# Biomarkers of Rapid Chronic Kidney Disease Progression in Type 2 Diabetes

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**Abstract**

We evaluated the performance of a large set of serum biomarkers in the prediction of rapid progression of chronic kidney disease in people with type 2 diabetes. We used a case-control design, nested within a prospective cohort of people with baseline eGFR 30-60 ml/min/1.73m$^2$. Cases (n=154) had a >40% eGFR decline within 3.5 years of follow-up and controls (n=153) maintained>95% of baseline eGFR at end of follow-up. We measured 207 serum biomarkers and used logistic regression with forward selection to select a subset of biomarkers that maximized prediction on top of clinical variables including age, sex, $HbA_{1c}$, eGFR and albuminuria. Nested cross-validation was used to determine the best number of biomarkers to retain and to evaluate predictive performance. 30 biomarkers showed significant associations with rapid progression ($p<0.0003$ adjusted for clinical characteristics). A panel of 14 biomarkers increased the area under the ROC curve from 0.706 (clinical data alone) to 0.868. Biomarkers selected included fibroblast growth factor-21, symmetric:asymmetric dimethylarginine ratio, beta 2-microglobulin, C16-acylcarnitine, and kidney injury molecule-1. Using more extensive clinical data, including pre-baseline eGFR slope improved prediction but to a lesser extent than biomarkers (Area under the ROC curve = 0.793). We report several novel associations of biomarkers with Chronic Kidney Disease progression and the utility of a sparse panel of biomarkers in improving prediction.

## Introduction

Kidney disease is a major cause of morbidity and mortality in patients with type 2 diabetes. (1) Developing new therapies to prevent kidney disease incidence and progression is a priority with many pharmaceutical companies currently having drugs in development. However, clinical trials in this area are challenging as there is a need to demonstrate prevention of progression of renal function decline or progression to end stage renal disease (ESRD) over a typical trial time horizon of a few years. Even from stage 3 Chronic Kidney Disease (CKD) the majority of patients progress only very slowly over say a five year horizon. Albuminuria and estimated Glomerular Filtration Rate (eGFR) status are currently our best means of identifying those at highest subsequent risk of ESRD. Identifying those at risk of more rapid progression would allow risk stratification and improved trial power and efficiency and would also enable targeting of new therapies as they become available.

There is considerable interest in developing biomarkers that would help in such prediction beyond the commonly used legacy biomarkers serum creatinine, albuminuria, and cystatin-C. Many evaluations of single or small sets of candidate biomarkers have been reported.(2, 3) In this study we explored a broad set of 207 serum protein and metabolite biomarkers, some candidate and some unbiased discovery biomarkers in 154 incident cases of rapid progression of renal function decline from CKD3 and 153 non-progressing controls from the Genetics of Diabetes Audit and Research Tayside Study (GO-DARTS) a Scottish type 2 diabetes cohort. Our aim was to identify a subset of biomarkers that together could maximise prediction of rapid progression of renal function decline on top of clinical history. The study is part of the Surrogate markers for Micro- and Macro-vascular hard endpoints for Innovative diabetes Tools programme, 'SUMMIT', funded by the Innovative

Medicines Initiative.(4) This is a collaborative endeavour across 19 academic centres and 6 pharmaceutical industry partners across Europe to identify novel biomarkers for the complications of diabetes in order to reduce bottlenecks in diabetes drug development. The study was designed to be closely aligned to the typical trial setting i.e. taking CKD 3 as the baseline state from which to improve prediction of further renal function decline.

## Results

Overall 12.5% of the Go-DARTS population with CKD3 at baseline lost >40% of their baseline eGFR within 3.5 years and were defined as cases. Follow-up eGFR data was available for a median of 5.5 (IQR 3.2, 6.0) years. Baseline demographics for the study population are shown in **Table 1**. Cases had longer diabetes duration, greater prevalence of albuminuria and retinopathy and a lower median eGFR at baseline than the controls.

## Data reduction steps

All 207 biomarkers measured are listed in **Online Supplementary Material** (**OSM) Table 1**. From this initial panel we removed forty-two biomarkers from further analysis as being uninformative (**OSM Table 2**), either because very few patients had detectable levels of the biomarker (n=22), or the biomarker was in tight correlation (r>0.9) with another biomarker (n=15) or because of too few results due to inadequate sample volume analysis (n=5). This left 165 biomarkers and we also evaluated the ratio of symmetric dimethylarginine (SDMA) to asymmetric dimethylarginine (ADMA). Many of the biomarkers measured had very strong correlations with each other (OSM Table 3).

## Univariate associations

The medians by case control status for all 166 biomarkers are shown in **OSM Table 4.** The volcano plot (**Figure 1**) shows the associations with rapid progression for all 166 biomarkers evaluated singly and adjusted for baseline age, sex, eGFR, albuminuria, HbA1c, and ACE Inhibitor and Angiotensin Receptor Blocker (ARB) use. Cystatin-C and beta 2-microglobulin which are strongly correlated (rho =0.82) had the strongest associations with similar effect sizes per standard deviate. We retained 62 biomarkers for further evaluation as having at least suggestive evidence for association in the initial cross validated logistic regression for each biomarker evaluated alone. **Table 2** lists the 30 biomarkers that reached Bonferroni adjusted significance level ($p<0.0003$) on adjustment for clinical covariates, examined singly with odds ratios for association with being a case.

## Biomarker panel performance

**Figure 2a** shows the performance on withdrawn data of the biomarker panels chosen by the forward selection process with a fixed termination criterion and those chosen by a top down selection process that was run with varying sparsity constraints i.e. set to terminate at different numbers of retained biomarkers. The Area under the ROC curve (AUROC) for a model including only clinical covariates was 0.706 (95% CI: 0.654, 0.772). The forward selection process selected 14 biomarkers as contributing to prediction improvement beyond the clinical covariates. This yielded a substantial increment in AUROC to 0.868 (95% CI: 0.832, 0.915). The difference in log-likelihoods computed on withdrawn data between the two

models was 35 natural log units, indicating that the addition of biomarkers significantly improved the prediction of the model. Using the top down approach, performance could be improved to varying degrees depending on the number of biomarkers selected reaching a maximum AUROC of 0.892 (95% CI: 0.859-0.934), but requiring 35 biomarkers to achieve this.

