Identification of Novel Circulating Biomarkers Predicting Rapid Decline in Renal Function in Type 2 Diabetes: The Fremantle Diabetes Study Phase II

Kirsten E. Peters,[1,2] Wendy A. Davis,[1] Jun Ito,[2] Kaye Winfield,[2] Thomas Stoll,[2] Scott D. Bringans,[2] Richard J. Lipscombe,[2] and Timothy M.E. Davis[1]

Diabetes Care 2017;40:1548–1555 | https://doi.org/10.2337/dc17-0911

OBJECTIVE

To assess the ability of plasma apolipoprotein (apo) A-IV (apoA4), apo C-III, CD5 antigen-like (CD5L), complement C1q subcomponent subunit B (C1QB), complement factor H–related protein 2, and insulin-like growth factor binding protein 3 (IBP3) to predict rapid decline in estimated glomerular filtration rate (eGFR) in type 2 diabetes.

RESEARCH DESIGN AND METHODS

Mass spectrometry was used to measure baseline biomarkers in 345 community-based patients (mean age 67.0 years, 51.9% males) from the Fremantle Diabetes Study Phase II (FDS2). Multiple logistic regression was used to determine clinical predictors of rapid eGFR decline trajectory defined by semiparametric group-based modeling over a 4-year follow-up period. The incremental benefit of each biomarker was then assessed. Similar analyses were performed for a ≥30% eGFR fall, incident chronic kidney disease (eGFR <60 mL/min/1.73 m^2), and eGFR decline of ≥5 mL/min/1.73 m^2/year.

RESULTS

Based on eGFR trajectory analysis, 35 participants (10.1%) were defined as "rapid decliners" (mean decrease 2.9 mL/min/1.73 m^2/year). After adjustment for clinical predictors, apoA4, CD5L, and C1QB independently predicted rapid decline (odds ratio 2.40 [95% CI 1.24–4.61], 0.52 [0.29–0.93], and 2.41 [1.14–5.11], respectively) and improved model performance and fit ($P < 0.001$), discrimination (area under the curve 0.75–0.82, $P = 0.039$), and reclassification (net reclassification index 0.76 [0.63–0.89]; integrated discrimination improvement 6.3% [2.1–10.4%]). These biomarkers and IBP3 contributed to improved model performance in predicting other indices of rapid eGFR decline.

CONCLUSIONS

The current study has identified novel plasma biomarkers (apoA4, CD5L, C1QB, and IBP3) that may improve the prediction of rapid decline in renal function independently of recognized clinical risk factors in type 2 diabetes.

[1]Medical School, Faculty of Health and Medical Sciences, The University of Western Australia, Fremantle, Western Australia, Australia
[2]Proteomics International, Perth, Western Australia, Australia

Corresponding author: Timothy M.E. Davis, tim.davis@uwa.edu.au.

Received 8 May 2017 and accepted 3 August 2017.

This article contains Supplementary Data online at http://care.diabetesjournals.org/lookup/suppl/doi:10.2337/dc17-0911/-/DC1.

Diabetes is the main cause of end-stage renal disease (ESRD), accounting for 40–50% of new cases in the U.S. and the largest annual health care expenditure compared with all other primary ESRD diagnoses (1,2). Up to one-third of adults with newly diagnosed type 2 diabetes have chronic kidney disease (CKD) (2), implying that it often develops during the course of prediabetes. Conventional assessment and monitoring of CKD is by measurement of albuminuria (urinary albumin-to-creatinine ratio [ACR] or urinary albumin excretion rate) and renal function (estimated glomerular filtration rate [eGFR]), but these measures are subject to substantial intraindividual variability over time that reflects intercurrent illness, hydration status, and medication changes (3). The relationship between ACR and eGFR is also variable, an example being the development of CKD (eGFR $<$60 mL/min/1.73 m^2) without albuminuria (4). In addition, the ability of baseline ACR and/or eGFR to predict the onset and progression of diabetic kidney disease (DKD) remains poor (5). Given the prognostic limitations of ACR and eGFR, there has been a focus on alternative biomarkers that could identify patients who are at increased risk of DKD. This includes studies of a range of plasma proteins (6–12), but most of these have been limited by small sample sizes; the exclusion of patients without albuminuria and/or CKD; and/or the inclusion of patients who are not representative of type 2 diabetes in the community, such as those participating in clinical trials or those who have been selected from hospital outpatient clinics.

