Kidney360 Publish Ahead of Print, published on May 21, 2021 as doi:10.34067/KID.0007552020

Multiplex Serum Biomarker Assays Improve Prediction of Renal and Mortality Outcomes in Chronic Kidney Disease.

William P. Martin[1], Chloe Conroy[2], Serika D. Naicker[3], Sarah Cormican[3,4], Tomás P. Griffin[3,5], Md Nahidul Islam[3], Eibhlin M. McCole[6], Ivan McConnell[7], John Lamont[7], Peter FitzGerald[7], John P. Ferguson[8], Ciarán Richardson[6], Susan E. Logue[3], Matthew D. Griffin[3,4]

[1]Diabetes Complications Research Centre, School of Medicine, Conway Institute, University College Dublin, Ireland.

[2]School of Medicine, University College Dublin, Ireland.

[3]Regenerative Medicine Institute (REMEDI) at CÚRAM Centre for Research in Medical Devices, School of Medicine, National University of Ireland, Galway, Ireland.

[4]Nephrology Services, Galway University Hospitals, Saolta University Health Care Group, Galway, Ireland.

[5]Centre for Diabetes, Endocrinology and Metabolism, Galway University Hospitals, Saolta University Health Care Group, Galway, Ireland.

[6]Randox Teoranta, Meenmore, Dungloe, Co. Donegal.

[7]Randox Laboratories Limited, 55 Diamond Road, Crumlin, Co. Antrim.

[8]HRB Clinical Research Facility, Galway, Ireland.

Correspondence to:
Prof. Matthew Griffin,
Regenerative Medicine Institute (REMEDI),
CÚRAM Centre for Research in Medical Devices,
School of Medicine,
College of Medicine, Nursing and Health Sciences,
National University of Ireland, Galway,
Ireland.
+353-91-495436
matthew.griffin@nuigalway.ie

Key Points

- Incorporation of 11 serum biomarkers alongside clinical variables improved prediction of adverse CKD outcomes over 5-year follow-up.
- Patients with the triad of high sTNFR1 and NGAL coupled with low C3a-desArg had particularly high adverse event rates during follow-up.
- Biomarkers were quantified on a single, clinical-grade analyser, with potential for improved translatability to the CKD outpatient setting.

Abstract

Background: We investigated the predictive value of 11 serum biomarkers for renal and mortality endpoints in people with chronic kidney disease (CKD).

Methods: Adults with CKD (n=139) were enrolled from outpatient clinics between February 2014 and November 2016. Biomarker quantification was performed using two multiplex arrays on a clinical-grade analyser. Relationships between biomarkers and renal and mortality endpoints were investigated by random forests and Cox proportional hazards regression.

Results: The cohort was 56% male. Mean age was 63 years and median [IQR] CKD-EPI eGFR was 33 [24-51] mL/min/BSA. Fifty-six (40%) people developed a composite endpoint defined as ≥40% decline in eGFR, doubling of serum creatinine, renal replacement therapy, or death over median follow-up of 5.4 [4.7-5.7] years. Prediction of the composite endpoint was better with random forests trained on serum biomarkers compared with clinical variables (area under the curve 0.81 vs 0.78). Predictive performance of biomarkers was further enhanced when considered alongside clinical variables (area under the curve 0.83 vs 0.81

for biomarkers alone). Patients (n=27, 19%) with high soluble tumour necrosis factor receptor-1 (≥3 ng/mL) and neutrophil gelatinase-associated lipocalin (≥156 ng/mL) coupled with low complement 3a des-arginine (<2,368 ng/mL) almost universally (96%) developed the composite renal and mortality endpoint. C-reactive protein (adjusted hazard ratio, 1.4; 95% confidence interval, 1.1 to 1.8), neutrophil gelatinase-associated lipocalin (adjusted hazard ratio, 2.8; 95% confidence interval, 1.3 to 6.1) and complement 3a des-arginine (adjusted hazard ratio, 0.6; 95% confidence interval, 0.4 to 0.96) independently predicted time to the composite endpoint.

Conclusions: Outpatients with the triad of high soluble tumour necrosis factor receptor-1 and neutrophil gelatinase-associated lipocalin coupled with low complement 3a des-arginine had high adverse event rates over 5-year follow-up. Incorporation of serum biomarkers alongside clinical variables improved prediction of CKD progression and mortality. Our findings require confirmation in larger, more diverse patient cohorts.

Introduction

Chronic kidney disease (CKD) is a growing public health problem, with its prevalence increasing by 29.3% since 1990 to affect 9.1% of the global population in 2017 (1). Although morbidity and mortality rates from other non-communicable diseases have declined over the past 3 decades, no such favourable trends exist for CKD (2). Death due to cardiovascular disease is over-represented amongst people with CKD and mortality rates increase as estimated glomerular filtration rate (eGFR) declines. In a meta-analysis of 21 general population cohorts incorporating over 1.2 million participants, eGFR independently predicted mortality risk in an almost linear fashion (3).

Communicating risk of adverse outcomes to patients with CKD is challenging, particularly at earlier, typically asymptomatic disease stages. Although eGFR and urine albumin-to-creatinine ratio (uACR) are strongly predictive of adverse outcomes in epidemiological studies, intra-individual variability weakens their prognostic value in clinical practice (4, 5). Measuring multiple circulating biomarkers simultaneously has the potential to uncover subgroups of patients with CKD who have differing risks of progressive renal functional decline and mortality. However, prognostication of adverse CKD outcomes with multiple biomarkers is challenged by the strong inter-correlation between biomarkers from diverse pathways, which may result in marginal improvements in predictive performance when additional biomarkers are studied (6-8). Additionally, many biomarker studies to date have enrolled specific subgroups of patients with CKD, for example diabetic kidney disease; predictive performance of circulating biomarkers across the spectrum of CKD severity and aetiology in real-world outpatient nephrology practice is underexplored.

We aimed to evaluate the performance of two multi-analyte serum biomarker arrays in

patients with CKD. Specifically, we aimed to ascertain the relationships of the 11 biomarkers to each other and to determine their individual and combined predictive value for renal and mortality outcomes. We hypothesised that clusters of patients with differing risks of adverse CKD outcomes could be identified based on serum biomarker profiles. Furthermore, we hypothesised that incorporation of multiple biomarkers into multivariate models would improve prediction of renal and mortality endpoints over 5-year follow-up compared with clinical variables alone in people with a broad range of CKD aetiologies and severity attending a tertiary referral nephrology centre.

Materials and Methods

Study Cohort

Adults with CKD stages 1 to 5 were enrolled from nephrology outpatient clinics at Galway University Hospitals between February 2014 and November 2016. As previously described (9), inclusion criteria were: age ≥18 years, diagnosis of CKD, absence of current infection, immunosuppression, cancer, acute cardiovascular event or haematological condition other than anaemia, haemoglobin ≥10 g/dL, not on renal replacement therapy (RRT), and no prior kidney transplant.

Clinical and laboratory data were recorded from enrolment to the end of follow-up on July 15th, 2020 in a secure, password-protected, web-based clinical database (Distiller®, SlidePath, Ireland). Longitudinal measurements of serum creatinine were extracted for each participant using the eMED*Renal*® system (Mediqal H.I., Aston, UK). An IDMS-traceable creatininase assay was used to measure creatinine (10). CKD-EPI eGFR was calculated from serum creatinine using standard formulae and expressed as mL/min/body surface area

(BSA). Second and subsequent creatinine values on a given day and creatinine values subsequent to RRT initiation were excluded. Individuals with <3 eGFR values were excluded from the dataset. Interim renal outcomes (≥40% decline in Chronic Kidney Disease-Epidemiology Collaboration [CKD-EPI] eGFR and doubling of serum creatinine) were defined at their first occurrence after study enrolment compared with baseline study values. RRT was defined as first requirement for dialysis or kidney transplantation after study enrolment. All-cause mortality after study enrolment was recorded. Time to event of renal and mortality endpoints was recorded for each patient. Duration of study follow-up was calculated as time from enrolment to date of last study follow-up or death. Duration of renal functional follow-up was calculated as time from enrolment to date of last eGFR determination. Annual eGFR slopes were calculated by linear regression of eGFR over time for individuals with ≥3 eGFR determinations over ≥1 year. Median log-transformed uACR was estimated from uPCR using the validated equation of Weaver et al. (11). Four-variable kidney failure risk equation (KFRE) scores for 2- and 5-year risks of progression to kidney failure were calculated according to regional (non-North American) formulae (12).

The study was approved by the Galway University Hospitals Clinical Research Ethics Committee (reference C.A. 885) and conducted in accordance with the 1964 Helsinki declaration. Participants provided written, informed consent.

Blood Sample Collection and Serum Biomarker Determinations

Serum was isolated from peripheral venous blood samples provided at enrolment. Eleven serum biomarkers were quantified using two CKD multiplex arrays on an Evidence Investigator® immunoassay analyser (Randox Teoranta, Donegal, Ireland). As physiological serum concentrations vary significantly for the 11 included biomarkers (from pg/mL to

μg/mL), lowly and highly abundant biomarkers were quantified on separate multiplex arrays to improve assay sensitivity and performance. A 7-analyte array measured low-abundance biomarkers: epidermal growth factor (EGF), interleukin-8, soluble tumour necrosis factor receptor-1 (sTNFR1) and receptor-2 (sTNFR2), fatty acid-binding protein-1 (FABP1), D-dimer, and macrophage inflammatory protein-1-alpha (MIP-1-alpha); a 4-analyte array measured highly abundant biomarkers: C-reactive protein, cystatin C, C3a with cleaved C-terminal arginine (C3a-desArg), and neutrophil gelatinase-associated lipocalin (NGAL). Additional details on serum isolation, the immunoassay procedure, the rationale behind biomarker selection, and assay performance and validation are provided in the **Supplemental Material**. Inter-assay coefficient of variation for individual biomarkers across multiplex array plate runs used to quantify biomarkers for the study cohort is presented in **Supplemental Table 1**. Inter-assay coefficient of variation was 8.0% for the 7-analyte array and 10.3% for the 4-analyte array, resulting in an overall inter-assay coefficient of variation of 9.2%.

Statistical Analyses

Descriptive and Inferential Statistics, Logistic Regression, and Clustering

RStudio® version 4.0.0 was used for analysis. A composite renal and mortality endpoint of ≥40% decline in eGFR, doubling of serum creatinine, RRT, or death was defined and used as the primary outcome for analyses. A renal-specific composite endpoint of ≥40% decline in eGFR, doubling of serum creatinine, or RRT was used in sensitivity analyses. Biomarkers were transformed using the natural logarithm for analysis. Cohort characteristics were summarised by descriptive statistics. Independent sample t-tests, Wilcoxon rank-sum tests, and χ^2 tests assessed for differences in clinical variables and biomarkers amongst those who did and did not develop the composite renal and mortality endpoint. P <0.05 was

considered statistically significant. Univariate relationships between serum biomarkers and the composite renal and mortality endpoint were investigated using logistic regression. Odds ratios from logistic regression models are expressed per one unit change in natural logarithm biomarker concentrations. Unsupervised clustering of patients based on biomarker concentrations was performed by principal components analysis (13).