The GO-DARTS dataset contains retrospective clinical data pre-baseline so we further examined the contribution of biomarkers to prediction beyond that achieved by using an extended set of clinical covariates data including longitudinal eGFR pre-baseline (see methods for full list). This extended clinical dataset showed a substantially higher prediction than the basic clinical covariate model (AUROC = 0.793 (95% CI: 0.738, 0.841) vs. 0.706 respectively). Addition of selected biomarkers improved prediction somewhat further with an increment in AUROC to 0.859 (95% CI: 0.816-0.902) with a panel of 7 biomarkers selected by forward selection, and a maximal AUROC of 0.871 (95% CI: 0.834-0.915) achieved with top down selection retaining 25 biomarkers (**Figure 2b**).

Replacing the dichotomous albuminuria variable with a continuous measure of urinary albumin concentration for the 220 individuals with data available using a single method did not improve the AUROC for either the restricted clinical covariate or extended clinical covariate models and the improvement in AUROCs due to the addition of biomarkers was of similar degree whether the clinical covariate model included the dichotomous or continuous albumin measure (data not shown).

**Figure 3a** shows the AUROC curves for the forward selection panel of 14 biomarkers and the top down selection of 35 biomarkers. However, a useful metric that summarises the potential value of biomarkers in selection of patients for a clinical trial is the "predicted event rate enrichment" achieved by using the biomarker panel in a given potential clinical trial population (**Figure3b**). For example in the Go-DARTS cohort just 12.5% of those meeting the eGFR baseline entry criterion of 30-60ml/min/1.73m$^2$ progressed to being a case within 3.5 years. Thus, without any selection by risk stratification (y axis of **Figure 3b**= 1) the expected cumulative incidence of progression is 12.5% (x-axis). The plot illustrates that selecting say the top 20% (y axis =0.2) of patients based on their score from a model combining clinical covariates and selected biomarkers could enrich the cumulative incidence of rapid progression to >60%.

**Components of the Selected Panels**

The 14 biomarkers selected by forward selection and their association with rapid progression adjusted for each other and clinical covariates in a logistic regression model are shown in **Table 3**. These biomarkers are for the most part a subset of the maximally predictive 35 biomarker panel selected by top down regression with the exception that cystatin-C was selected with top down, whereas beta-2-microglobulin, was selected instead in forward selection. Also adrenomedullin showed only weak association with progression when adjusted for the other biomarkers and was not included in the top down selection (see **OSM Table 5**).

The 7 biomarkers selected in the forward selection panel on top of the extended clinical covariates are a subset of the 14 biomarker panel– Kidney Injury Molecule 1

(KIM-1), SDMA:ADMA ratio, beta 2-microglobulin, alpha-1 antitrypsin (2), C-16 acylcarnitine, fibroblast growth factor-21 (FGF-21) and uracil.

**Discussion**

In this study of individuals with type 2 diabetes and CKD3 we found that within a large set of candidate and global discovery biomarkers, at least 62 showed some evidence for association with subsequent rapid renal function decline. Many of these biomarkers showed high correlations with each other and with clinical covariates so that a much sparser set of 14 biomarkers contained most of the predictive information beyond clinical covariates. We found that the increment in prediction with these biomarkers was of sufficient magnitude that it would be useful for risk stratification into clinical trials. Some of the biomarkers identified in the most predictive panels are already known to be associated with eGFR (i.e.beta-2-microglobulin and cystatin-C), whereas others have little or no prior data (e.g. SDMA:ADMA ratio, FGF-21, uracil). The best biomarker panel for prediction consisted of the restricted clinical covariates along with 35 biomarkers; however, this was only modestly better than the best sparse model which only required measurement of 14 biomarkers. The expense and logistics associated with validation and subsequent utilization of any new panel means that the 14 biomarker panel represents a more pragmatic approach. This panel of biomarkers now warrants further evaluation in other cohorts.

We used clinical creatinine measurements to calculate eGFR in this study and as there is considerable variability in this measure it is unsurprising that the in-sample measure of creatinine was also selected in our biomarker panels. Space does not

permit a detailed discussion of each of the 14 biomarkers selected and some of the associations have been described elsewhere. (5, 6) Here we focus on some of the more novel associations.

One of the strongest predictive biomarkers in our study was the ratio of SDMA:ADMA. SDMA and ADMA are released during proteolytic breakdown of nuclear proteins and both have been studied as biomarkers for cardiovascular disease (CVD).(7-9) SDMA is primarily excreted via the kidney and is strongly associated with renal function(7) but there is also some evidence that the protein methyltransferase PRMT5 that synthesises SDMA regulates interleukin-2-gene expression (10) suggesting that higher levels of SDMA might also reflect inflammation. We did not find any association of SDMA with inflammatory biomarkers such as C-reactive protein but there was a positive correlation (rho=0.39) with interleukin-2 receptor 1-alpha levels. However rather than a bioactive effect of SDMA itself, the observed prediction in our study may simply indicate that SDMA accumulates when filtration falls and thus it is predictive because it is a good biomarker of filtration. In contrast to SDMA we found that ADMA was only weakly inversely correlated with eGFR but more strongly correlated with arginine. The ratio may be a biomarker of these complex interactions and we note that models including the ratio yielded higher AUROCs than models including ADMA and SDMA separately.

KIM-1 is a protein expressed on the apical membrane of proximal tubule cells. Its ectodomain is shed into the lumen and serves as a urinary biomarker of kidney injury though there have been mixed results for it as a prognostic biomarker in diabetic

kidney disease.(11, 12) Studies have shown that KIM-1 expression is increased in the glomerulus in diabetic animal models (13) and is elevated in their plasma.(14) Recently, it was reported that shed KIM-1 also serves as a blood biomarker of kidney injury in humans, since plasma KIM-1 levels were higher in patients with acute kidney injury (AKI) than in healthy controls or post-cardiac surgery patients without AKI.(2) In that study serum KIM-1 level at baseline in type 1 diabetes patients strongly predicted rate of eGFR loss and risk of ESRD during 5-15 years of follow-up.