The progression of DKD is traditionally analyzed using the hard end point of ESRD, with the doubling of serum creatinine level (corresponding to a 57% reduction in eGFR) accepted as a useful surrogate. However, these end points accrue relatively slowly, with large longitudinal studies required to capture sufficient outcomes. Recent interest has turned to alternative eGFR-based metrics that can be used over shorter time periods. The U.S. Food and Drug Administration has proposed a 30–40% eGFR decline over 2 or 3 years as a suitable surrogate end point in clinical trials as it is strongly and consistently associated with ESRD (13). The Kidney Disease: Improving Global Outcomes (KDIGO) guidelines (14) recommend an annual eGFR decline of 5 mL/min/1.73 m^2 as an alternative for the assessment of DKD progression. Serial measurements of serum creatinine defining the trajectories of kidney function over time, such as through latent class analysis, have been shown to capture the dynamic nature of eGFR, with rapid declining trajectories associated with all-cause mortality (15) and major cardiovascular events (16) in individuals with type 2 diabetes.

The aim of the present longitudinal observational study was, therefore, to assess the ability of a selection of novel groupings of plasma protein biomarkers to predict rapid declining eGFR in a representative community-based cohort of individuals with type 2 diabetes. The incremental benefit of biomarkers added to clinical prediction models was determined across the following four clinically relevant definitions of DKD progression: *1*) rapidly declining eGFR trajectory, *2*) incident CKD, *3*) an eGFR decline of $\geq$30% over 4 years (or 7.5%/year), and *4*) an annual eGFR decline of $\geq$5 mL/min/1.73 m^2.

RESEARCH DESIGN AND METHODS

Patients

We used data from the longitudinal observational Fremantle Diabetes Study Phase II (FDS2), details of which have been published (17). Of 1,551 patients with type 2 diabetes recruited to the FDS2 between 2008 and 2011, 345 had attended three biennial assessments (baseline, year 2, and year 4) between 2008 and 2014 and had complete data on urine ACR, eGFR, and medication, including the use of renin-angiotensin-aldosterone system (RAAS) inhibitors. Fasting plasma samples collected from this subgroup and stored at −80°C were used in the present FDS2 substudy. The FDS2 protocol was approved by the South Metropolitan Area Health Service Human Research Ethics Committee. All subjects gave informed consent before participation.

Renal Outcomes

The Chronic Kidney Disease Epidemiology Collaboration equation was used to calculate eGFR (18). The primary outcome of interest was the eGFR trajectory based on the strong association between this measure and adverse outcomes in type 2 diabetes (15,16). Trajectories were modeled using finite mixtures of suitably defined probability distributions, as described previously (15). The modeling identified four linear trajectories of eGFR change over time ("low," "medium," "high," and "rapid declining"). For further analysis by trajectory, patients with a rapid declining trajectory ("rapid decliners") were compared with those with a nonrapid declining trajectory (called "nonrapid decliners," pooling data from patients in the "low," "medium," and "high" trajectories).

In a series of additional analyses, the utility of the biomarkers for predicting rapid kidney decline by alternative definitions were investigated, including *1*) incident CKD (eGFR $<$60 mL/min/1.73 m^2 at year 4 in individuals who had an eGFR $\geq$60 mL/min/1.73 m^2 at baseline), *2*) eGFR decline of $\geq$30% between study entry and year 4 (7.5%/year) (13), and *3*) an annual decline in eGFR of $\geq$5 mL/min/1.73 m^2 calculated as (baseline eGFR − year 4 eGFR)/(time between baseline and year 4) (14). Microalbuminuria and macroalbuminuria were defined as a first-morning urinary ACR of $\geq$3 mg/mmol and $\geq$30 mg/mmol, respectively.

Biomarker Discovery and Verification

Biomarker discovery has been described previously (19). In brief, a list of candidate biomarkers was determined by a proteomics mass spectrometry–based discovery and validation workflow. All samples were measured using targeted mass spectrometry, known as multiple reaction monitoring (MRM). Changes in relative peptide abundance were measured against an ^{18}O-labeled reference plasma to give peak area ratios for each biomarker. The robustness of the MRM assay was demonstrated by the relative quantitative analysis of intraday and interday reference plasma controls and a synthetic stable isotope–labeled peptide (intraday coefficient of variation 5.9%, interday coefficient of variation 8.1%). In previously reported studies, the MRM assay PromarkerD (Proteomics International, Perth, Australia) was developed that identified a panel of simultaneously measured biomarkers of DKD (19) that included apolipoprotein A-IV (apoA4), apolipoprotein C-III (apoC3), CD5 antigen-like (CD5L), complement C1q subcomponent subunit B (C1QB), complement factor H–related protein 2 (CFHR2), and insulin-like growth factor–binding protein 3 (IBP3) (20).