Decision Tree and Random Forest Classification Models

A supervised machine learning approach with binary classification random forests was used to explore the value of all biomarkers considered together in predicting the composite renal and mortality endpoint. Random forests have several advantages over logistic regression, including seamless handling of regressor collinearity and automatic selection and fitting of non-linear relationships and statistical interactions. A binary classification decision tree evaluating classification of the composite renal and mortality endpoint by serum biomarkers was generated using default parameters of the R function 'rpart' to illustrate the complementary information provided by multiple biomarkers (14, 15). Biomarkers were inputted to the decision tree and random forest models as continuous variables. Biomarker thresholds were selected by recursive binary splitting to maximise node purity in each tree (the number of individuals from a single class, who either did or did not develop the composite renal and mortality endpoint). Binary classification random forests (5,000 trees per model), with the composite renal and mortality endpoint as the response variable, were fit (16). Three model types were created: clinical variables alone (age, gender, hypertension, diabetes, and eGFR), biomarkers alone, and clinical variables plus biomarkers. A leave-one-out cross-validation approach was implemented, which consisted of excluding one individual in turn from training the random forest model. Subsequently, the trained random

forest model predicted the class of the individual excluded during model training. This process was repeated iteratively for each individual in the dataset such that a predicted class was assigned to each study participant by each of the three random forest model types. The leave-one-out cross-validation procedure was only performed for random forest models and not for other model types.

Area under the curve (AUC) values were calculated for each of the three model types using the predicted probability of having developed the composite renal and mortality endpoint and the individual's actual recorded composite renal and mortality endpoint status as inputs to the function 'roc' in the R package pROC (17). Receiver operating characteristic curves were plotted using the function 'ggroc'. Model performance metrics (sensitivity, specificity, positive predictive value, and negative predictive value) were calculated across three probability thresholds (10%, 30%, and 50%) for labelling patients as having developed the composite renal and mortality endpoint. For example, an individual with a predicted probability of the composite renal and mortality endpoint by a random forest model of 45% would be labelled as having developed the composite renal and mortality endpoint by the first two thresholds evaluated, but not the latter. An estimate of variable importance to the random forest models (mean decrease in accuracy) was calculated from mean values of each model iteration during the leave-one-out cross-validation procedure.

Cox Proportional Hazards Regression Models

Multivariable Cox proportional hazards regression models were created to investigate relationships between biomarkers and time to renal and mortality and renal-specific composite endpoints. The primary Cox model analysis investigated the value of biomarkers when considered in addition to clinical variables in predicting time to the composite renal and mortality endpoint. For this analysis, two models were constructed: a clinical model

adjusting for age, gender, hypertension, diabetes, and eGFR; and a clinical plus biomarker model additionally incorporating all log-transformed biomarker values. Backward elimination of non-significant biomarker effects from the clinical plus biomarker model was subsequently performed using stepAIC (18). Biomarkers for which the p-values for their hazard ratios were greater than 0.05 were manually excluded from the Akaike Information Criterion (AIC)-selected model to create a final parsimonious clinical plus biomarker model. Clinical variables were manually retained in the final model. Hazard ratios from Cox models are expressed per one unit change in natural logarithm biomarker concentrations. Comparisons of Cox model adequacy (clinical model versus clinical plus biomarker model) were assessed using likelihood ratio χ^2 tests.

Cox models were constructed using the R package survival (19). The R package survminer was used to create Forest plots and to plot fully adjusted survival curves from the multivariate clinical plus biomarker Cox model for the composite renal and mortality endpoint according to biomarker tertiles, which were calculated using the function 'ntile' from the R package dplyr (20, 21). We tested each Cox model for proportionality assumptions using Schoenfeld residuals. AIC values for fitted Cox models were obtained using extractAIC (22).

Sensitivity Analyses

A series of sensitivity analyses was performed to further evaluate the predictive value of serum biomarkers incorporated in random forest and Cox models outlined above. Random forest models trained on clinical variables, alone or in combination with biomarkers, were additionally adjusted for baseline uACR. Baseline uACR was included in random forest models in two separate analyses: firstly, random forest models were performed in the

subgroup with baseline uACR data available and secondly, after imputation of missing baseline uACR data using the function 'rfImpute' in the R package randomForest (16). Imputation of missing uACR data was only performed for sensitivity analyses involving random forests.

With respect to the final parsimonious clinical plus biomarker Cox model of the composite renal and mortality endpoint outlined above, the predictive value of biomarkers in this model was separately evaluated in Cox models in the subgroup with CKD stages 3-5/eGFR <60 mL/min/BSA and in the subgroup with baseline uACR data available. The value of these biomarkers for predicting time to a renal-specific composite endpoint was also evaluated using Cox models, both in the full study cohort and in the subgroup with baseline uACR data available.

Results

Baseline Characteristics and Serum Biomarker Concentrations

Baseline characteristics and serum biomarker concentrations of the study cohort (n=139) stratified by development (n=56) or not (n=83) of the composite renal and mortality endpoint are presented in **Table 1**. The study population had a mean age of 63 years, 56% were male, and median [IQR] eGFR was 33 [24 - 51] mL/min/BSA. Of those sampled, study participants had moderate proteinuria with a median [IQR] uACR of 127 [22 - 479] mg/g and uPCR of 327 [124 - 814] mg/g. After calculating uACR from uPCR (11), baseline uACR data were available for 113 (81%) individuals for whom the median [IQR] uACR was 144 [22 - 532] mg/g. Characteristics of individuals with available and missing baseline uACR data (after conversion of uPCR to uACR where available) are presented in **Supplemental Table 2**. Those with missing uACR data trended to be older (68$\pm$14 vs 62$\pm$17 years, p=0.06);

otherwise no significant differences between those with and without baseline uACR data were observed. Over 25% and 80% of the study cohort had diabetes mellitus and hypertension, respectively, while glomerulonephritis (22%) and diabetic kidney disease (17%) were the two most documented CKD aetiologies. Median [IQR] 2- and 5-year risks of progression to kidney failure were 1.4 [0.3 – 6.5] and 5.4 [1.2 – 23.1] % respectively.

Compared with individuals who did not develop the composite renal and mortality endpoint, those who did were older (67±15 vs 60±17 years, p=0.01), more likely to be male (71% vs 46%, p=0.005), had a higher prevalence of diabetes mellitus (43% vs 13%, p<0.001), lower eGFR (26 [18 - 34] vs 43 [31 - 60] mL/min/BSA, p<0.001), higher uACR (283 [83 - 993] vs 104 [19 - 269] mg/g, p<0.001), and higher 5-year KFRE scores (22.5 [6.1 – 55.1] vs 2.1 [0.3 – 9.0] %, p<0.001). Concentrations of several serum biomarkers were higher in those who developed the composite renal and mortality endpoint, including sTNFR1, sTNFR2, NGAL, cystatin C, and to a lesser extent C-reactive protein, FABP1, and MIP-1-alpha. Conversely, concentrations of EGF and C3a-desArg were lower in those who developed the composite renal and mortality endpoint. Of note, the median sTNFR2 concentration (1.4 [0.8 – 2.2] ng/mL) was lower than that of sTNFR1 (3.0 [2.1 – 4.6] ng/mL) in the study cohort, which is the opposite to what has been observed in several studies (23-25). However, sTNFR1 and sTNFR2 concentrations remained strongly correlated with each other (Pearson r correlation 0.73, p<0.001) (**Supplemental Figure 1**).

Incidence of Renal and Mortality Endpoints

Median [IQR] duration of study follow-up was 5.4 [4.7 – 5.7] years. Median duration of renal functional follow-up (study enrolment to date of final eGFR determination) was 4.7 [3.5 – 5.2] years, with participants having a median of 22 [12 - 34] eGFR values (**Table 2**). The

median rate of decline in eGFR was -0.9 [-2.3 – 0.5] mL/min/BSA/year. Renal functional decline was greater in those who developed the composite renal and mortality endpoint compared with those who did not (-2.2 [-3.9 - -1.4] vs 0 [-1 – 1.6] mL/min/BSA/year, p<0.001). Relevant study endpoints including ≥40% decline in CKD-EPI eGFR, doubling of serum creatinine, RRT, and death occurred in 38 (68%), 14 (25%), 21 (38%), and 15 (27%), respectively. Fifty-six (40%) individuals developed the composite renal and mortality endpoint while 47 (34%) developed the renal-specific composite endpoint.

Univariate Relationships Between Serum Biomarkers and the Composite Endpoint

Figure 1 presents biomarkers with significant associations with the composite renal and mortality endpoint by univariate logistic regression. EGF (odds ratio [OR], 0.5; 95% confidence interval [95% CI], 0.3 to 0.9) and C3a-desArg (OR, 0.6; 95% CI, 0.4 to 0.96) were inversely associated with, while MIP-1-alpha (OR, 1.8; 95% CI, 1.2 to 2.8) and C-reactive protein (OR, 1.6; 95% CI, 1.2 to 2.2) were positively associated with, the composite renal and mortality outcome. Furthermore, sTNFR1 (OR, 25.9; 95% CI, 8.6 to 94.5), sTNFR2 (OR, 13.7; 95% CI, 4.5 to 47.4), NGAL (OR, 4.8; 95% CI, 2.4 to 10.6), and cystatin C (OR, 10.4; 95% CI, 4.0 to 31.5) were strongly associated with the composite renal and mortality endpoint.

Clustering of Patients Based on Serum Biomarkers

Unsupervised clustering of patients by principal components analysis identified shifts in biomarkers by CKD stage and by the composite renal and mortality endpoint (**Figure 2**, panels A and B), with the loading plot (**Figure 2**, panel C) illustrating which biomarkers were important in this regard. Expression of several biomarkers clustered together in a predictable fashion based on a priori knowledge. sTNFR1 and sTNFR2 clustered together, as did two biomarkers which were inversely associated with the composite renal and mortality

endpoint: EGF and C3a-desArg. Patients separated based on biomarker expression along principal components 1 and 2 by both CKD stage and by development of the composite renal and mortality endpoint. Biomarkers clustered in the upper left corner (EGF and C3a-desArg) were more strongly expressed in earlier stage CKD and in those who did not develop the composite renal and mortality endpoint (**Figure 2**, panel C and **Table 1**). Conversely, biomarkers in the far right (NGAL, cystatin C, sTNFR1, and sTNFR2) were more strongly expressed in advanced CKD and in those who developed the composite renal and mortality endpoint (**Figure 2**, panel C and **Table 1**).

Prediction of a Renal and Mortality Composite Endpoint by Serum Biomarkers using Supervised Machine Learning (Random Forest Classification Models)

C3a-desArg reclassified risk of the composite renal and mortality endpoint amongst those with high sTNFR1 and NGAL (**Figure 3**, panel A). Those with high sTNFR1 (≥3 ng/mL), high NGAL (≥156 ng/mL), but also high C3a-desArg (≥2,368 ng/mL) had a 44% risk of the composite renal and mortality endpoint. Conversely, individuals with high sTNFR1 (≥3 ng/mL), high NGAL (≥156 ng/mL), and low C3a-desArg (<2,368 ng/mL), which accounted for 19% of the cohort, almost universally (96%) developed the composite renal and mortality endpoint.

Receiver operating characteristic (ROC) curves and AUC values for classification of the composite renal and mortality endpoint by random forests trained on clinical variables alone, biomarkers alone, and clinical variables plus biomarkers are presented in **Figure 3**, panel B. An incremental improvement in predictive performance was observed between models trained on biomarkers compared with those trained on clinical variables (AUC 0.81 vs 0.78). Predictive performance was further enhanced after inclusion of clinical variables

alongside biomarkers to train the models (AUC 0.83 vs 0.81 for biomarkers alone). Baseline eGFR was the most important predictor of the composite renal and mortality endpoint in models trained on clinical variables alone, while, when incorporated alongside biomarkers, eGFR and cystatin C were ranked as the third and fourth most important variables, respectively (**Figure 3**, panels C-E). sTNFR1 and NGAL were the 2 most important variables to prediction of the composite renal and mortality endpoint, both when biomarkers were considered alone and alongside clinical variables.