We identified associations with rapid progression of two fibroblast growth factor members -FGF-21 and FGF-23. FGF-21 is a 181 amino acidpolypeptide secreted predominantly by the liver and adipose tissue and has been shown to play an important role in lipid and energy metabolism.(15, 16) Previous studies have reported cross sectional associations with eGFR. (17) Median serum FGF-21 levels were >7-15-fold higher in dialysis patients than controls (18, 19) but fell after short term angiotensin blockade.(19) In cross sectional studies FGF-21 levels were also elevated with albuminuria even when eGFR was >60 ml/min.(20) FGF-21 was independently associated with urinary albumin in type 2 diabetes.(20) The kidney has relatively low levels of FGF-21 (21) and FGF-21 activity depends on the tissue specific expression of its co-factor Klotho β, which is predominantly in the liver and adipose tissue rather than the kidney. Thus, association between FGF-21 and renal disease progression may reflect simple accumulation in renal disease. However, it might also reflect an anti-fibrotic response; it was shown that FGF-21 prevented the expression of pro-fibrotic cytokines, including TGF-β1 in the kidney.(22) FGF-23 is a 32-kD bone derived hormone with several known endocrine functions in the kidney,

including the promotion of urinary phosphate excretion and the inhibition of the hydroxylation of 25-hydroxyvitamin D.(23) Elevated FGF-23 was an independent risk factor for end-stage renal disease in patients with relatively preserved kidney function and for mortality across the spectrum of CKD.(24) Recently it was shown in 13,448 subjects of the Atherosclerosis Risk in Communities study (ARIC), that higher serum level of FGF-23 were associated with increased risk of incident ESRD, independent of the baseline level of kidney function and a number of other risk factors.(25)

C16-acylcarnitine was one of the strongest predictors of rapid progression in our study. Previously in the KORA cohort acylcarnitines and especially the ratio of serine to glutarylcarnitine were associated with eGFR(26) and in FinnDiane urinary acylcarnitines were associated with albumin levels.(27) Higher plasma acylcarnitines were also predictive of ESRD in a small study of people with type 1 diabetes.(28) We found that C16-acylcarnitine was only weakly inversely correlated with eGFR (rho=-0.11) and only modestly associated with cystatin C (rho=0.31) yet was strongly associated with rapid progression.

Our study found other novel associations with renal disease progression that warrant further investigation including alpha 1 antitrypsin, which has been identified as a potential urinary biomarker for renal disease.(29, 30) We should note this measure had 50% of values missing at random, however a sensitivity analysis restricted to data without imputation showed the univariate odds ratio for it was essentially unchanged (1.77 in the imputed data vs. 1.74 in the unimputed data). Other

biomarkers such as hydroxyproline, creatine, uracil and Fatty Acid Binding Protein Heart reported here currently have no direct explanation for the associations.

We also showed that much of the increment in prediction gained with biomarkers could be obtained with the use of more extensive historical clinical data. However, typically in clinical trials there is little historical data available. In using this more extensive data we did not do any variable selection, instead we fitted a model using all the variables available that were likely to be relevant. There is no consensus on a risk prediction model for renal disease progression. A recent review of risk prediction models for patients with CKD revealed limited data with a wide range of end points including ESRD, incident CVD, and mortality.(31) Risk prediction models for clinical purposes will also become more important as new treatments arise for prevention of progression of renal disease in diabetes and biomarkers may be useful additions to clinical covariates again where extensive past medical history, including historical eGFR measures are not available.

The strengths of this study are that we have measured a large number of biomarkers covering numerous pathophysiological pathways. We have also made use of k-fold cross-validation and machine learning methods that avoid the problem of over-fitting when testing large numbers of associations and increase the generalisability of findings to other settings. Though further studies of the generalisability of findings are warranted.

There are also weaknesses. The sample size is modest and only one cohort has been studied and validation in external cohorts is needed. We used a dichotomous

variable for albuminuria due to no single method used for all samples to assess albuminuria status. As a result there is potential for residual confounding due to albuminuria. However, a sensitivity analysis of the sub-group with albumin concentration data measured by a single method showed that the increment in AUROC achieved by adding biomarkers to the clinical covariates was not reduced by the use of a continuous measure of albuminuria rather than the dichotomous variable. Thus, we think it is reasonable to conclude that the biomarkers are not materially affected by residual confounding. Another weakness is that we only have limited data from blinded duplicate samples due to limited volume availability though in general the repeatability data was good (**see OSM page 4**). We also note that the effect of errors in measurement act to reduce the power to detect associations rather than introduce false positive associations. Thirdly, we did not have a measure of every biomarker in every sample which required us to impute missing values. However, the degree of missing at random was not high with only 6 biomarkers in the study having ≥30% of values imputed and a sensitivity analysis examining individual biomarker associations after adjustment for clinical covariates in the non-imputed data showed consistent associations with those seen in the imputed dataset.

In keeping with our aim to identify biomarkers that might improve clinical trial stratification we have restricted the study to individuals with CKD3 at baseline. It is not possible to conclude that biomarkers that are associated with progression at this stage will also be predictive of progression in individuals with CKD1 or 2 and studies are needed to examine biomarkers in such individuals. It should also be noted that panels of biomarkers predicting from earlier stages of renal disease are more likely

to include biomarkers that are in the causal pathway whereas panels predicting from CKD3 may include biomarkers altered secondarily due to declines in glomerular filtration. The study also does not identify biomarkers that are necessarily specific for renal decline due to diabetic kidney disease as the underlying cause of renal disease in people with type 2 diabetes is highly heterogeneous. (32)

In conclusion we identified a panel of biomarkers that substantially improved the prediction of rapid progression of renal decline in people with diabetes and identified novel associations of biomarkers that warrant further investigation for relevance to pathogenesis of kidney disease in type 2 diabetes.

## Methods

### Data and Sample Sources

All samples for this study came from the Go-DARTS cohort. Go-DARTS is a hospital clinic and primary care based sample of people diagnosed with type 2 diabetes in the Tayside region of Scotland. Adults attending primary and secondary care in the area were invited to participate and enrolled in the study between December 1998 and May 2009 and are continuously followed up using linked electronic health care data.(33) The final sample comprises ~75% of all those with type 2 diabetes residing in Tayside. Diabetes status was based on a clinical record of a diagnosis of diabetes and was validated by checking against the clinical record data, on-going prescription and biochemistry laboratory data for results in keeping with the presence of diabetes. Patients gave a blood sample at study entry and agreed to have their routine and diabetes specific clinical and mortality records ascertained prospectively. Covariate data including prescription information, blood pressure and anthropometry

results were obtained by extraction from the ongoing primary care and hospital diabetes electronic records. Laboratory data is supplied directly to the Go-DARTS database so we have access to all serum creatinine values measured as part of routine clinical practice both before and after study enrollment. 90% of study participants had ≥1 eGFR measure per year of available follow-up. The study complied with the Declaration of Helsinki guidelines and informed consent was obtained from all study participants.