Statistical Analyses

Statistical analyses were performed in SPSS for Windows (version 22; SPSS Inc., Chicago, IL) and RStudio software (version 1.0.136). A two-tailed level of significance

of $P < 0.05$ was used throughout. Data are presented as proportions, mean ± SD, geometric mean (SD range), or, in the case of variables that did not conform to a normal or $\log_e$-normal distribution (ln), median and interquartile range (IQR). All biomarker peak area ratios were ln-transformed prior to analysis. For independent samples, two-way comparisons for proportions were performed by Fisher exact test, for normally distributed variables by Student *t* test, and for non-normally distributed variables by Mann-Whitney *U* test.

Multivariate logistic regression analysis (forward conditional variable selection with $P < 0.05$ for entry and $P > 0.10$ for removal) was used to investigate independent predictors of each definition of rapid decline. All clinically plausible variables with bivariate $P \leq 0.20$ were considered for entry in a forward stepwise manner. After the most parsimonious clinical model was defined (clinical model), all plasma biomarker concentrations were considered for entry in a forward stepwise manner (clinical model plus biomarker model 1). To assess the prognostic performance of a combined panel of biomarkers, the significant biomarkers from each definition of rapid decline were forced into a series of additional models (clinical model plus biomarker model 2). Only participants with complete data were included in each model.

Measures of model fit, calibration, discrimination, and reclassification were used to assess the incremental benefit of biomarkers to each clinical model for predicting the risk of rapid decline. Model fit was determined using the likelihood ratio test (LRT), with higher χ^2 values indicating better global fit. Model calibration was determined using the Hosmer-Lemeshow goodness-of-fit test, with larger *P* values (>0.05) showing good agreement between observed and predicted outcomes. Observed probabilities were plotted against predicted probabilities over deciles of predictions (from Hosmer-Lemeshow tests) for each model to assess calibration. Model discrimination was assessed by the area under the curve (AUC) of the receiver operating characteristic. The Youden index was used to determine the optimal cutoff for maximum sensitivity and specificity in each model. Improvement in AUC after the addition of the biomarkers was calculated using the method of DeLong et al. (21). Internal validation was performed using 1,000 bootstrap resamples to adjust for statistical optimism/overfitting. The optimism-corrected AUC, calibration slope, and intercept are a more approximate estimate of the performance of the model in an external sample (22).

Continuous/category-free net reclassification improvement (NRI >0) was used to assess model reclassification because no established risk cutoffs warrant the use of categorical NRI (23). The overall NRI shows the proportion of upward and downward movement in predicted probabilities when the biomarkers are added to the clinical model. Absolute integrated discrimination improvement (IDI) was used to determine the average increase in predicted probabilities for those who were rapid decliners and the reduction in those who were not rapid decliners after the addition of the biomarkers (24). The relative IDI (rIDI) was calculated as the ratio of IDI over the discrimination slope of the clinical model. The NRI and IDI were assessed overall and separately in rapid decliners (NRI in rapid decliners [NRI_R]; IDI in rapid decliners [IDI_R]) and in nonrapid decliners (NRI in nonrapid decliners [NRI_{NR}]; IDI in nonrapid decliners [IDI_{NR}]).

RESULTS

Cohort Characteristics

The patient characteristics of the present FDS2 subgroup at study entry are summarized in Table 1. The 345 participants had a mean ± SD age of 67.0 ± 9.4 years, 51.9% were males, and their median diabetes duration was 9.0 years (IQR 3.0–15.2 years). The mean baseline eGFR was 80.6 ± 18.8 mL/min/1.73 m^2, 13.0% had CKD, 33.9% had microalbuminuria, and 4.1% had macroalbuminuria. Most (71.0%) were receiving treatment with RAAS inhibitors. Over the 4 years encompassed by the present substudy, the mean annual decline in eGFR was 1.7 ± 2.5 mL/min/1.73 m^2. There were no participants with a baseline eGFR of <15 mL/min/1.73 m^2.