Additional random forest performance metrics stratified by three predicted probability thresholds (10%, 30%, and 50%) for classifying the composite renal and mortality endpoint are presented in **Table 3**. At a low (10%) predicted probability threshold, inclusion of serum biomarkers improved sensitivity for classification of the composite endpoint at the expense of reduced specificity. At the 30% predicted probability threshold, models trained on serum biomarkers again improved sensitivity for classification of the composite endpoint while also achieving specificity values comparable to models trained on clinical variables alone. At both of these thresholds, highest sensitivity for classification of the composite endpoint was observed when serum biomarkers were considered alongside clinical variables. At the 50% predicted probability threshold, favourable trends towards improved sensitivity and specificity were observed in models trained on serum biomarkers, albeit the absolute magnitude of improvement was smaller than at lower predicted probability thresholds.

Sensitivity analyses were performed to test the extent to which improved classification of the composite renal and mortality endpoint by random forest models incorporating serum biomarkers persisted after inclusion of uACR alongside other clinical variables. While AUC values of random forest models trained on clinical variables alone did improve after

incorporation of baseline uACR using two different approaches, the highest AUC values continued to be observed in random forest models trained on both clinical variables plus serum biomarkers (**Supplemental Figure 2**, panels A-B).

Prediction of Time to Renal and Mortality and Renal-Specific Composite Endpoints by Serum Biomarkers (Cox Models)

The AIC value for the Cox model incorporating all clinical and biomarker variables was 382.92, which reduced to 374.83 after stepwise backward elimination of six biomarkers in the following order: epidermal growth factor, FABP1, cystatin C, interleukin-8, MIP-1-alpha, and sTNFR2. Three biomarkers were included in the final clinical plus biomarker model: C-reactive protein, NGAL, and C3a-desArg. Compared with a Cox model incorporating only clinical variables (AIC 385.31), this parsimonious clinical plus biomarker model (AIC 375.86) improved prediction of time to the composite renal and mortality endpoint (p=0.001). C-reactive protein (adjusted hazard ratio [aHR], 1.4; 95% CI, 1.1 to 1.8) and NGAL (aHR, 2.8; 95% CI, 1.3 to 6.1) were positively associated with the composite renal and mortality endpoint (**Figure 4**, panel A). C3a-desArg was inversely associated with the composite renal and mortality endpoint (aHR, 0.6; 95% CI, 0.4 to 0.96). **Figure 4**, panels B-D present survival without the composite renal and mortality endpoint in the fully adjusted multivariate clinical plus biomarker Cox model stratified by tertiles of C-reactive protein (B), NGAL (C) and C3a-desArg (D).

Several sensitivity analyses were performed to further interrogate these findings. In Cox models performed in the subgroup of patients with CKD stages 3-5/eGFR <60 mL/min/BSA, serum biomarkers improved prediction of time to the composite renal and mortality endpoint (p=0.001). C-reactive protein, NGAL, and C3a-desArg remained independently

predictive of time to the composite renal and mortality endpoint, with hazard ratios similar to those obtained in the full study cohort observed (**Supplemental Figure 3**, panel A). In Cox models additionally adjusted for uACR, which were performed in the subgroup of patients with baseline uACR available, serum biomarkers improved prediction of time to the composite renal and mortality endpoint (p=0.001). 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) remained predictive of the composite renal and mortality endpoint (**Supplemental Figure 3**, panel B).

Sensitivity analyses were also performed to test the predictive value of the serum biomarkers with respect to a renal-specific composite outcome, defined as ≥40% decline in CKD-EPI eGFR, doubling of serum creatinine, or RRT. Compared with a Cox model incorporating only clinical variables (AIC 329.01), the clinical plus biomarker model (AIC 325.00) improved prediction of time to the renal-specific composite endpoint (p=0.02). NGAL (aHR, 3.0; 95% CI, 1.3 to 6.7) and C3a-desArg (aHR, 0.5; 95% CI, 0.3 to 0.9) independently predicted time to the renal-specific composite endpoint (**Supplemental Figure 4**, panel A). In Cox models additionally adjusted for uACR, serum biomarkers that improved prediction of time to the renal-specific composite endpoint (p=0.03). NGAL (aHR, 2.7; 95% CI, 1.1 to 6.3) remained predictive of the renal-specific composite endpoint (**Supplemental Figure 4**, panel B).

Discussion

This study provides insight into the value of 11 serum biomarkers measured using two multiplex biochip arrays in the identification of outpatients with CKD at high-risk of accelerated renal functional decline and mortality. Biomarkers were quantified on a single, clinical-grade platform, which offers a route for improved translatability to the CKD

outpatient setting where biomarkers may enhance prognostication afforded by existing clinical risk prediction tools (12, 26). Global shifts in serum biomarker profiles, reflecting changes in both protective factors and injury markers, were evident with declining kidney function and in those who subsequently developed a composite renal and mortality endpoint defined on the basis of CKD progression, need for RRT, or death. We demonstrate, using random forests and multivariate Cox models, that incorporation of biomarkers alongside clinical variables improves prediction of CKD progression and mortality.

We employed diverse statistical methods including univariate logistic regression (for ease-of-interpretability and visual representation), principal components analysis (to illustrate clustering of patients based on serum biomarker profiles), random forest models (to accurately characterise the predictive value of the biomarkers for adverse outcomes, not least due to seamless handling of regressor collinearity), and Cox models (to incorporate time-to-event data). Replication of the overarching results (improved predictive performance of models incorporating serum biomarkers for adverse CKD outcomes) using a variety of statistical approaches adds to the robustness of the present study findings.

Annual decline in kidney function was moderate in our study cohort at -0.9 mL/min/BSA/year, which was significantly lower than a local cohort of patients with type 2 diabetic kidney disease (mean eGFR 47 mL/min/BSA) attending our hospital for multi-disciplinary CKD care, in whom eGFR trajectories ranged from -6 mL/min/BSA/year pre-intervention to -3 mL/min/BSA/year post-intervention (10). However, the latter cohort of patients exclusively had type 2 diabetes of median 10 years' duration and were enrolled up to a decade before patients in the current study (10). eGFR slope trajectory of the current cohort was more similar to, albeit still lower than, a group of patients with type 2 diabetes and CKD (mean eGFR 42 mL/min/BSA) enrolled in Dublin, Ireland during 2014-2015 in whom

annual change in eGFR was -2 mL/min/BSA/year (27). Thus, improvements in CKD management and a lower prevalence of diabetes (25%) in the present study cohort may explain the modest rates of renal functional decline observed.

Interestingly, patients with the triad of high sTNFR1 (≥3 ng/mL) and NGAL (≥156 ng/mL) coupled with low C3a-desArg (<2,368 ng/mL) almost universally (96%) developed an adverse renal outcome or died during follow-up. This finding requires validation in a larger external cohort, but does suggest that this biomarker signature may be useful for enrolment of high-risk patients with CKD into prospective studies and may also prove useful as a means of guiding treatment intensification in nephrology practice (28). The discriminant value of sTNFR1 and NGAL for adverse outcomes was improved by C3a-desArg, highlighting that proteins with diverse functions in innate immune responses and inflammation may have complementary prognostic benefit.

To the best of our knowledge, circulating C3a-desArg has not previously been explored as a biomarker of adverse CKD outcomes. C3a-desArg, also known as acylation-stimulating protein, is an adipokine that binds to the complement 5a receptor-2 to regulate metabolic processes, specifically stimulation of triglyceride accumulation and synthesis in adipocytes and glucose uptake in pancreatic β-cells (29-31). The majority of C3a-desArg is produced by the alternative complement pathway (32). However, adiponectin activates C1q, which generates C3a and C5a fragments via classical pathway activation (33). Increased plasma C3a-desArg has been documented in patients with nephrotic syndrome, and may be implicated in the pathogenesis of the associated dyslipidaemia (34, 35). In the present study, circulating C3a-desArg was inversely associated with adverse CKD outcomes. We hypothesise that decreased C3a-desArg lowers glucose uptake and decreases clearance of triglycerides and fatty acids, thereby promoting glucose intolerance, hyperinsulinaemia, and

renal glucotoxicity and liptoxicity (36). Decreased C3a-desArg may partly occur due to obesity-associated hypoadiponectinaemia (33, 36). By extension, adverse renal outcomes in those with low C3a-desArg in our cohort may be partly accounted for by loss of the protective effects of adiponectin on glomerular podocytes – if so, this may be reversed by intentional weight loss strategies (37).

Random forests trained on serum biomarkers were superior to models trained on clinical variables in terms of prognosticating CKD progression and death. Predictive performance of the biomarkers was further enhanced when incorporated alongside clinical variables. sTNFR1 and NGAL were the two most important biomarkers to classification by random forests, reaffirming their predictive value which has been demonstrated across multiple cohorts (23-25, 27, 38-40). C-reactive protein ranked as the fifth most important variable to random forest classification of the composite renal and mortality endpoint, both in models trained on serum biomarkers alone and in models trained on both clinical variables and biomarkers, which further endorses its role in prognosticating the risk of renal functional decline in CKD (41, 42). Both C-reactive protein and NGAL independently predicted time to the composite renal and mortality endpoint, which persisted after additional adjustment for baseline albuminuria and in the subgroup of patients with baseline eGFR <60 mL/min/BSA. Serum NGAL also independently predicted time to a renal-specific composite endpoint, which persisted after additional adjustment for albuminuria.

The study cohort had a high prevalence of glomerulonephritis at 22%. Indeed, glomerulonephritis was the most documented CKD aetiology in the study cohort. A significant proportion of biomarker research in CKD has focused on the subgroup of patients with DKD, although the CRIC cohort has investigated biomarker prognostication in study populations with CKD who do not exclusively have diabetes (43). Immunosuppressive

therapy in people with glomerulonephritis has the potential to modify associations between biomarkers and adverse CKD outcomes (44), however active immunosuppression was an exclusion criterion for the present study cohort. Several of the biomarkers which demonstrated the strongest independent predictive value for adverse CKD outcomes in the present study cohort have also demonstrated associations with histopathological markers of disease severity and risk of renal functional decline in biopsy-proven glomerular diseases such as IgA nephropathy and lupus nephritis, including sTNFR1, NGAL, and C-reactive protein (45-48).

Absolute sTNFR2 concentrations were lower than those of sTNFR1 in the study cohort, which is in contrast to other studies evaluating the prognostic value of both biomarkers in patients with CKD (23-25). At the time of development of the multiplex arrays, limited international reference material was available by which to standardise and harmonise the assays. More generally, assay standardisation and harmonisation remains a significant challenge in biomarker research (49). Nevertheless, the multiplex arrays used in the current study are specific for the 11 target biomarkers contained therein. Indeed, sTNFR1 and sTNFR2 concentrations were strongly correlated with each other and the two biomarkers clustered together by principal components analysis. Furthermore, sTNFR1 and sTNFR2 were the two biomarkers which were most strongly associated with development of the composite renal and mortality endpoint by univariate logistic regression, and both biomarkers ranked as important variables to correct classification of the composite renal and mortality endpoint by random forest models. Thus, the multiplex arrays are specific for both sTNFR1 and sTNFR2 targets. Although quantification of sTNFR2 by the multiplex arrays appears to provide absolute serum concentrations that are lower than those reported by

others using stand-alone immunoassays, this did not affect interpretation of the prognostic value of either sTNFR1 or sTNFR2 in the study cohort.