**Rapid Progressor Phenotype**

The phenotype for this study was designed around the typical enrolment criteria into trials for assessing reno-protective drugs. We identified all individuals with CKD3 (i.e. an eGFR of 30-60ml/min/1.73m$^2$) at enrolment. People were classified as cases if they lost >40% of their baseline eGFR within 3.5 years of follow-up and as controls if their most recent eGFR measure was >95% of baseline after follow-up of >3.5 years and had no fall in eGFR to <80% of baseline at any time during follow-up. Individuals were excluded if they had not received anti-hypertensive treatment within 1 year of baseline (to eliminate people not receiving active management) or had a history of hospital admission for acute renal failure (as assessed by hospital admission data) during follow-up. EGFR was calculated using the serum creatinine measured at the clinical laboratory (principally measured with alkaline picrate based methods) using the MDRD4 equation eGFR = 186 x (creatinine in mmol/l/88.4)$^{-1.154}$ x (age$^{-0.203}$) x 0.742 (if female)x (1.210 if black).(34)

**Biomarker Measurements**

Biomarkers were selected either on the basis of hypothesis-driven rationale (i.e. published biomarkers from relevant pathophysiological pathways such as kidney function, tubular intestinal injury, glomerular injury, endothelial dysfunction, oxidative stress, inflammation, fibrosis, cardiovascular dysfunction, metabolic disorders) or hypothesis-free as part of global discovery.

We used three platforms; 1) ELISA kits were used to measure 5 candidates at the University Heart Center Hamburg biomarker laboratory 2) Luminex technology was used to perform multiplexed, microsphere-based assays for 58 biomarkers by combining optical classification schemes, biochemical assays, flow cytometry and advanced digital signal processing as described **(35)** at the CLIA certified Myriad RBM laboratory (Austin TX, USA). Some of the biomarkers measured on this platform were selected specifically due to high interest for example KIM-1, Cystatin-C, while others were included due to being plexed with biomarkers of high interest for example beta-amyloid 42); and 3) liquid chromatography (LC) electrospray tandem mass spectrometry (MSMS) platforms for targeted metabolite and tryptic peptide analyses were used to yield quantitation of 144 metabolites and peptides at the WellChild Laboratory (Kings College London, UK). Here we made use of the extensive biomarker platform that has been developed, to measure biomarkers in which we had specific interest (e.g SDMA, NAG) and at the same time acquire data on a broader set of metabolites and tryyptic peptides dereived from plasma proteins for which we had no prior evidence. Further details of methods and sample quality control data for the 207 biomarkers measured is given in the **OSM methods** section and **OSM table 1**.

**Clinical Covariates**

Clinical covariates were recorded at the study day visit and included Body Mass Index (BMI) and blood pressure (the average of two readings). $HbA_{1c}$ and serum creatinine were measured on the day of sampling as part of routine clinical testing by standard clinical laboratory methods. Albuminuria was assessed by either a urinary albumin concentration on a spot urine or a 24hour urinary protein concentration with evidence of albuminuria based on the highest level of albuminuria (normo, micro or macroalbuminuria) recorded in the 5 years prior to baseline. Smoking status was based on patient report at study enrolment. Prior CVD was based on the presence of an ICD-9 or ICD-10 code consistent with a major CVD event prior to sampling. Medication was based on primary care prescribing data at study enrolment. Retinopathy status was derived from the retinal screening examination grade closest to study enrolment. We used all measures of serum creatinine up to the time of sampling to calculate a weighted historical eGFR with greatest weight given to the more recent measures. For analysis we considered a basic set of clinical covariates (age, sex, eGFR, albuminuria, $HbA_{1c}$, ACE Inhibitor use and ARB use) as well as an extensive set which also included blood pressure, the weighted average of past eGFRs over a median of 7.2 years, diabetes duration, BMI, prior CVD, insulin use and use of antihypertensive drugs.

**Data cleaning and imputation**

The data from the biomarker laboratories was cleaned and imputed before analysis. We used a sparse iterative regression model for imputation (see **OSM methods**). We used imputation for two issues: left censoring i.e. values below detection limit and for completely missing at random values. All data were Gaussianized prior to analysis

**Data analysis**

We applied two complementary approaches to biomarker selection: forward selection using logistic regression, and sparse logistic regression with the L1 (LASSO) regularization penalty (36) (see **OSM methods**). Prior to selection models we included two filter steps: step one identified all biomarkers with a correlation of >0.9 and for each pair retained a single biomarker (see **OSM table 2**)- where one of the pair of biomarkers was of high prior interest we selected it over the non-high prior interest biomarker (this was the case with the retention of N-Terminal Prohormone B type Natriuretic Peptide over Malondialdehyde-Modified Low-Density Lipoprotein), but otherwise the choice of which biomarker in the pair to retain was random; and step two used the training set data to identify biomarkers with univariate association with the outcome and selected the 50 biomarkers with the strongest associations. We assessed prediction in models where we included or omitted this second filtering step and showed that the best performance was seen with the filtered models.

We used nested k-fold cross validation for learning the parameters of the selection models and actually performing the selection of the biomarker panels. This learning was done on the training fold data (and inner folds defined within it), while the test fold is reserved exclusively for testing the performance of the biomarker panel by computing AUROCs. By only testing the performance on test data not used for selection this yields an unbiased estimate of the AUROC. We used the AUROC on test data as the performance criterion. The highest-scoring method was re-applied to select the final biomarker panel using the complete dataset, and summary statistics of the resulting biomarkers were reported. We used difference in log likelihood computed on withdrawn data to determine whether there was a significant difference between pairs of models using a threshold of a difference of 1 natural log

units as a cut point for statistical significance (see **OSM page 4**). We also calculated the positive predictive value of the test where the probability of a case being correctly identified as such is plotted against the percentile of the score, to demonstrate how using different cut points of the model score might alter probability of identifying those at risk for progression.

All data preparation and analyses were performed using R version 2.15.2.

**Disclosures**

HCL, MC, BF and DD are co-inventors of a patent on biomarkers as predictors in rapid decline of renal function pending. SH is an employee with Sanofi Aventis and is a co-inventor of a patent on biomarkers as predictors in rapid decline of renal function pending; MJB is an employee of Pfizer and a shareholder in Pfizer and is a co-inventor of a patent on biomarkers as predictors in rapid decline of renal function pending; RND and CT were contracted on a fee for service basis for the measurement of biomarkers included in this study with payment coming from EFPIA partners and IMI-JU as part of the SUMMIT project, and outside the submitted work are also founding directors of SpOtOn Clinical Diagnostics Ltd; RND has also received non-financial support from Pfizer Ltd and is a co-inventor of a patent on biomarkers as predictors in rapid decline of renal function pending; EN is employee with F. Hoffmann-La Roche Ltd, Switzerland and is a co-inventor of a patent on biomarkers as predictors in rapid decline of renal function pending FA is a director of data analysis company Pharmatics Limited and is a co-inventor of a patent on biomarkers as predictors in rapid decline of renal function pending ; PMM is a stakeholder in Pharmatics Limited and is a co-inventor of a patent on biomarkers as

predictors in rapid decline of renal function pending. HMC reports grants and personal fees from Pfizer Inc., grants and institutional consultancy fees from Sanofi Aventis, Regeneron and Novartis Pharmaceuticals and is a co-inventor of a patent on biomarkers as predictors in rapid decline of renal function pending.