Rapidly Declining eGFR Trajectory

Based on latent class analysis, 35 individuals (10.1%) were in the rapid declining eGFR trajectory group with a mean annual decline in eGFR of 2.9 mL/min/1.73 m^2. The remaining 310 individuals (low, medium, and high eGFR trajectories) had a mean decline of 1.6 mL/min/1.73 m^2/year. Baseline clinical and demographic characteristics in these two subgroups are shown in Table 1. Individuals with a rapidly declining eGFR trajectory were older and had longer diabetes duration and higher serum triglyceride, uric acid, and creatinine concentrations; lower eGFR and total cholesterol; and a greater prevalence of ischemic heart disease and diuretic medication use than those with nonrapid decline. Baseline apoA4, apoC3, C1QB, and CFHR2 levels were higher in rapid decliners compared with nonrapid decliners.

The results of three multivariate prognostic models (clinical and clinical plus biomarkers 1 and 2) are shown in Table 2. In the clinical model, diuretic use, older age, longer diabetes duration, and lower serum HDL cholesterol level were independent predictors of rapid eGFR decline. After adjusting for the most parsimonious clinical model, higher apoA4 and C1QB levels and lower CD5L levels were significant independent predictors. Duration of diabetes became a nonsignificant predictor after the addition of the biomarkers. The addition of the biomarkers to the clinical model improved model fit (ΔLRT χ^2 = 19.16, $P < 0.001$), calibration (Hosmer-Lemeshow test $P = 0.11$), discrimination (AUC increase from 0.75 to 0.82, $P = 0.039$), sensitivity and specificity (increased from 82.4% to 88.2% and from 63.4% to 68.5%, respectively), and risk classification (Fig. 1). A calibration plot of the observed probabilities against the predicted probabilities over deciles of predictions (from Hosmer-Lemeshow tests) for each model shows acceptable calibration with data close to the 45° line (Supplementary Fig. 1). The bootstrapped optimism-corrected AUCs were 0.73 and 0.78, respectively, for the clinical and clinical plus biomarker models (Table 2). There was no improvement in calibration intercept and slope.

The biomarkers improved risk classification when added to the clinical prediction model (overall NRI 0.76 [95% CI 0.63–0.89]). Of the 34 rapid decliners, 24 (70.6%) were reclassified as being at higher risk and 10 (29.4%) as being at lower risk (NRI_R 0.41 [0.11–0.72]). Of the 292 nonrapid decliners, 95 (32.5%) were reclassified to higher risk and 197 (67.5%) to lower risk (NRI_{NR} 0.35 [0.24–0.46]). The absolute IDI indicates that there was a significant increase in predicted probabilities for those who were rapid decliners (IDI_R 5.6% [1.5–9.6%]) and a reduction in

Table 1—Baseline demographic and clinical characteristics of 345 participants with type 2 diabetes by rapid eGFR decline defined by eGFR trajectory