Our study cohort was relatively small which limited statistical power, but event rates of renal and mortality outcomes were high and findings were replicated using random forests and Cox models. Ethnicity data were missing, although the majority (>95%) of patients attending nephrology clinics in Ireland are Caucasian (10, 50). The predictive value of our multiplex arrays requires validation in a larger, more ethnically diverse cohort. Additionally, a head-to-head comparison of the value of the KFRE equation with that of the multiplex biomarker arrays for prediction of kidney failure should be performed in a subsequent, larger-scale study (12). Furthermore, prediction of incident CKD by the multiplex biomarker arrays in populations without established CKD should be evaluated and compared with existing clinical risk prediction tools such as the equations developed by the CKD Prognosis Consortium (26). Baseline albuminuria data were missing for over half of the cohort. We calculated log-transformed uACR from uPCR using a validated equation (11), providing uACR values for over 80% of participants. Individuals with missing uACR data trended to be older but there were no significant differences between those with available and missing uACR data in terms of CKD stage or aetiology, suggesting that uACR data was largely missing at random. Furthermore, we performed sensitivity analyses to demonstrate persistence of the predictive value of serum biomarkers in random forest and Cox models despite additional adjustment for albuminuria.

In summary, simultaneous measurement of 11 serum biomarkers using novel multiplex biochip array technology was technically feasible and robust. Parallel assessment of multiple biomarkers provided complementary prognostic value. In particular, patients with the triad of high sTNFR1, high NGAL, and low C3a-desArg almost universally developed an adverse

renal endpoint or died over 5-year follow-up. Incorporation of serum biomarkers alongside clinical variables improved prediction of CKD progression and mortality. Our findings provide a strong basis for focusing the content of multi-analyte biomarker panels as risk prediction tools in the CKD outpatient setting, and for confirmation of their clinical value and cost-effectiveness in larger, more diverse patient cohorts.

Abbreviations

95% CI: 95% confidence interval

aHR: adjusted hazard ratio

AIC: Akaike Information Criterion

AUC: area under the curve

BSA: body surface area

C3a-desArg: complement protein 3a des-arginine (cleaved at C-terminal arginine

CKD: chronic kidney disease

CKD-EPI: Chronic Kidney Disease-Epidemiology Collaboration

EGF: epidermal growth factor

eGFR: estimated glomerular filtration rate

FABP1: fatty acid-binding protein-1

IQR: interquartile range

KFRE: kidney failure risk equation

MIP-1-alpha: macrophage inflammatory protein-1-alpha

NGAL: neutrophil gelatinase-associated lipocalin

OR: odds ratio

RRT: renal replacement therapy

SD: standard deviation

sTNFR1: soluble tumour necrosis factor receptor-1

sTNFR2: soluble tumour necrosis factor receptor-2

T1: tertile 1

T2: tertile 2

T3: tertile 3

uACR: urine albumin-to-creatinine ratio

uPCR: urine protein-to-creatinine ratio

Disclosures

E.M. McCole and C. Richardson are employed by Randox Teoranta and do not own any shares. I. McConnell and J. Lamont are employed by Randox Laboratories Limited and do not own any shares. J. Lamont is named as an inventor on a patent filed on the results. P. Fitzgerald reports the following: Ownership Interest: Randox Laboratories Limited. T. Griffin reports the following: Honoraria: Novonordisk, Sanofi. M. Griffin reports the following: Honoraria: American Society of Nephrology, National Institutes of Health, Hebei Medical University, China; Scientific Advisor or Membership: Editorial Boards for JASN, Kidney International, Transplantation, Frontiers in Antigen Presenting Cell Biology and Frontiers in Renal Pharmacology, Section Editor- Mayo Clinic Proceedings. All remaining authors have nothing to disclose.

Funding

Primary funding for the study derived from an Enterprise Ireland Innovation Partnership grant with co-funding from Randox Teoranta Ltd. (grant number IP-2013-0248, MDG and SL). WPM's and SC's contributions were performed within the Irish Clinical Academic Training (ICAT) Programme, supported by the Wellcome Trust and the Health Research Board (Grant Number 203930/B/16/Z), the Health Service Executive National Doctors Training and Planning and the Health and Social Care, Research and Development Division, Northern Ireland. SDN was supported by a Molecular Medicine Ireland Clinical and Translational Research Scholarship funded by the Irish Government's Programme for Research in Third Level Institutions, Cycle5 and by the College of Medicine, Nursing and Health Sciences of the National University of Ireland Galway. TPG was supported by a Hardiman Scholarship from the College of Medicine, Nursing and Health Science of the National University of Ireland, Galway and a bursary from the Irish Endocrine Society/Royal College of Physicians of Ireland. JF is supported by a Health Research Board Emerging Investigator Award (grant number EIA 2017-017). The work was also supported by grants from the European Commission [Horizon 2020 Collaborative Health Project NEPHSTROM (grant number 634086; WPM, TPG, and MDG) and from Science Foundation Ireland [REMEDI Strategic Research Cluster (grant number 09/SRC-B1794; MDG) and CÚRAM

Research Centre (grant number 13/RC/2073; MDG)] and by the European Regional Development Fund.

Acknowledgments

The authors wish to sincerely thank all of the patients who participated in the study and acknowledge, with gratitude, the doctors, nurses, and phlebotomists at Merlin Park University Hospital, Galway who contributed to enrolment of study subjects and collection of blood samples. The authors acknowledge the support of the UCD School of Medicine Summer Student Research Awards (SSRA) programme in facilitating CC's contribution to the study, including data collection and interpretation.

Author Contributions:

W Martin: Conceptualization; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Writing - original draft; Writing - review and editing

C Conroy: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing - original draft; Writing - review and editing

S Naicker: Data curation; Formal analysis; Investigation; Methodology; Writing - review and editing

S Cormican: Data curation; Formal analysis; Investigation; Methodology; Supervision; Writing - review and editing

T Griffin: Conceptualization; Formal analysis; Methodology; Supervision; Writing - review and editing

M N Islam: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing - review and editing

E McCole: Data curation; Formal analysis; Investigation; Methodology; Writing - review and editing

I McConnell: Conceptualization; Formal analysis; Funding acquisition; Investigation; Methodology; Resources; Writing - review and editing

J Lamont: Conceptualization; Formal analysis; Funding acquisition; Methodology; Resources; Writing - review and editing

P FitzGerald: Conceptualization; Formal analysis; Funding acquisition; Methodology; Writing - review and editing

J Ferguson: Conceptualization; Formal analysis; Funding acquisition; Methodology; Supervision; Writing - review and editing

C Richardson: Conceptualization; Formal analysis; Funding acquisition; Methodology; Project administration; Supervision; Writing - review and editing

S Logue: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Writing - review and editing

M Griffin: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Writing - review and editing

Supplemental Material

Blood Sample Collection and Serum Isolation.

Immunoassay Procedure for Serum Biomarker Determinations.

Biomarker Selection, Assay Performance, and Validation.

Supplemental Table 1. Inter-Assay Coefficient of Variation of Multiplex Serum Biomarker Arrays.

Supplemental Table 2. Characteristics of Study Participants with Available and Missing Baseline uACR Data.

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

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.

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).

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).

References

1. GBD Chronic Kidney Disease Collaboration: Global, regional, and national burden of chronic kidney disease, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet,* 395**:** 709-733, 2020 10.1016/s0140-6736(20)30045-3
2. Jager KJ, Fraser SDS: The ascending rank of chronic kidney disease in the global burden of disease study. *Nephrology Dialysis Transplantation,* 32**:** ii121-ii128, 2017 10.1093/ndt/gfw330
3. Matsushita K, van der Velde M, Astor BC, Woodward M, Levey AS, de Jong PE, Coresh J, Gansevoort RT: Association of estimated glomerular filtration rate and albuminuria with all-cause and cardiovascular mortality in general population cohorts: a collaborative meta-analysis. *Lancet (London, England),* 375**:** 2073-2081, 2010 10.1016/S0140-6736(10)60674-5
4. Waikar SS, Rebholz CM, Zheng Z, Hurwitz S, Hsu CY, Feldman HI, Xie D, Liu KD, Mifflin TE, Eckfeldt JH, Kimmel PL, Vasan RS, Bonventre JV, Inker LA, Coresh J: Biological Variability of Estimated GFR and Albuminuria in CKD. *American journal of kidney diseases : the official journal of the National Kidney Foundation,* 72**:** 538-546, 2018 10.1053/j.ajkd.2018.04.023
5. Levey AS, Becker C, Inker LA: Glomerular filtration rate and albuminuria for detection and staging of acute and chronic kidney disease in adults: a systematic review. *Jama,* 313**:** 837-846, 2015 10.1001/jama.2015.0602
6. Colhoun HM, Marcovecchio ML: Biomarkers of diabetic kidney disease. *Diabetologia,* 61**:** 996-1011, 2018 10.1007/s00125-018-4567-5
7. Colombo M, Valo E, McGurnaghan SJ, Sandholm N, Blackbourn LAK, Dalton RN, Dunger D, Groop P-H, McKeigue PM, Forsblom C, Colhoun HM, on behalf of the FinnDiane Study G, the Scottish Diabetes Research Network Type 1 Bioresource C: Biomarker panels associated with progression of renal disease in type 1 diabetes. *Diabetologia,* 62**:** 1616-1627, 2019 10.1007/s00125-019-4915-0
8. Looker HC, Colombo M, Hess S, Brosnan MJ, Farran B, Dalton RN, Wong MC, Turner C, Palmer CNA, Nogoceke E, Groop L, Salomaa V, Dunger DB, Agakov F, McKeigue PM, Colhoun HM: Biomarkers of rapid chronic kidney disease progression in type 2 diabetes. *Kidney international,* 88**:** 888-896, 2015 https://doi.org/10.1038/ki.2015.199
9. Naicker SD, Cormican S, Griffin TP, Maretto S, Martin WP, Ferguson JP, Cotter D, Connaughton EP, Dennedy MC, Griffin MD: Chronic Kidney Disease Severity Is Associated With Selective Expansion of a Distinctive Intermediate Monocyte Subpopulation. *Frontiers in Immunology,* 9, 2018 10.3389/fimmu.2018.02845
10. Martin WP, Griffin TP, Lappin DW, Griffin DG, Ferguson JP, O'Brien T, Griffin MD: Influence of Referral to a Combined Diabetology and Nephrology Clinic on Renal Functional Trends and Metabolic Parameters in Adults With Diabetic Kidney Disease. *Mayo Clinic proceedings Innovations, quality & outcomes,* 1**:** 150-160, 2017 10.1016/j.mayocpiqo.2017.07.003
11. Weaver RG, James MT, Ravani P, Weaver CGW, Lamb EJ, Tonelli M, Manns BJ, Quinn RR, Jun M, Hemmelgarn BR: Estimating Urine Albumin-to-Creatinine Ratio from Protein-to-Creatinine Ratio: Development of Equations using Same-Day Measurements. *Journal of the American Society of Nephrology,* 31**:** 591-601, 2020 10.1681/asn.2019060605