MW, CNP, LG, and VS report no conflicts of interest.

## Authorship

All authors meet the ICMJE criteria for authorship

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## Figure Legends

### Figure 1: Volcano Plot of Association of 166 Biomarkers with Rapid Progression of eGFR decline

The labelled points are where there was a level of significance -log10(pvalue) >9 or a fold-change greater than +/- >0.6.

### Figure 2: Performance Metrics of Models by Number of Biomarkers Retained and Selection Method

Model performance plots showing the AUROC achieved with the forward selection panel (shown by filled blue square) compared with the performance of the top down selected panels (shown by yellow diamonds) with the number of retained biomarkers allowed to vary up to 35 biomarkers.

A) On top of the AUROC achieved by age, sex, HbA1c, albuminuria, eGFR. ACE Inhibitor and Angiotensin Receptor Blocker use alone
B) On top of the AUROC achieved by an extended set of clinical covariates including longitudinal eGFR (see methods for full list)

### Figure 3: Performance of Panels of Biomarkers Chosen by Forward Selection and Top Down Selection Compared with Clinical Data Alone

Performance plots for the best overall and sparse biomarker models including clinical covariates age, sex, HbA1c, albuminuria, eGFR and ACE Inhibitor and Angiotensin Receptor Blocker use (red line), clinical covariates and forward selection biomarkers (blue line) and clinical covariates and 35 biomarker panel (yellow line)

A) Area Under the ROC curves
B) Positive Predictive Value Plot

**Table 1: Baseline demographics for cases and controls**

| | Control | | Case | |
|---|---|---|---|---|
| | Frequency/ Median | Interquartile Range | Frequency/ Median | Interquartile Range |
| Female sex (%) | 64.3 | - | 57.5 | - |
| Age (years) | 72 | 66, 76 | 74 | 69, 80 |
| Diabetes Duration (years) | 7.2 | 3.5, 11.0 | 9.1 | 5.1, 15.4 |
| Body Mass Index (kg/m$^2$) | 29.5 | 26.1, 34.4 | 30.6 | 27.1, 34.8 |
| Systolic Blood Pressure (mmHg) | 144.3 | 129.8, 153.4 | 144.0 | 131.0, 158.5 |
| Diastolic Blood Pressure (mmHg) | 73.3 | 66.5, 79.4 | 71.0 | 63.5, 78.0 |
| $HbA_{1c}$ (%) | 7.1 | 6.4, 8.2 | 7.3 | 6.5, 8.4 |
| Baseline eGFR (ml/min/1.73m$^2$) | 51.3 | 44.9, 54.6 | 48.2 | 40.5, 54.8 |
| Weighted Average eGFR (ml/min/1.73m$^2$) | 57.8 | 52.6, 63.5 | 50.7 | 44.8, 56.7 |
| Insulin Use (%) | 25.3 | - | 30.7 | - |
| Antihypertensive Use (%) | 95.5 | - | 96.1 | - |
| Diabetic Retinopathy (%) | 55.2 | - | 74.5 | - |
| Smoking (%) | | | | |
| Current smoking | 11.7 | - | 11.1 | - |
| Ex smoker | 40.3 | - | 58.2 | - |
| Never smoker | 48.1 | - | 30.7 | - |
| Prior CVD (%) | 21.43 | - | 28.1 | - |
| Albuminuria * (%) | 18.8 | - | 45.1 | - |
| Median follow-up (years) | 5.8 | 5.5, 6.2 | 3.2 | 2.2, 5.7 |
| Median time to caseness (years) | - | - | 1.8 | 1.2, 2.3 |

* Albuminuria status relates to the presence of microalbuminuria or macroalbuminuria at the time of sampling or any time in the prior 5 years.

Data was complete except for: BMI missing for 2 people, SBP missing for 3 people, DBP missing for 2 people, HbA1c missing for 1 person, and drug treatment missing for 1 person.

**Table 2: Thirty Biomarkers Significantly Associated with Rapid Progressionof eGFR Examined Singly and Adjusted for Clinical Covariates.**

| | Control Median | Control IQR | Case Median | Case IQR | OR | 95% CI | p.value |
|---|---|---|---|---|---|---|---|
| Adrenomedullin (ng/ml) | 2.2 | 1.7, 2.6 | 2.9 | 2.4, 3.6 | 2.94 | 2.10, 4.22 | <0.00001 |
| Alpha-1 Antitrypsin (2) | 161.37 | 127.79, 187.81 | 186.38 | 165.60, 214.51 | 1.77 | 1.36, 2.33 | <0.00001 |
| Alpha-1-Microglobulin (ug/ml) | 17.0 | 14.0, 20.0 | 21.0 | 19.0, 24.3 | 3.31 | 2.31, 4.88 | <0.00001 |
| Beta-2-Microglobulin (ug/ml) | 2.0 | 1.7, 2.4 | 2.7 | 2.4, 3.4 | 6.11 | 3.90, 10.05 | <0.00001 |
| C16-acylcarnitine (nM/l) | 284.14 | 227.66, 355.38 | 305.26 | 280.32, 395.84 | 1.68 | 1.29, 2.21 | 0.00015 |
| Creatinine (uM/l) | 98.18 | 80.49, 112.60 | 113.1 | 91.16, 127.90 | 3.43 | 1.97, 6.36 | <0.00001 |
| Cystatin-C (ng/ml) | 1340 | 1140, 1510 | 1680 | 1490, 1900 | 6.34 | 3.88, 10.97 | <0.00001 |
| Fibroblast Growth Factor 21 (ng/ml) | 0.25 | 0.16, 0.43 | 0.40 | 0.30, 0.65 | 2.06 | 1.56, 2.80 | <0.00001 |
| Fibroblast growth factor 23 (ng/ml) | 0.08 | 0.05, 0.13 | 0.12 | 0.08, 0.22 | 1.85 | 1.37, 2.55 | <0.00001 |
| Growth Derived Factor 15 (pg/ml) | 2328 | 1761, 3355 | 3785 | 2681, 5555 | 2.30 | 1.69, 3.20 | <0.00001 |
| High Sensitivity Troponin T (pg/ml) | 5.29 | 2.89, 12.89 | 16.53 | 9.78, 26.75 | 3.15 | 2.11, 4.85 | <0.00001 |
| Interleukin-2 receptor alpha (pg/ml) | 2493 | 2075, 3152 | 3174 | 2710, 4180 | 2.45 | 1.79, 3.43 | <0.00001 |
| Kidney Injury Molecule-1 (ng/ml) | 0.05 | 0.04, 0.08 | 0.09 | 0.07, 0.16 | 2.60 | 1.88, 3.68 | <0.00001 |
| Lysine (uM/l) | 217.67 | 190.63, 244.12 | 203.9 | 174.87, 215.00 | 0.55 | 0.42, 0.72 | <0.00001 |
| Methylmalonic acid (nM/l) | 270 | 220, 350 | 366 | 310, 460 | 2.09 | 1.56, 2.87 | <0.00001 |