	N	All	Nonrapid decliners	Rapid decliners	*P*
Number (%)	345	345	310 (89.9)	35 (10.1)	
Age (years)	345	67.0 ± 9.4	66.6 ± 9.3	70.3 ± 8.8	0.028
Male sex (%)	345	51.9	51.6	54.3	0.86
BMI (kg/m^2)	345	31.0 ± 5.5	31.0 ± 5.6	30.7 ± 5.1	0.75
Waist circumference (cm)	345	102.7 ± 13.5	102.5 ± 13.7	104.7 ± 12.1	0.36
Ethnic background (% AC/SE/OE/ Asian/Ab/other)	345	64.9/11.0/7.0/3.2/0.3/13.6	64.5/11.3/7.4/3.5/0.3/12.9	68.6/8.6/2.9/0.0/0.0/20.0	0.64
Age at diabetes diagnosis (years)	345	57.1 ± 10.9	57.1 ± 11.1	57.2 ± 9.5	0.95
Diabetes duration (years)*	345	9.0 [3.0–15.2]	8.2 [3.0–15.0]	13.3 [6.0–19.9]	0.014
Fasting plasma glucose (mmol/L)†	344	7.1 (5.5–9.2)	7.1 (5.5–9.1)	7.6 (5.6–10.3)	0.16
HbA_{1c} (%)	345	7.0 ± 1.0	6.9 ± 1.0	7.2 ± 1.3	0.18
HbA_{1c} (mmol/mol)	345	53 ± 10.9	52 ± 11.1	55 ± 13.9	0.18
Serum total cholesterol (mmol/L)	344	4.3 ± 1.0	4.3 ± 1.0	4.1 ± 1.0	0.31
Serum HDL cholesterol (mmol/L)	344	1.28 ± 0.31	1.29 ± 0.31	1.16 ± 0.31	0.020
Serum triglycerides (mmol/L)†	344	1.5 (0.9–2.3)	1.4 (0.9–2.3)	1.7 (1.0–2.8)	0.044
Serum uric acid (mmol/L)†	344	0.34 (0.26–0.44)	0.34 (0.26–0.43)	0.38 (0.31–0.47)	0.005
Serum creatinine (μmol/L)†	345	75 (56–101)	73 (55–97)	98 (76–127)	<0.001
Urinary ACR (mg/mmol)†	345	2.9 (0.9–8.8)	2.8 (0.9–8.7)	3.4 (1.1–10.4)	0.35
eGFR (mL/min/1.73 m^2)	345	80.6 ± 18.8	82.9 ± 17.4	59.9 ± 18.1	<0.001
eGFR categories (% G1/G2/ G3a/G3b/G4)‡	345	37.7/49.3/6.4/5.5/1.2	40.6/50.6/3.5/3.9/1.3	11.4/37.1/31.4/20.0/0.0	<0.001
CKD stage (% 0/1/2/3)§	345	57.1/30.7/6.1/6.1	60.0/30.6/4.5/4.8	31.4/31.4/20.0/17.1	<0.001
Systolic blood pressure (mmHg)	345	147 ± 20	147 ± 20	148 ± 24	0.80
Diastolic blood pressure (mmHg)	345	80 ± 12	80 ± 12	77 ± 11	0.08
Neuropathy (%)	345	73.6	73.5	74.2	>0.99
PAD (%)	345	17.4	17.1	20.0	0.64
CVD (%)	345	5.5	4.5	14.3	0.033
IHD (%)	345	25.5	24.5	34.3	0.22
Alcohol consumption (standard drinks/day)*	326	0.1 [0.0–1.5]	0.1 [0.0–1.2]	0.3 [0.0–1.5]	0.47
Smoking status (% never/ex-/current)	345	47.2/47.0/5.8	47.1/46.5/6.5	48.6/51.4/0.0	0.36
Any physical activity (%)	341	94.4	95.1	88.6	0.12
Diabetes treatment (%)					
Diet	345	29.3	30.3	20.0	0.24
OHA	345	49.0	49.0	48.6	1.00
Insulin ± OHA	345	21.7	20.6	31.4	0.19
Antihypertensive medication (%)	345	79.7	78.4	91.4	0.08
Diuretic	345	34.8	32.3	57.1	0.005
ACE-I	345	44.3	43.2	54.3	0.22
ARB	345	33.9	33.2	40.0	0.45
β-Blocker	345	22.3	21.9	25.7	0.67
Calcium channel blocker	345	26.1	25.5	31.4	0.43
Other	345	4.3	4.2	5.7	0.66
Lipid-lowering medication (%)	345	73.9	73.2	80.0	0.54
Aspirin use (%)	344	72.4	43.2	48.6	0.59
Plasma biomarkers (peak area ratios)†					
apoA4	345	1.17 (0.57–2.42)	1.12 (0.54–2.30)	1.78 (0.96–3.32)	<0.001
apoC3	345	0.86 (0.33–2.28)	0.83 (0.32–2.17)	1.21 (0.43–3.36)	0.031
CD5L	344	2.37 (1.17–4.79)	2.39 (1.18–4.82)	2.23 (1.08–4.61)	0.59
C1QB	328	0.41 (0.22–0.77)	0.40 (0.22–0.73)	0.53 (0.25–1.12)	0.012
CFHR2	344	0.95 (0.53–1.72)	0.92 (0.52–1.60)	1.35 (0.66–2.78)	<0.001
IBP3	335	0.97 (0.58–1.64)	0.96 (0.58–1.59)	1.08 (0.56–2.09)	0.22

Rapid decliners were defined by eGFR trajectories as described in RESEARCH DESIGN AND METHODS. All values are mean ± SD, unless noted otherwise. Ab, Aboriginal; AC, Anglo-Celtic; ACE-I, ACE inhibitor; ARB, angiotensin receptor blocker; CVD, cerebrovascular disease; IHD, ischemic heart disease; OE, other European; OHA, oral hypoglycemic agent; PAD, peripheral arterial disease; SE, southern European. *Median [IQR]. †Geometric mean (SD range). ‡eGFR categories: G1 ≥90; G2 60–89; G3a 45–59; G3b 30–44; G4 15–29 mL/min/1.73 m^2. §CKD stage defined by KDIGO 2012 guidelines (39).