12. Tangri N, Grams ME, Levey AS, Coresh J, Appel LJ, Astor BC, Chodick G, Collins AJ, Djurdjev O, Elley CR, Evans M, Garg AX, Hallan SI, Inker LA, Ito S, Jee SH, Kovesdy CP, Kronenberg F, Heerspink HJL, Marks A, Nadkarni GN, Navaneethan SD, Nelson RG, Titze S, Sarnak MJ, Stengel B, Woodward M, Iseki K, Consortium ftCP: Multinational Assessment of Accuracy of Equations for Predicting Risk of Kidney Failure: A Meta-analysis. *Jama,* 315**:** 164-174, 2016 10.1001/jama.2015.18202
13. Kassambara A, Mundt F: factoextra: Extract and Visualize the Results of Multivariate Data Analyses. R package version 1.0.7. https://CRAN.R-project.org/package=factoextra. 2020.
14. Therneau T, Atkinson B: rpart: Recursive Partitioning and Regression Trees. R package version 4.1-15. https://CRAN.R-project.org/package=rpart. 2019.
15. Borkovec M, Madin N: ggparty: 'ggplot' Visualizations for the 'partykit' Package. R package version 1.0.0. https://CRAN.R-project.org/package=ggparty. 2019.
16. Liaw A, Wiener M: Classification and Regression by randomForest. R News 2(3), 18--22., 2002.
17. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez J-C, Müller M: pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinformatics,* 12**:** 77, 2011 10.1186/1471-2105-12-77
18. Venables WN, Ripley BD: Modern Applied Statistics with S. Fourth Edition. Springer, New York. ISBN 0-387-95457-0., 2002.
19. Therneau TM: A Package for Survival Analysis in R. R package version 3.1-12. https://CRAN.R-project.org/package=survival. 2020.
20. Kassambara A, Kosinski M, Biecek P: survminer: Drawing Survival Curves using 'ggplot2'. R package version 0.4.6. https://CRAN.R-project.org/package=survminer. 2019.
21. Wickham H, Averick M, Bryan J, Chang W, D'Agostino McGowan L, Francois R, Grolemund G, Hayes A, Henry L, Hester J, Kuhn M, Lin Pedersen T, Miller E, Milton Bache S, Muller K, Ooms J, Robinson D, Paige Seidel D, Spinu V, Takahashi K, Vaughan D, Wilke C, Woo K, Yutani H: Welcome to the Tidyverse. *Journal of Open Source Software,* 4**:** 1686, 2019. https://doi.org/10.21105/joss.01686
22. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/. 2020.
23. Niewczas MA, Gohda T, Skupien J, Smiles AM, Walker WH, Rosetti F, Cullere X, Eckfeldt JH, Doria A, Mayadas TN, Warram JH, Krolewski AS: Circulating TNF Receptors 1 and 2 Predict ESRD in Type 2 Diabetes. *Journal of the American Society of Nephrology,* 23**:** 507, 2012 10.1681/ASN.2011060627
24. Coca SG, Nadkarni GN, Huang Y, Moledina DG, Rao V, Zhang J, Ferket B, Crowley ST, Fried LF, Parikh CR: Plasma Biomarkers and Kidney Function Decline in Early and Established Diabetic Kidney Disease. *J Am Soc Nephrol,* 28**:** 2786-2793, 2017 10.1681/ASN.2016101101
25. Schrauben SJ, Shou H, Zhang X, Anderson AH, Bonventre JV, Chen J, Coca S, Furth SL, Greenberg JH, Gutierrez OM, Ix JH, Lash JP, Parikh CR, Rebholz CM, Sabbisetti V, Sarnak MJ, Shlipak MG, Waikar SS, Kimmel PL, Vasan RS, Feldman HI, Schelling JR: Association of Multiple Plasma Biomarker Concentrations with Progression of Prevalent Diabetic Kidney Disease: Findings from the Chronic Renal Insufficiency Cohort (CRIC) Study. *Journal of the American Society of Nephrology,* 32**:** 115, 2021 10.1681/ASN.2020040487

26. CKD Prognosis Consortium: Development of Risk Prediction Equations for Incident Chronic Kidney Disease. *Jama,* 322**:** 2104-2114, 2019 10.1001/jama.2019.17379
27. Martin WP, Tuohy C, Doody A, Jackson S, Canavan RJ, Slattery D, Twomey PJ, McKenna MJ, le Roux CW, Docherty NG: Parallel assessment of albuminuria and plasma sTNFR1 in people with type 2 diabetes and advanced chronic kidney disease provides accurate prognostication of the risks of renal decline and death. *Scientific reports,* 10**:** 14852-14852, 2020 10.1038/s41598-020-71684-6
28. Yamanouchi M, Skupien J, Niewczas MA, Smiles AM, Doria A, Stanton RC, Galecki AT, Duffin KL, Pullen N, Breyer MD, Bonventre JV, Warram JH, Krolewski AS: Improved clinical trial enrollment criterion to identify patients with diabetes at risk of end-stage renal disease. *Kidney international,* 92**:** 258-266, 2017 10.1016/j.kint.2017.02.010
29. Cui W, Paglialunga S, Kalant D, Lu H, Roy C, Laplante M, Deshaies Y, Cianflone K: Acylation-stimulating protein/C5L2-neutralizing antibodies alter triglyceride metabolism in vitro and in vivo. *American journal of physiology Endocrinology and metabolism,* 293**:** E1482-1491, 2007 10.1152/ajpendo.00565.2006
30. Cui W, Lapointe M, Gauvreau D, Kalant D, Cianflone K: Recombinant C3adesArg/acylation stimulating protein (ASP) is highly bioactive: a critical evaluation of C5L2 binding and 3T3-L1 adipocyte activation. *Molecular immunology,* 46**:** 3207-3217, 2009 10.1016/j.molimm.2009.08.013
31. Kolev M, Kemper C: Keeping It All Going-Complement Meets Metabolism. *Frontiers in immunology,* 8**:** 1-1, 2017 10.3389/fimmu.2017.00001
32. Paglialunga S, Fisette A, Yan Y, Deshaies Y, Brouillette JF, Pekna M, Cianflone K: Acylation-stimulating protein deficiency and altered adipose tissue in alternative complement pathway knockout mice. *American journal of physiology Endocrinology and metabolism,* 294**:** E521-529, 2008 10.1152/ajpendo.00590.2007
33. Peake PW, Shen Y, Walther A, Charlesworth JA: Adiponectin binds C1q and activates the classical pathway of complement. *Biochemical and biophysical research communications,* 367**:** 560-565, 2008 10.1016/j.bbrc.2007.12.161
34. Ozata M, Oktenli C, Gulec M, Ozgurtas T, Bulucu F, Caglar K, Bingol N, Vural A, Ozdemir IC: Increased Fasting Plasma Acylation-Stimulating Protein Concentrations in Nephrotic Syndrome. *The Journal of Clinical Endocrinology & Metabolism,* 87**:** 853-858, 2002 10.1210/jcem.87.2.8243
35. Tang JH, Wen Y, Wu F, Zhao XY, Zhang MX, Mi J, Cianflone K: Increased plasma acylation-stimulating protein in pediatric proteinuric renal disease. *Pediatric nephrology (Berlin, Germany),* 23**:** 959-964, 2008 10.1007/s00467-007-0738-1
36. Escasany E, Izquierdo-Lahuerta A, Medina-Gomez G: Underlying Mechanisms of Renal Lipotoxicity in Obesity. *Nephron,* 143**:** 28-32, 2019 10.1159/000494694
37. Martin WP, Docherty NG, Le Roux CW: Impact of bariatric surgery on cardiovascular and renal complications of diabetes: a focus on clinical outcomes and putative mechanisms. *Expert review of endocrinology & metabolism***:** 1-12, 2018 10.1080/17446651.2018.1518130
38. Niewczas MA, Pavkov ME, Skupien J, Smiles A, Md Dom ZI, Wilson JM, Park J, Nair V, Schlafly A, Saulnier PJ, Satake E, Simeone CA, Shah H, Qiu C, Looker HC, Fiorina P, Ware CF, Sun JK, Doria A, Kretzler M, Susztak K, Duffin KL, Nelson RG, Krolewski AS: A signature of circulating inflammatory proteins and development of end-stage renal disease in diabetes. *Nature medicine,* 25**:** 805-813, 2019 10.1038/s41591-019-0415-5

39. Guo L, Zhu B, Yuan H, Zhao W: Evaluation of serum neutrophil gelatinase-associated lipocalin in older patients with chronic kidney disease. *Aging Med (Milton),* 3**:** 32-39, 2020 10.1002/agm2.12098
40. Bolignano D, Lacquaniti A, Coppolino G, Donato V, Campo S, Fazio MR, Nicocia G, Buemi M: Neutrophil Gelatinase-Associated Lipocalin (NGAL) and Progression of Chronic Kidney Disease. *Clinical Journal of the American Society of Nephrology,* 4**:** 337, 2009 10.2215/CJN.03530708
41. Mc Causland FR, Claggett B, Burdmann EA, Eckardt KU, Kewalramani R, Levey AS, McMurray JJ, Parfrey P, Remuzzi G, Singh AK, Solomon SD, Toto RD, Pfeffer MA: C-Reactive Protein and Risk of ESRD: Results From the Trial to Reduce Cardiovascular Events With Aranesp Therapy (TREAT). *American journal of kidney diseases : the official journal of the National Kidney Foundation,* 68**:** 873-881, 2016 10.1053/j.ajkd.2016.07.022
42. Fu EL, Franko MA, Obergfell A, Dekker FW, Gabrielsen A, Jernberg T, Carrero JJ: High-sensitivity C-reactive protein and the risk of chronic kidney disease progression or acute kidney injury in post-myocardial infarction patients. *American heart journal,* 216**:** 20-29, 2019 10.1016/j.ahj.2019.06.019
43. Hannan M, Ansari S, Meza N, Anderson AH, Srivastava A, Waikar S, Charleston J, Weir MR, Taliercio J, Horwitz E, Saunders MR, Wolfrum K, Feldman HI, Lash JP, Ricardo AC: Risk Factors for CKD Progression. *Clinical Journal of the American Society of Nephrology***:** CJN.07830520, 2020 10.2215/CJN.07830520
44. Kędzierska K, Sindrewicz K, Sporniak-Tutak K, Gołembiewska E, Zair L, Sieńko J, Stańczyk-Dunaj M, Baranowska-Bosiacka I, Ciechanowski K: Does Immunosuppressive Therapy Affect Markers of Kidney Damage? *Annals of transplantation,* 21**:** 137-144, 2016 10.12659/aot.895275
45. Srivastava A, Schmidt IM, Palsson R, Weins A, Bonventre JV, Sabbisetti V, Stillman IE, Rennke HG, Waikar SS: The Associations of Plasma Biomarkers of Inflammation with Histopathologic Lesions, Kidney Disease Progression, and Mortality - The Boston Kidney Biopsy Cohort Study. *Kidney Int Rep*, 10.1016/j.ekir.2020.12.025
46. Oh YJ, An JN, Kim CT, Yang SH, Lee H, Kim DK, Joo KW, Paik JH, Kang S-W, Park JT, Lim CS, Kim YS, Lee JP: Circulating Tumor Necrosis Factor α Receptors Predict the Outcomes of Human IgA Nephropathy: A Prospective Cohort Study. *PloS one,* 10**:** e0132826, 2015 10.1371/journal.pone.0132826
47. Torres-Salido MT, Cortés-Hernández J, Vidal X, Pedrosa A, Vilardell-Tarrés M, Ordi-Ros J: Neutrophil gelatinase-associated lipocalin as a biomarker for lupus nephritis. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association,* 29**:** 1740-1749, 2014 10.1093/ndt/gfu062
48. Kaartinen K, Syrjänen J, Pörsti I, Hurme M, Harmoinen A, Pasternack A, Huhtala H, Mustonen J: Inflammatory markers and the progression of IgA glomerulonephritis. *Nephrology Dialysis Transplantation,* 23**:** 1285-1290, 2008 10.1093/ndt/gfm782
49. Vesper HW, Myers GL, Miller WG: Current practices and challenges in the standardization and harmonization of clinical laboratory tests. *The American journal of clinical nutrition,* 104 Suppl 3**:** 907S-912S, 2016 10.3945/ajcn.115.110387
50. Martin WP, Bauer J, Coleman J, Dellatorre-Teixeira L, Reeve JLV, Twomey PJ, Docherty NG, O'Riordan A, Watson AJ, le Roux CW, Holian J: Obesity is common in chronic kidney disease and associates with greater antihypertensive usage and proteinuria:

evidence from a cross-sectional study in a tertiary nephrology centre. *Clinical obesity*: e12402, 2020 10.1111/cob.12402