| | Control Median | Control IQR | Case Median | Case IQR | OR | 95% CI | p.value |
|---|---|---|---|---|---|---|---|
| N-acetylaspartate (nM/l) | 296.58 | 239.50, 378.61 | 341.62 | 306.09, 452.19 | 1.76 | 1.33, 2.37 | 0.00013 |
| N-terminal prohormone of brain natriuretic peptide (pg/ml) | 552.5 | 247.00, 1152.50 | 1487.23 | 607.00, 3170.00 | 2.10 | 1.54, 2.94 | <0.00001 |
| Osteopontin (ng/ml) | 15 | 11, 23 | 26 | 18, 33 | 2.58 | 1.82, 3.78 | <0.00001 |
| Sialic acid (uM/l) | 1.09 | 0.93, 1.31 | 1.37 | 1.19, 1.76 | 2.43 | 1.73, 3.52 | <0.00001 |
| Symmetric Dimethylarginine (nM/l) | 564 | 499.00, 647.50 | 662.91 | 578.00, 786.00 | 2.49 | 1.72, 3.69 | <0.00001 |
| SDMA:ADMA | 1.06 | (0.93, 1.22) | 1.23 | 1.13, 1.49 | 2.63 | 1.86, 3.81 | <0.00001 |
| Tamm-Horsfall Urinary Glycoprotein (ug/ml) | 0.04 | 0.03, 0.05 | 0.03 | 0.02, 0.03 | 0.46 | 0.33, 0.62 | <0.00001 |
| Thrombomodulin (ng/ml) | 5.39 | 4.60, 6.40 | 6.5 | 5.75, 7.40 | 2.00 | 1.48, 2.73 | <0.00001 |
| Tissue Inhibitor of Metalloproteinases 1 (ng/ml) | 170 | 150, 192 | 188 | 172, 218 | 2.02 | 1.52, 2.74 | <0.00001 |
| Trefoil Factor 3 (ug/ml) | 0.17 | 0.13, 0.22 | 0.27 | 0.21, 0.37 | 4.17 | 2.81, 6.42 | <0.00001 |
| Tryptophan (uM/l) | 57.31 | 50.65, 64.25 | 52.21 | 43.10, 56.76 | 0.54 | 0.41, 0.72 | <0.00001 |
| Tumor Necrosis Factor Receptor I (pg/ml) | 2639 | 1985, 3217 | 3440 | 2852, 4130 | 2.41 | 1.76, 3.37 | <0.00001 |
| Tumor Necrosis Factor Receptor 2 (ng/ml) | 9.7 | 8.30, 12.00 | 13 | 10.00, 16.00 | 2.55 | 1.84, 3.63 | <0.00001 |
| Uracil (nM/l) | 119.26 | 94.25, 152.80 | 136.54 | 121.23, 172.55 | 1.76 | 1.35, 2.35 | <0.00001 |

| | Control Median | Control IQR | Case Median | Case IQR | OR | 95% CI | p.value |
|---|---|---|---|---|---|---|---|
| Vascular Cell Adhesion Molecule-1 (ng/ml) | 603 | 530, 747 | 724 | 612, 885 | 1.77 | 1.34, 2.36 | <0.00001 |

Odds ratios (OR) are per standard deviate.

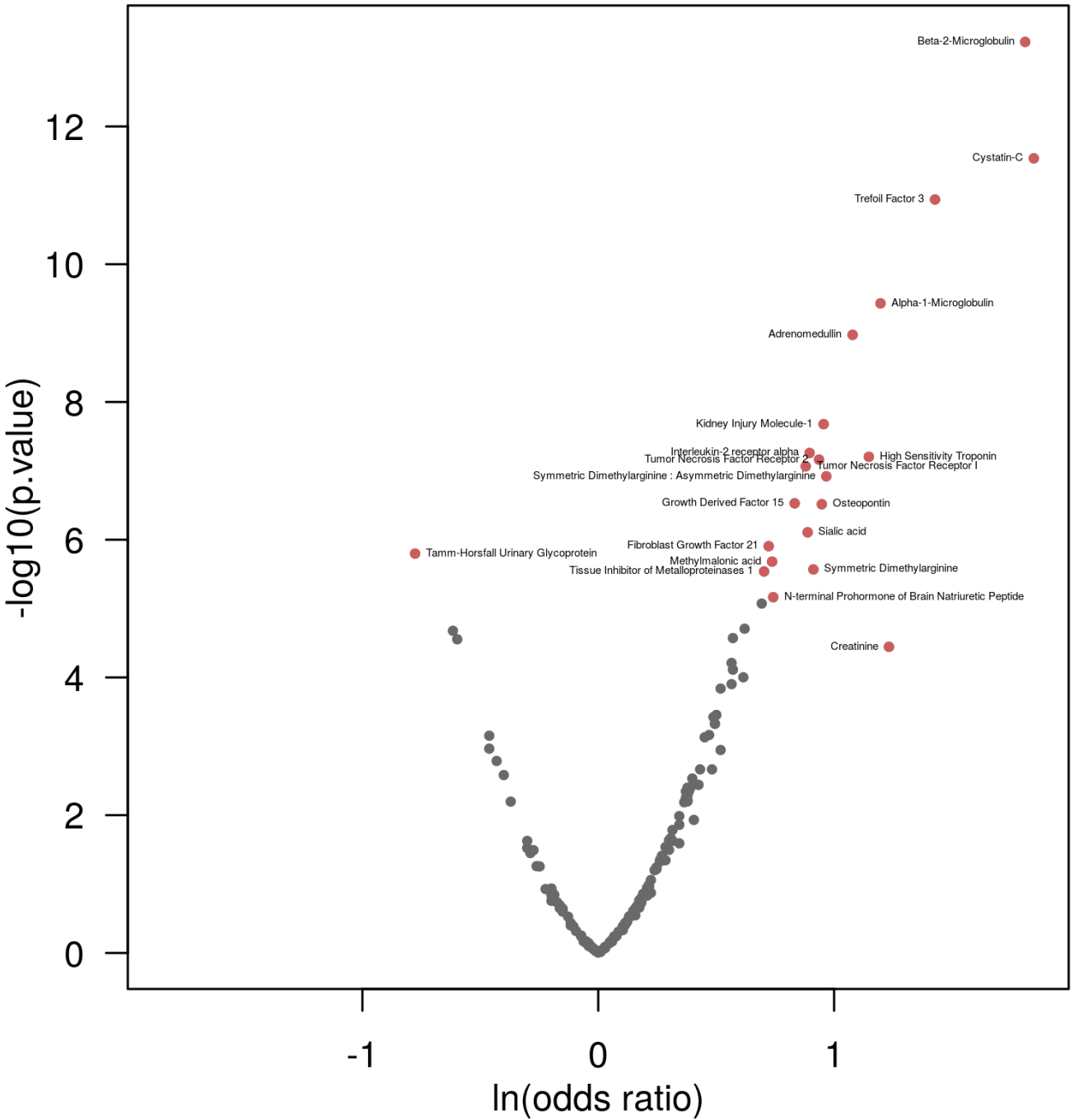
Clinical covariates adjusted for were: age, sex, baseline eGFR, Albuminuria status, $HbA_{1c}$, use of ACE inhibitors and use of ARBs