Table 2—Performance of the clinical and clinical plus biomarkers prediction models for rapid eGFR decline defined by eGFR trajectory

	Clinical model (*N* = 326)	Clinical plus biomarkers model 1 (*N* = 326)	Clinical plus biomarkers model 2 (*N* = 316)§
Variable			
Diuretic use	2.41 (1.14–2.08), 0.021	2.59 (1.17–5.70), 0.019	2.52 (1.11–5.74), 0.028
Age (per 10 years)	1.67 (1.06–2.64), 0.027	1.75 (1.08–2.83), 0.022	1.73 (1.04–2.87), 0.034
Diabetes duration (per 5 years)	1.28 (1.02–1.60), 0.033	1.22 (0.94–1.57), 0.129	1.19 (0.92–1.56), 0.192
HDL cholesterol (per mmol/L)	0.10 (0.02–0.45), 0.003	0.08 (0.02–0.38), 0.001	0.11 (0.02–0.51), 0.005
ln(apoA4)*	NI	2.40 (1.24–4.61), 0.009	2.93 (1.40–6.16), 0.004
ln(C1QB)*	NI	2.41 (1.14–5.11), 0.021	2.65 (1.19–5.92), 0.017
ln(CD5L)*	NI	0.52 (0.29–0.93), 0.027	0.50 (0.27–0.92), 0.027
ln(IBP3)*	NI	NI	0.80 (0.37–1.74), 0.573
Performance measure			
LRT χ^2 test, *P*	25.41, <0.001	44.57, <0.001	44.13, <0.001
ΔLRT χ^2 test, *P*	Reference	19.16, <0.001	21.21, <0.001
H-L test χ^2, *P*	6.95, 0.54	13.0, 0.11	6.37, 0.61
Sensitivity (%)†	82.4	88.2	84.4
Specificity (%)†	63.4	68.5	72.2
Positive predictive value (%)†	20.8	24.6	25.5
Negative predictive value (%)†	96.9	98.0	97.6
AUC (95% CI)	0.75 (0.66–0.84)	0.82 (0.76–0.88)	0.83 (0.77–0.89)
ΔAUC, *P*	Reference	0.07, 0.039	0.08, 0.023
Optimism-corrected AUC‡	0.73	0.78	0.79
Calibration intercept‡	−0.17	−0.28	−0.34
Calibration slope‡	0.89	0.82	0.79
NRI (>0)	Reference	0.76 (0.63–0.89)	0.82 (0.68–0.95)
NRI_R	Reference	0.41 (0.11–0.72)	0.44 (0.13–0.75)
NRI_{NR}	Reference	0.35 (0.24–0.46)	0.38 (0.27–0.49)
Absolute IDI (%)	Reference	6.3 (2.1–10.4)	7.4 (3.1–11.6)
IDI_R	Reference	5.6 (1.5–9.6)	6.6 (2.5–10.8)
IDI_{NR}	Reference	0.7 (−0.2 to 1.5)	0.8 (−0.2 to 1.7)
rIDI (%)	Reference	68.5	64.5

Only participants with complete data were included in each model. The most parsimonious clinical model was derived as described in RESEARCH DESIGN AND METHODS, followed by inclusion of biomarkers with significant independent predictive value (clinical plus biomarkers model 1); then all significant biomarkers across the four definitions of rapid eGFR decline were forced into the clinical model (clinical plus biomarkers model 2). Values are given as OR (95% CI), *P*, unless otherwise indicated. AUC, area under the curve; H-L, Hosmer-Lemeshow; IDI, integrated discrimination index; NI, not included. *A 2.72-fold change in mean peak area ratio of apoA4, C1QB, CD5L, or IBP3 corresponds to a change of 1 in ln-transformed (apoA4, C1QB, CD5L, or IBP3), respectively. †Based on optimal cutoff defined by Youden index. ‡Based on internal validation by bootstrap resampling. §The performance of this model was compared with the clinical model applied to the same 316 individuals.

those who were not (IDI_{NR} 0.7% [−0.2 to 1.5]) after the addition of the biomarkers, resulting in an overall gain in predictive ability of the model (overall IDI 6.3% [2.1–10.4%]) (Table 2). Similarly, the rIDI showed improvement compared with the clinical model after the biomarkers were added (rIDI 68.5%).