Table 1. Baseline Characteristics and Serum Biomarker Concentrations of the Study Cohort Stratified by Development of a Composite Renal and Mortality Endpoint (n=139).[a,b,c]

Characteristic	Data available (n (%))	Total cohort (n=139)	Did not develop composite endpoint (n=83)	Developed composite endpoint (n=56)
Clinical parameters				
Age (mean±SD; years)	139 (100)	63±17	60±17	67±15
Male (n (%))	139 (100)	78 (56)	38 (46)	40 (71)
Diabetes mellitus (n (%))	139 (100)	35 (25)	11 (13)	24 (43)
Hypertension (n (%))	139 (100)	115 (83)	64 (77)	51 (91)
Coronary artery disease (n (%))	139 (100)	18 (13)	8 (10)	10 (18)
CKD stage (n (%))	139 (100)			
Grade 1		5 (4)	5 (6)	0 (0)
Grade 2		17 (12)	16 (19)	1 (2)
Grade 3a		22 (16)	19 (23)	3 (5)
Grade 3b		42 (30)	25 (30)	17 (30)
Grade 4		46 (33)	16 (19)	30 (54)
Grade 5		7 (5)	2 (2)	5 (9)
CKD aetiology (n (%))	139 (100)			
Diabetes		23 (17)	8 (10)	15 (27)
Hypertension		12 (9)	9 (11)	3 (5)
Glomerulonephritis		30 (22)	23 (28)	7 (13)
Congenital		8 (6)	7 (8)	1 (2)
Polycystic kidney disease		6 (4)	3 (4)	3 (5)
Obstructive		6 (4)	2 (2)	4 (7)
Interstitial		7 (5)	5 (6)	2 (4)
Other/unknown		47 (34)	26 (31)	21 (38)
Laboratory data				
Serum creatinine (mean±SD; mg/dL)	139 (100)	2.0±1.0	1.6±0.6	2.6±1.1
CKD-EPI eGFR (median [IQR]; mL/min/BSA)	139 (100)	33 [24 - 51]	43 [31 - 60]	26 [18 - 34]
uACR (median [IQR]; mg/g)	58 (42)	127 [22 - 479]	33 [18 - 144]	504 [92 - 1249]
uPCR (median [IQR]; mg/g)	76 (55)	327 [124 - 814]	301 [124 - 637]	345 [181 - 1381]
Merged uACR (median [IQR]; mg/g)[d]	113 (81)	144 [22 - 532]	104 [19 - 269]	283 [83 - 993]
Haemoglobin (mean±SD; g/dL)	138 (99)	13.0±1.7	13.4±1.9	12.3±1.2

KFRE estimates (median [IQR]; %)	113 (81)			
2-year		1.4 [0.3 - 6.5]	0.6 [0.1 - 2.4]	6.4 [1.6 - 18.7]
5-year		5.4 [1.2 - 23.1]	2.1 [0.3 - 9.0]	22.5 [6.1 - 55.1]
Multiplex biomarker values				
C-reactive protein (median [IQR]; ng/mL)	139 (100)	3035 [1607 - 8244]	2610 [1416 - 5296]	4612 [2303 - 15181]
Cystatin C (median [IQR]; ng/mL)	139 (100)	3715 [2575 - 5000]	3241 [2115 - 4384]	4984 [3662 - 5739]
C3a-desArg (median [IQR]; ng/mL)	139 (100)	1762 [1010 - 2877]	2240 [1099 - 3392]	1467 [947 - 2178]
D-dimer (median [IQR]; ng/mL)	138 (99)	81 [41 - 153]	73 [34 - 143]	103 [49 - 172]
EGF (median [IQR]; pg/mL)	138 (99)	91 [54 - 126]	109 [58 - 134]	72 [47 - 104]
FABP1 (median [IQR]; ng/mL)	139 (100)	1.6 [0.7 - 3.0]	1.3 [0.6 – 2.7]	1.9 [1.2 – 3.8]
Interleukin-8 (median [IQR]; pg/mL)	139 (100)	3.7 [2.6 - 8.5]	3.9 [2.5 – 8.6]	3.6 [2.7 – 7.3]
MIP-1-alpha (median [IQR]; pg/mL)	130 (94)	4.8 [2.6 - 10.6]	3.9 [2.3 – 6.6]	7.5 [3.7 – 15.1]
NGAL (median [IQR]; ng/mL)	137 (99)	167 [116 - 253]	137 [104 - 194]	213 [173 - 327]
sTNFR1 (median [IQR]; ng/mL)	136 (98)	3.0 [2.1 - 4.6]	2.4 [1.9 – 3.2]	4.6 [3.5 – 6.8]
sTNFR2 (median [IQR]; ng/mL)	138 (99)	1.4 [0.8 - 2.2]	1.1 [0.6 – 1.6]	2.1 [1.3 – 2.5]

[a]BSA = body surface area; CKD-EPI = Chronic Kidney Disease-Epidemiology Collaboration; C3a-desArg = complement protein C3a (cleaved at C-terminal arginine); EGF = epidermal growth factor; eGFR = estimated glomerular filtration rate; FABP1 = fatty acid-binding protein-1; IQR = interquartile range; KFRE = kidney failure risk equation (4-variable; non-North America); MIP-1-alpha = macrophage inflammatory protein-1-alpha; NGAL = neutrophil gelatinase-associated lipocalin; SD = standard deviation; sTNFR1 = soluble tumour necrosis factor receptor-1; sTNFR2 = soluble tumour necrosis factor receptor-2; uACR = urine albumin-to-creatinine ratio; uPCR = urine protein-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]Composite endpoint: ≥40% decrease in CKD-EPI eGFR, doubling of serum creatinine, renal replacement therapy, or mortality.

[d]Merged uACR represents a combination of measured uACR and calculated uACR from uPCR using the validated equation of Weaver et al. uACR values on the natural log scale were exponentiated such that presented values are in absolute units in mg/g.

Table 2. Duration of Follow-up and Incidence of Renal and Mortality Endpoints During the Study Period (n=139).[a,b]

Characteristic	Data available (n (%))	Total cohort (n=139)	Did not develop composite endpoint (n=83)	Developed composite endpoint (n=56)
Number of eGFR measurements (median [IQR])	139 (100)	22 [12 - 34]	19 [11 - 29]	27 [15 - 42]
Duration of renal functional follow-up (median [IQR]; years)[c]	139 (100)	4.7 [3.5 - 5.2]	4.9 [4.1 – 5.2]	4.1 [1.8 – 5.2]
Duration of study follow-up (median [IQR]; years)[d]	139 (100)	5.4 [4.7 - 5.7]	5.4 [4.8 – 5.7]	5.4 [4.4 – 5.7]
Slope of CKD-EPI eGFR (median [IQR]; mL/min/BSA/year)[e]	129 (93)	-0.9 [-2.3 - 0.5]	0 [-1 – 1.6]	-2.2 [-3.9 - -1.4]
≥40% decrease in CKD-EPI eGFR (n (%))	139 (100)	38 (27)	0 (0)	38 (68)
Doubling of serum creatinine (n (%))	139 (100)	14 (10)	0 (0)	14 (25)
Required RRT (n (%))	139 (100)	21 (15)	0 (0)	21 (38)
Death from any cause (n (%))	139 (100)	15 (11)	0 (0)	15 (27)

[a]BSA = body surface area; CKD-EPI = Chronic Kidney Disease-Epidemiology Collaboration; eGFR = estimated glomerular filtration rate; IQR = interquartile range; RRT = renal replacement therapy; SD = standard deviation.

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

[c]Duration between date of study enrolment and date of final eGFR determination. eGFR values subsequent to RRT initiation were excluded.

[d]Duration between date of study enrolment and date of last study follow-up or date of death.

[e]Slope of CKD-EPI eGFR was calculated only for individuals with 3 or more eGFR values over at least 1 year.

Table 3. Random Forest Model Performance Metrics for Prediction of the Composite Renal and Mortality Endpoint (n=126).[a,b,c]

Parameter	Predicted Probability Threshold >10%			Predicted Probability Threshold >30%			Predicted Probability Threshold >50%		
	Clinical	Biomarker	Clinical + Biomarker	Clinical	Biomarker	Clinical + Biomarker	Clinical	Biomarker	Clinical + Biomarker
Sensitivity	0.92	0.96	0.98	0.79	0.85	0.88	0.69	0.71	0.69
Specificity	0.36	0.24	0.21	0.69	0.65	0.71	0.81	0.83	0.82
Positive predictive value	0.47	0.44	0.43	0.61	0.60	0.65	0.69	0.72	0.70
Negative predictive value	0.87	0.90	0.94	0.84	0.88	0.90	0.81	0.82	0.81

[a]Three types of random forest classification models were implemented to evaluate prediction of the composite renal and mortality endpoint: clinical variables alone (age, gender, hypertension, diabetes, and baseline eGFR), serum biomarkers alone, and clinical variables plus serum biomarkers.

[b]A leave-one-out cross-validation approach was implemented, which consisted of excluding one individual from training the random forest model. Subsequently, the trained random forest model predicted the class of the individual excluded during model training. This process was repeated iteratively for each individual in the dataset such that a predicted class was assigned to each study participant by each of the three random forest model types.

[c]Model performance metrics were calculated across three probability thresholds (10%, 30%, and 50%) for labelling patients as having developed the composite renal and mortality endpoint. For example, an individual with a predicted probability of the composite renal and mortality endpoint by a random forest model of 45% would be labelled as having developed the composite renal and mortality endpoint by the first two thresholds evaluated, but not the latter.

Figure Legends

Figure 1. Univariate associations between serum biomarkers and a composite renal and mortality endpoint.

A-B: Variables which exhibited a significant inverse relationship with the composite renal and mortality endpoint, including epidermal growth factor (A) and C3a-desArg (B).

C-D: Biomarkers with a modest positive relationship with the composite renal and mortality endpoint, including MIP-1-alpha (C) and C-reactive protein (D).

E-H: Biomarkers which displayed a strong positive relationship with the composite renal and mortality endpoint, including sTNFR1 (E), sTNFR2 (F), NGAL (G), and cystatin C (H).

Individuals who developed the composite renal and mortality endpoint are identified as 100% on the y-axis and as triangular points. Individuals who did not develop the composite renal and mortality endpoint are identified as 0% on the y-axis and as circular points.

Biomarkers which displayed an inverse association with the composite renal and mortality endpoint are coloured in blue, those with a strong positive association are coloured in red, and those with modest positive associations are presented in a shade intermediate between both colours.

Log-transformation of serum biomarkers was performed prior to modelling.

Odds ratios from logistic regression models are expressed per one unit change in natural logarithm biomarker concentrations.

95% confidence interval is represented by navy shading surrounding pink regression curve.

The size of individual data points is scaled by baseline eGFR to illustrate relationships between serum biomarkers and kidney function at enrolment. Larger points = higher eGFR; smaller points = lower eGFR.

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

Figure 2. Principal components analysis illustrates relationships between biomarker expression, CKD stage, and a composite renal and mortality endpoint.

A: Unsupervised clustering of patients by principal components analysis identifies global shifts in biomarkers across categorical CKD stages, grouped as grades 1 and 2 (eGFR ≥60 mL/min/BSA), grade 3 (eGFR 30-59 mL/min/BSA), and grades 4 and 5 (eGFR <30 mL/min/BSA).