**Table 3: Association of 14 Biomarkers Contributing to Prediction of Rapid Progression in Forward Selection Adjusted for Each Other and Clinical Covariates***

| | Odds Ratio per Standard Deviate | 95% Confidence Interval | P-value |
|---|---|---|---|
| Symmetric Dimethylarginine : Asymmetric Dimethylarginine | 8.36 | 3.83, 20.40 | <0.0001 |
| Creatinine | 3.52 | 1.54, 8.76 | 0.0042 |
| Beta-2-Microglobulin | 3.19 | 1.56, 6.84 | 0.0019 |
| Symmetric Dimethylarginine | 0.32 | 0.13, 0.72 | 0.0075 |
| Alpha-1 Antitrypsin (2) | 2.05 | 1.38, 3.14 | 0.0006 |
| Kidney Injury Molecule-1 | 1.93 | 1.18, 3.27 | 0.0111 |
| Uracil | 1.84 | 1.22, 2.84 | 0.0046 |
| N-terminal prohormone of brain natriuretic peptide | 1.84 | 1.15, 3.01 | 0.0123 |
| C16-acylcarnitine | 1.76 | 1.16, 2.73 | 0.0090 |
| Hydroxyproline† | 1.73 | 1.12, 2.72 | 0.0151 |
| Fibroblast Growth Factor 21 | 1.69 | 1.06, 2.75 | 0.0288 |
| Fatty Acid-Binding Protein heart † | 0.63 | 0.38, 1.02 | 0.0588 |
| Creatine† | 0.65 | 0.41, 1.01 | 0.0590 |
| Adrenomedullin | 1.07 | 0.56, 2.04 | 0.8370 |

*Clinical covariates included: age, sex, baseline eGFR, Albuminuria status, $HbA_{1c}$, and use of ACE Inhibitors or Angiotensin Receptor Blockers† biomarker not statistically significant in univariate analyses adjusted only for clinical covariates

**Figure 1**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Cystatin-C
Trefoil Factor 3
Alpha-1-Microglobulin
Adrenomedullin
Kidney Injury Molecule-1
Interleukin-2 receptor alpha
Tumor Necrosis Factor Receptor 2
High Sensitivity Troponin
Tumor Necrosis Factor Receptor I
Symmetric Dimethylarginine : Asymmetric Dimethylarginine
Growth Derived Factor 15
Osteopontin
Sialic acid
Fibroblast Growth Factor 21
Tamm-Horsfall Urinary Glycoprotein
Methylmalonic acid
Tissue Inhibitor of Metalloproteinases 1
Symmetric Dimethylarginine
N-terminal Prohormone of Brain Natriuretic Peptide
Creatinine
-log10(p.value)
12
10
8
6
4
2
0
-1
0
1
ln(odds ratio)