Significant biomarkers from each of the four definitions of rapid eGFR decline (apoA4, C1QB, CD5L, and IBP3; see below) were combined into a final clinical plus biomarker model 2. The addition of IBP3 to apoA4, C1QB, and CD5L in predicting rapidly declining eGFR trajectory provided further incremental improvements in model fit, discrimination, and reclassification (Table 2). In addition, microalbuminuria was forced into all clinical prediction models but failed to provide significant predictive power when considered as either a categorical variable (ACR ≥3 mg/mmol) or a continuous variable (data not shown).

Alternative Definitions of Rapid eGFR Decline

There is limited consensus in measuring the progression of DKD, hence the baseline clinical and demographic characteristics according to alternative definitions of rapid eGFR decline (incident CKD, eGFR decline ≥30%, and annual decline in eGFR ≥5 mL/min/1.73 m^2) were compared (Supplementary Tables 1–3). A series of additional clinical and clinical plus biomarker models were developed, and their predictive performance was assessed (Supplementary Tables 4–6).

During 4.1 years (IQR 3.7–4.4 years) of follow-up, CKD developed in 37 individuals (12.3%) with the clinical model identifying ischemic heart disease, lower baseline eGFR, and total cholesterol level as independent predictors. After adjustment, higher apoA4 added significantly to the clinical model (clinical plus biomarker model 1), improving model fit, calibration, discrimination, and reclassification (Supplementary Fig. 1 and Supplementary Table 4). The addition of biomarkers C1QB, CD5L, and IBP3 to apoA4 (clinical plus biomarker model 2) showed further incremental improvements in model reclassification for predicting incident CKD (Supplementary Table 4).

During follow-up, there were 30 individuals (8.7%) with a fall in eGFR of ≥30% over 4 years. These participants had a mean decrease in eGFR of 6.3 vs. 1.3 mL/min/1.73 m^2/year in the 315 individuals with <30% decline ($P < 0.001$). The clinical model for predicting this renal end point included ischemic heart disease, diuretic use, older age, increased diastolic blood pressure, and lower

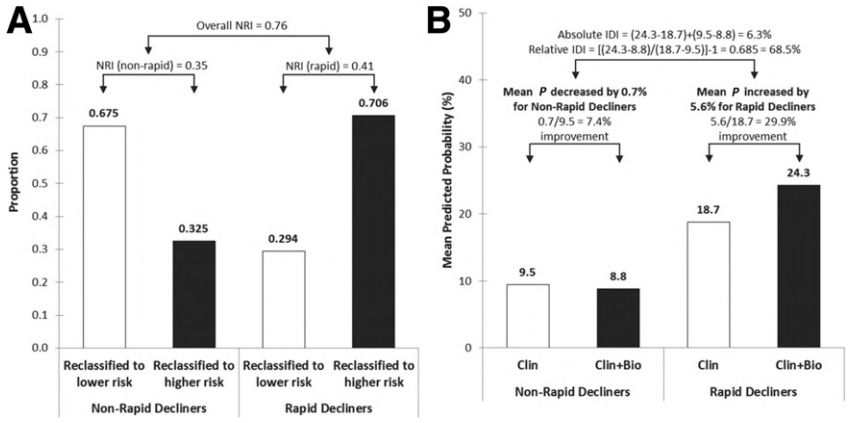

Figure 1—Graphical depiction of NRI (*A*) and IDI (*B*) for rapid eGFR decline defined by eGFR trajectory. The NRI plot shows the proportion of individuals reclassified to higher or lower risk by rapid decline status after the addition of biomarkers to the clinical model. The IDI plot shows the mean predicted probability of rapid and nonrapid decliners according to the clinical (Clin) and clinical plus biomarker (Clin+Bio) models.

serum total cholesterol. After adjustment, higher apoA4 and lower IBP3 levels added significantly to the predictive ability of the clinical model (clinical plus biomarker model 1) (Supplementary Fig. 1 and Supplementary Table 5). The addition of C1QB and CD5L to apoA4 and IBP3 (clinical plus biomarker model 2) showed further incremental improvements in model reclassification (Supplementary Table 5).

