP3052939B1/en?oq=EP+3052939B1
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3. Liaw A, Wiener M: Classification and Regression by randomForest. R News 2(3), 18-22, 2002.