**Figure 2**

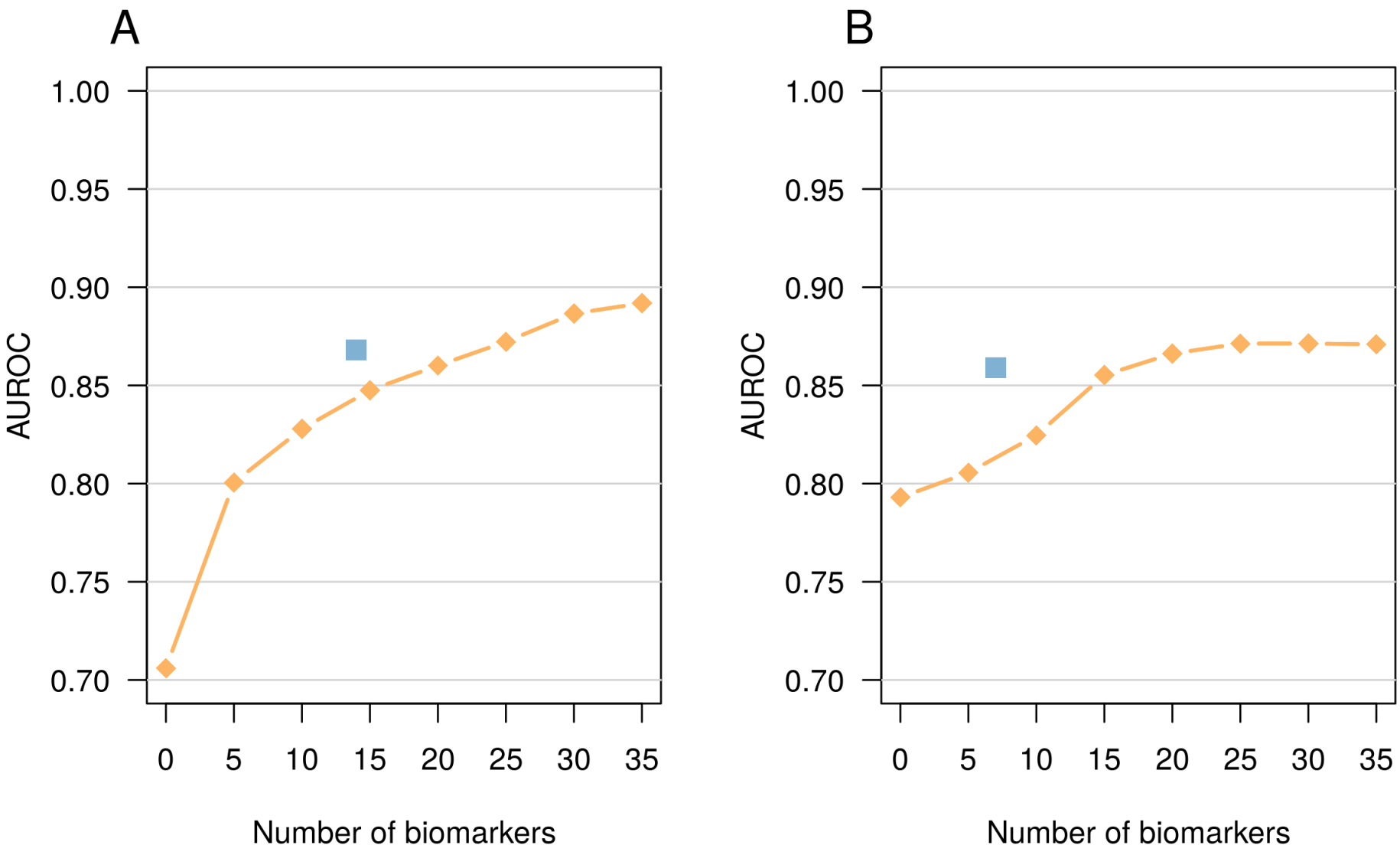
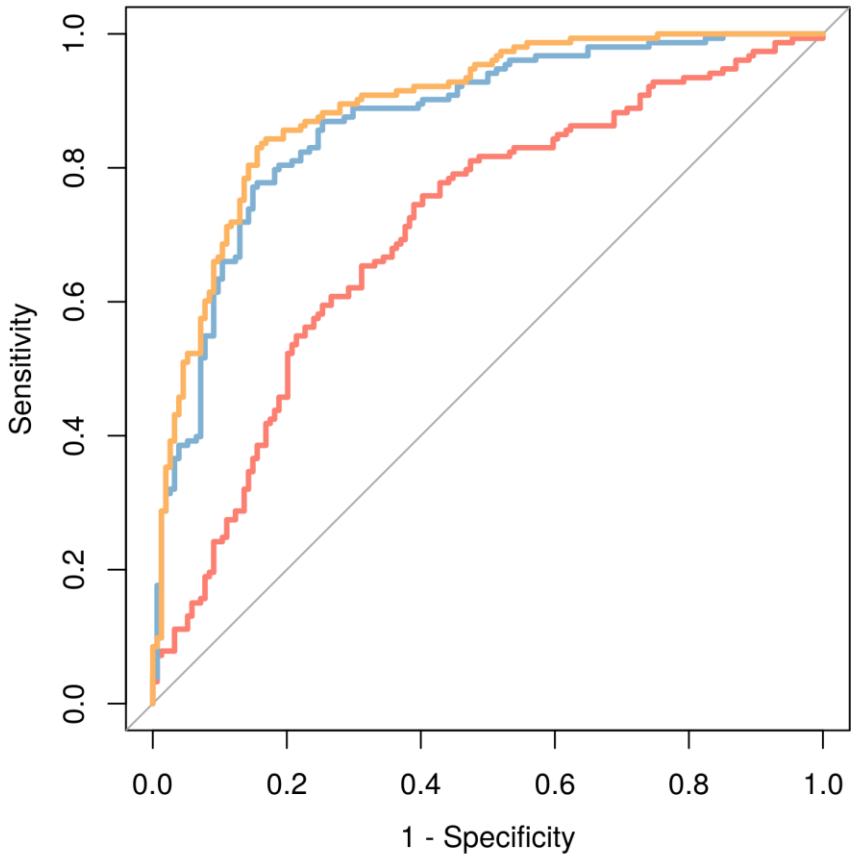

A
B
AUROC
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0
5
10
15
20
25
30
35
Number of biomarkers

**Figure 3**

**A**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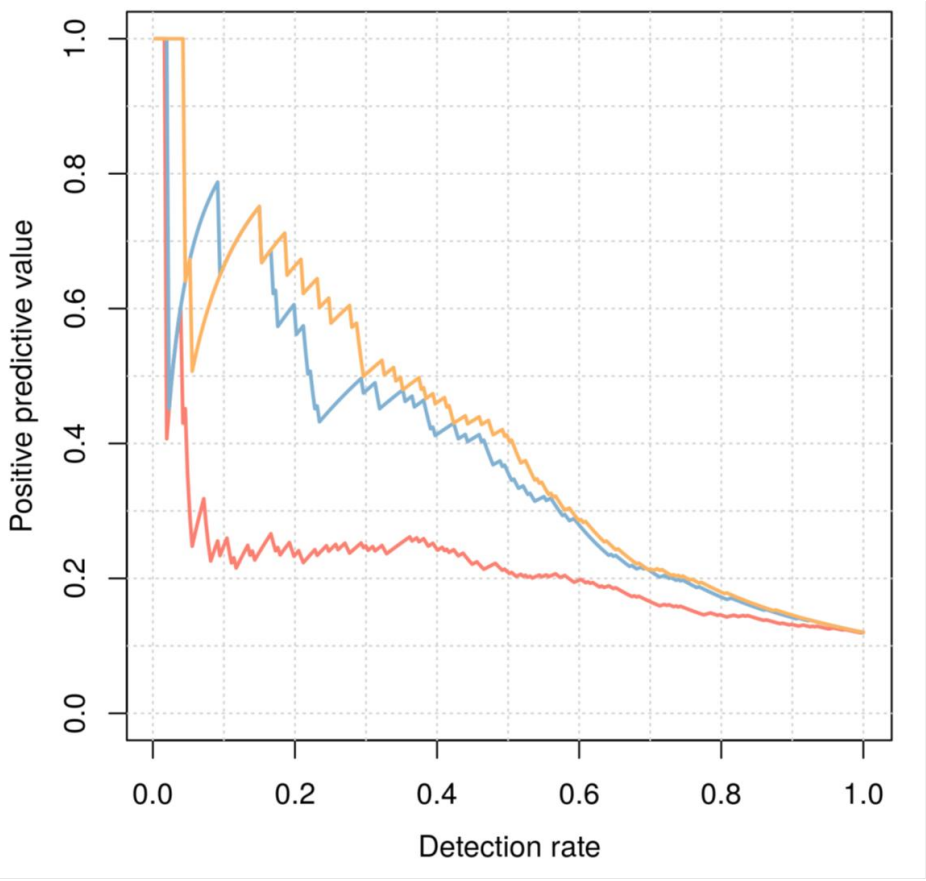
0.4
0.6
0.8
1.0
1 - Specificity

**B**


Positive predictive value
0.0
0.2
0.4
0.6
0.8
1.0
Detection rate