B: Unsupervised clustering of patients by principal components analysis identifies global shifts in biomarker expression profiles between those who did and did not develop a composite renal and mortality endpoint during follow-up.

C: A loadings plot from principal components analysis reveals the influential biomarkers which drive the shifts in biomarker expression across CKD stages and when stratified by the composite renal and mortality endpoint. Individuals with advanced CKD who developed the composite renal and mortality endpoint had higher expression of biomarkers in the right of the plot, including sTNFR1, sTNFR2, NGAL, and cystatin C. Individuals with earlier stage CKD who did not develop the composite renal and mortality endpoint had higher expression of protective factors in the upper left corner of the plot including C3a-desArg and epidermal growth factor.

The colour of the points illustrates the directionality of the relationships between biomarkers and the composite renal and mortality endpoint (blue = inverse, red = strong positive, while modest positive relationships are coloured in between both). The shape of the points represents statistical significance of the relationship between biomarkers and the composite renal and mortality endpoint by univariate logistic regression (circle = statistically significant, triangle = not statistically significant).

In panels A, B, and C, the x- and y-axes represent principal components 1 and 2, respectively.

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

Figure 3. A supervised machine learning approach (random forest classifier) illustrates the added predictive value of multiple serum biomarkers for a composite renal and mortality **endpoint, both when considered alone and in addition to conventional clinical variables.**

A: Decision tree classification of the composite renal and mortality endpoint by serum biomarkers in the study cohort. The decision tree highlights the predictive value of simultaneously assessing multiple serum biomarkers. In this decision tree, the 3 biomarkers are ranked by their proximate level of importance to correct classification of the composite renal and mortality endpoint, from sTNFR1 (highest) to C3a-desArg (lowest).

Individuals with low sTNFR1 values (<3 ng/mL) had a relatively low risk of the composite renal and mortality endpoint (12%). However, not all individuals with high sTNFR1 values had the same risk of the composite renal and mortality endpoint. Those with high sTNFR1 (≥3 ng/mL) coupled with low NGAL values (<156 ng/mL) had a 39% risk of the composite renal and mortality endpoint, while those with high sTNFR1 (≥3 ng/mL), high NGAL (≥156 ng/mL), but also high C3a-desArg values (≥2,368 ng/mL) had a 44% risk of the composite renal and mortality endpoint. Conversely, individuals with the triad of high sTNFR1 (≥3 ng/mL), high NGAL (≥156 ng/mL), and low C3a-desArg (<2,368 ng/mL), which accounted for approximately 20% of the study cohort, almost universally (96%) developed the composite renal and mortality endpoint during follow-up.

Biomarker values in the decision tree are coloured in a continuous gradient from left to right from blue (lower risk) to red (higher risk).

B: 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, and baseline eGFR) (green), serum biomarkers alone (orange), and clinical variables plus serum biomarkers (purple). A leave-one-out cross-validation approach was implemented for the random forest models. The plot illustrates incremental improvements in correct prediction of the composite renal and mortality endpoint across the 3 model types.

AUC values and associated 95% confidence intervals for the 3 model types are presented in the inset table: 0.78 for clinical variables alone, 0.81 for serum biomarkers alone, and 0.83 for clinical variables plus serum biomarkers.

C-E: Dotplots of variable importance across the 3 random forest classification models of the composite renal and mortality endpoint: clinical variables alone (age, gender, hypertension, diabetes, and baseline eGFR) (C), serum biomarkers alone (D), and clinical variables plus biomarkers (E). eGFR was the most important clinical variable, while sTNFR1 and NGAL were the biomarkers which provided the most predictive value to the models.

All 5 clinical variables are presented (C), while the top 8 most important variables are presented for the biomarkers alone and clinical variables plus biomarker models (D and E).

The dots are coloured in a continuous gradient from navy (lower variable importance) to yellow (higher variable importance).

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

Figure 4. C-reactive protein, NGAL and C3a-desArg independently predict time to development of a composite renal and mortality endpoint (Cox proportional hazards regression).

A: Forest plot of parsimonious Cox proportional hazards regression model incorporating clinical variables and serum biomarkers predictive of a composite renal and mortality endpoint (n=126).

C-reactive protein (adjusted hazard ratio [aHR], 1.4; 95% confidence interval [95% CI], 1.1 to 1.9) and NGAL (aHR, 2.7; 95% CI, 1.3 to 5.8) 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, 0.5; 95% CI, 0.3 to 0.9).

B-D: Plots of survival without the composite renal and mortality endpoint derived from the fully adjusted, multivariate clinical plus biomarker Cox proportional hazards regression

model presented in the Forest plot in panel A, stratified by tertiles of serum C-reactive protein (B), NGAL (C) and C3a-desArg (D).

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.

Figure 1

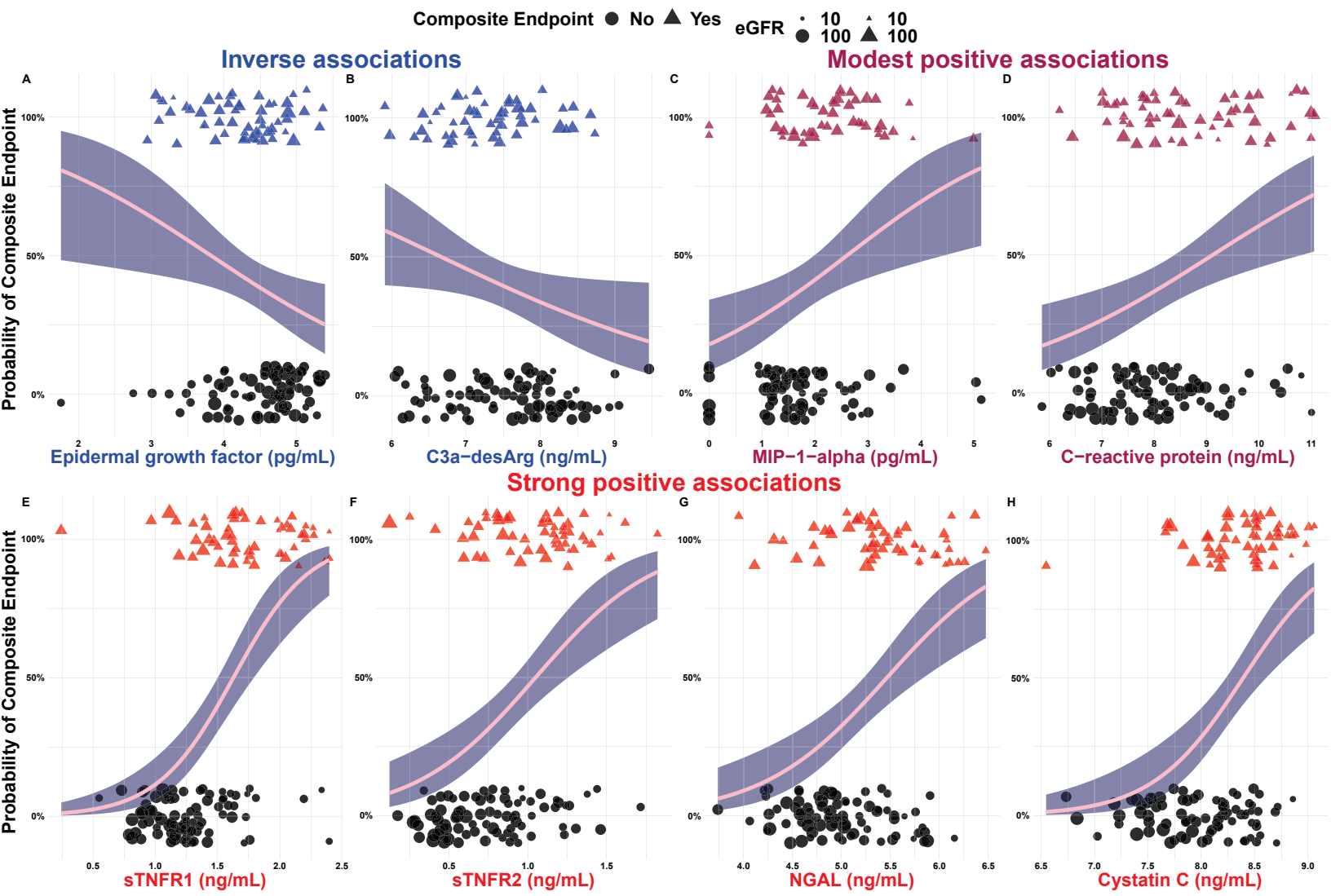

Composite Endpoint No Yes
eGFR 10 10 100 100
Inverse associations
Modest positive associations
A
B
C
D
Probability of Composite Endpoint
100%
50%
0%
Epidermal growth factor (pg/mL)
C3a−desArg (ng/mL)
MIP−1−alpha (pg/mL)
C−reactive protein (ng/mL)
Strong positive associations
E
F
G
H
sTNFR1 (ng/mL)
sTNFR2 (ng/mL)
NGAL (ng/mL)
Cystatin C (ng/mL)

Figure 2

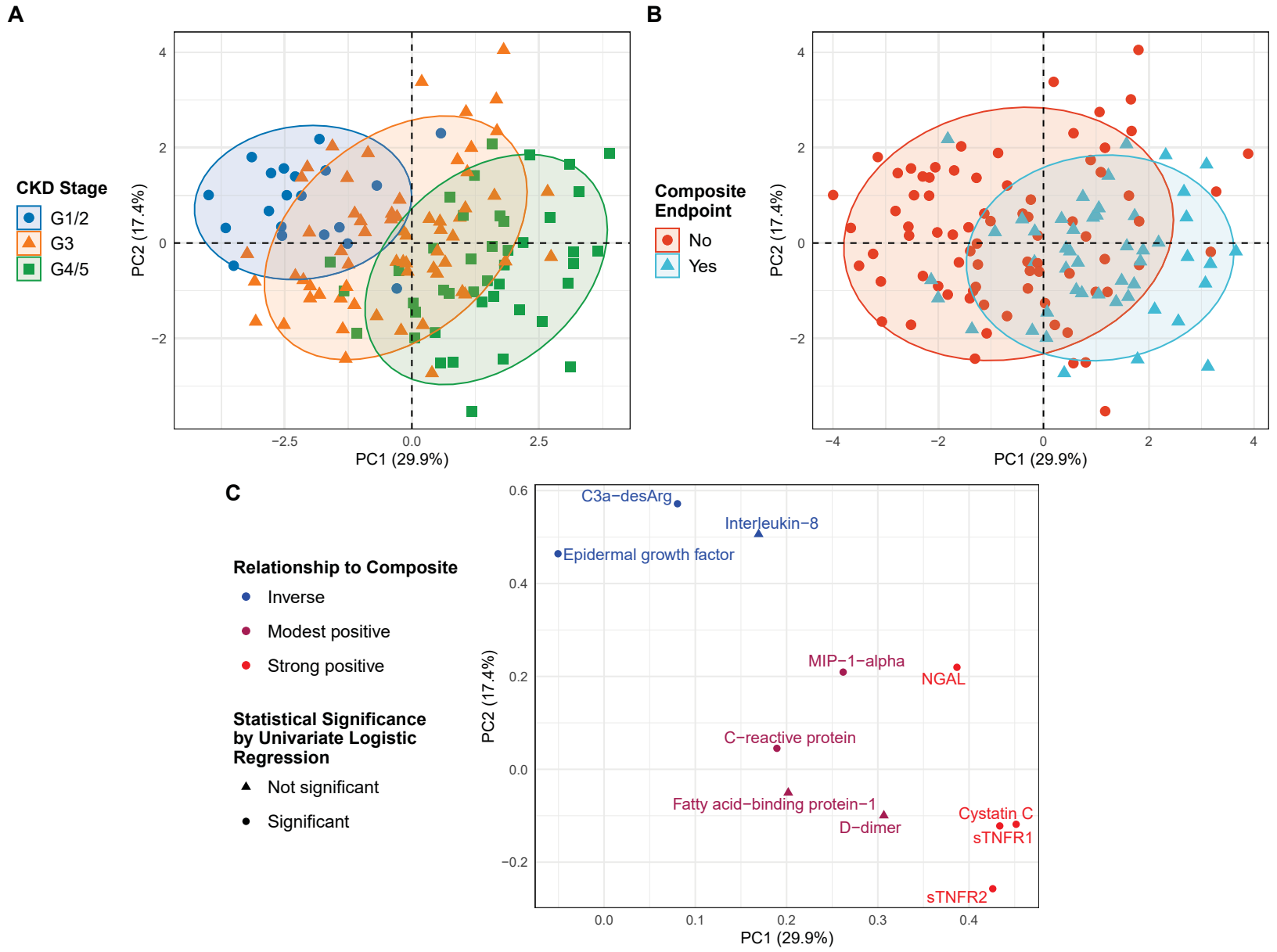

A
CKD Stage
G1/2
G3
G4/5
PC2 (17.4%)
PC1 (29.9%)
B
Composite Endpoint
No
Yes
PC2 (17.4%)
PC1 (29.9%)
C
Relationship to Composite
Inverse
Modest positive
Strong positive
Statistical Significance by Univariate Logistic Regression
Not significant
Significant
C3a−desArg
Interleukin−8
Epidermal growth factor
MIP−1−alpha
NGAL
C−reactive protein
Fatty acid−binding protein−1
D−dimer
Cystatin C
sTNFR1
sTNFR2
PC2 (17.4%)
PC1 (29.9%)

Figure 3

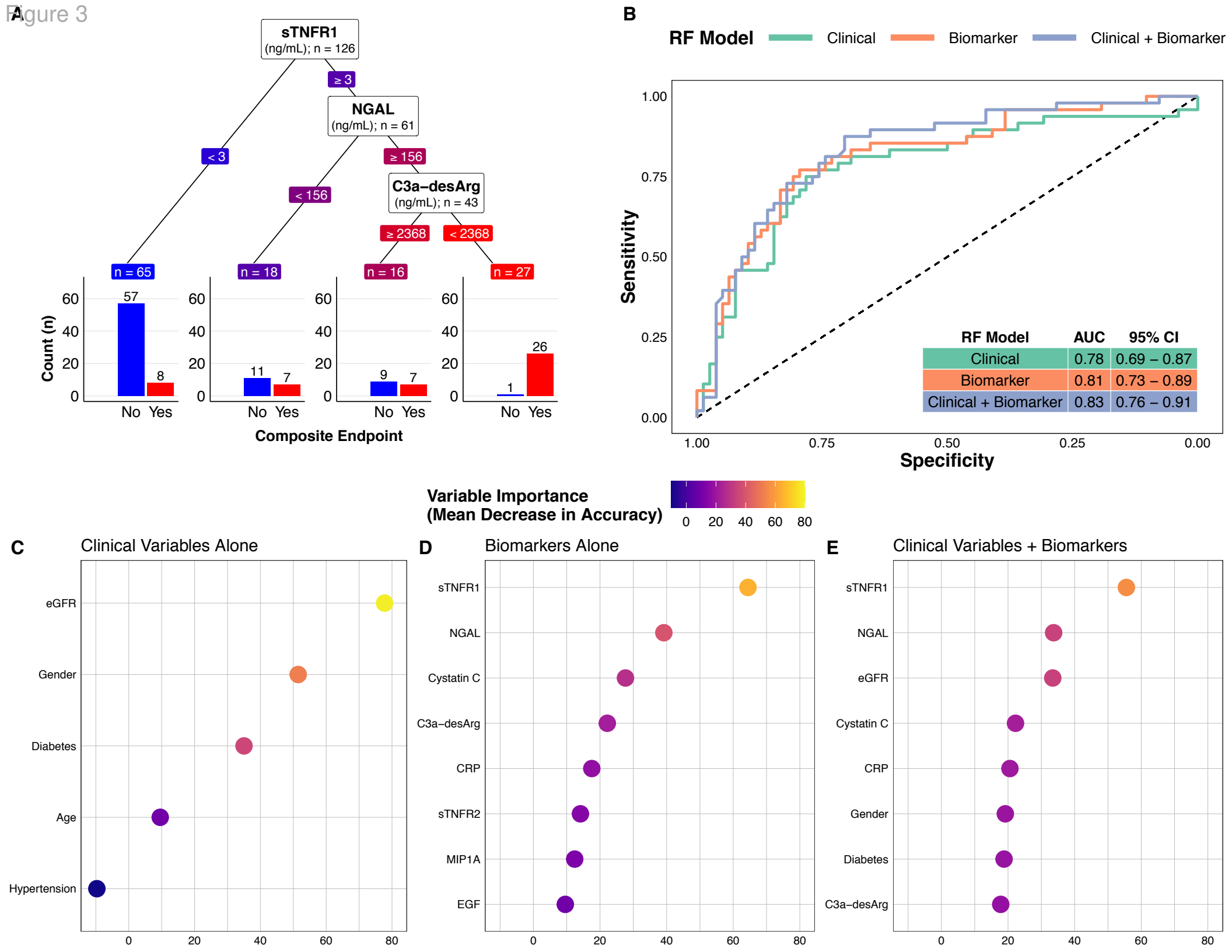
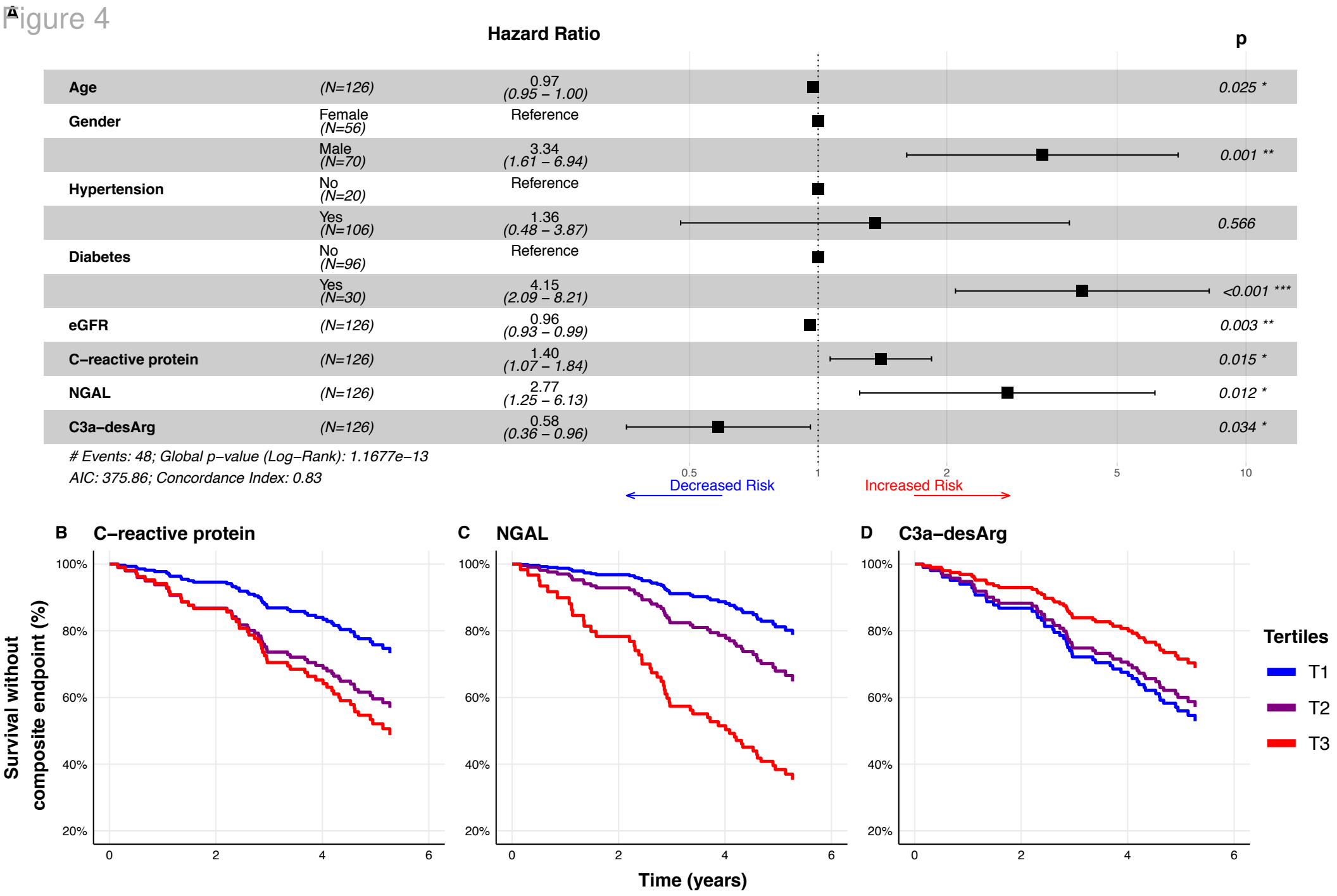

A
sTNFR1
(ng/mL); n = 126
≥ 3
< 3
NGAL
(ng/mL); n = 61
≥ 156
< 156
C3a–desArg
(ng/mL); n = 43
≥ 2368
< 2368
n = 65
n = 18
n = 16
n = 27
Count (n)
60
40
20
0
57
8
11
7
9
7
1
26
No
Yes
Composite Endpoint
B
RF Model
Clinical
Biomarker
Clinical + Biomarker
Sensitivity
1.00
0.75
0.50
0.25
0.00
Specificity
RF Model
AUC
95% CI
Clinical
0.78
0.69 – 0.87
Biomarker
0.81
0.73 – 0.89
Clinical + Biomarker
0.83
0.76 – 0.91
Variable Importance
(Mean Decrease in Accuracy)
0
20
40
60
80
C
Clinical Variables Alone
eGFR
Gender
Diabetes
Age
Hypertension
D
Biomarkers Alone
sTNFR1
NGAL
Cystatin C
C3a–desArg
CRP
sTNFR2
MIP1A
EGF
E
Clinical Variables + Biomarkers
sTNFR1
NGAL
eGFR
Cystatin C
CRP
Gender
Diabetes
C3a–desArg

Figure 4


A
Hazard Ratio
p
Age (N=126) 0.97 (0.95 – 1.00) 0.025 *
Gender
Female (N=56) Reference
Male (N=70) 3.34 (1.61 – 6.94) 0.001 **
Hypertension
No (N=20) Reference
Yes (N=106) 1.36 (0.48 – 3.87) 0.566
Diabetes
No (N=96) Reference
Yes (N=30) 4.15 (2.09 – 8.21) <0.001 ***
eGFR (N=126) 0.96 (0.93 – 0.99) 0.003 **
C–reactive protein (N=126) 1.40 (1.07 – 1.84) 0.015 *
NGAL (N=126) 2.77 (1.25 – 6.13) 0.012 *
C3a–desArg (N=126) 0.58 (0.36 – 0.96) 0.034 *
Events: 48; Global p–value (Log–Rank): 1.1677e–13
AIC: 375.86; Concordance Index: 0.83
0.5
1
2
5
10
Decreased Risk
Increased Risk
B C–reactive protein
C NGAL
D C3a–desArg
Survival without composite endpoint (%)
100%
80%
60%
40%
20%
0
2
4
6
Time (years)
Tertiles
T1
T2
T3