There was an annual decline in eGFR of $\geq$5 mL/min/1.73 m^2 in 28 individuals (8.1%), which was predicted by the presence of ischemic heart disease, increased diastolic blood pressure, and increased HbA_{1c}. After adjustment, a lower IBP3 level added significantly to the clinical prediction model (clinical plus biomarker model 1) and improved model performance (Supplementary Fig. 1 and Supplementary Table 6). The addition of biomarkers apoA4, C1QB, and CD5L to IBP3 (clinical plus biomarker model 2) showed further incremental improvements in model fit, discrimination, and reclassification for predicting annual eGFR decline $\geq$5 mL/min/1.73 m^2 (Supplementary Table 6).

CONCLUSIONS

The present substudy from the longitudinal observational FDS2 has extended preliminary developmental data relating to a novel panel of plasma proteins (PromarkerD) (19) to confirm that these biomarkers have prognostic use in DKD. Four biomarkers, apoA4, CD5L, C1QB, and IBP3, predicted measures of rapid decline in eGFR over a 4-year follow-up period in community-based patients with type 2 diabetes. Their prognostic use was independent of conventional clinical variables, and they added significant predictive value as assessed from indices of model fit, calibration, discrimination, and reclassification.

Other published data support the PromarkerD panel as a DKD prognostic index. There is a reported association between higher circulating levels of apoA4 and renal impairment in individuals without diabetes (25,26), and there is evidence for increased renal apoA4 excretion in individuals with diabetic nephropathy (27). Raised apoA4 levels are an early marker of mild to moderate DKD (25), predict CKD progression in patients recruited from renal outpatient clinics (28), and are strongly associated with CKD independently of known risk factors in the general population (26). C1QB is deposited in the kidneys in C1q nephropathy, leading to renal damage via inflammatory and immune responses, which is consistent with data from a rodent model showing significantly increased C1QB levels in the kidneys of diabetic versus control animals (29). CD5L or apoptosis inhibitor of macrophage protein is implicated in immune and inflammatory responses. Plasma CD5L is normally present in high concentrations via interaction with IgM (30). In acute kidney injury in mice, CD5L dissociates from IgM and is excreted in the urine, with recovery from acute kidney injury possible after CD5L interacts with kidney injury molecule 1 (31). In the current study, lower circulating levels of CD5L were observed in patients with rapidly declining kidney function, suggesting either increased renal excretion or, alternatively, a defect in CD5L or IgM expression.

The one biomarker for which the present data appear inconsistent with the published literature is IBP3, a regulator of insulin-like growth factor 1 that has been implicated in the development of diabetic nephropathy. In the current study, lower circulating levels of IBP3 were observed in participants with an eGFR decline of $\geq$30% or $\geq$5 mL/min/1.73 m^2/year in apparent contrast to a previous study (32) showing an association between increased IBP3 level with low baseline eGFR in individuals with type 2 diabetes. However, in this latter study, the average eGFR reduction over 7 years was only 2 mL/min/1.73 m^2, there was no rapid renal decline subgroup, and there was association between IBP3 level and longitudinal eGFR trends. There were also differences in patient characteristics (our participants were older and had a higher baseline eGFR, and more were female and were receiving treatment with RAAS inhibitors at baseline) and study design (including the method of eGFR calculation) compared with the current study. It is possible that the lower levels of IBP3 observed in the current study are due to increased renal excretion via already damaged glomeruli or increased serum proteolysis (33).

A range of other potential biomarkers have been studied in the context of DKD (6). The current study aligns with recent studies describing the prognostic utility of tumor necrosis factor receptor (TNFR) 1 and TNFR2 (9,12,34,35). Our biomarker panel provided a similar discriminative ability (sensitivities of 83–94% and specificities of 72–80% for clinical plus biomarker model 2) to published data on TNFR1/TNFR2 for predicting renal decline (sensitivities of 68–72% and specificities of 81–86%) (36). In another of the TNFR1/TNFR2 studies (9), patients were classified by quartiles of biomarker concentration. Adopting this approach showed that most rapid decliners in the current study were in the highest apoA4/C1QB quartile or the lowest CD5L/IBP3 quartile (data not shown), but these results should be interpreted with caution because of small cell numbers. A recent study of 1,135 participants with type 2 diabetes with baseline renal function similar to those in the

current study (12) showed that plasma adrenomedullin, TNFR1, and N-terminal pro B-type natriuretic peptide were associated with rapid eGFR decline, independent of established risk factors, and that they significantly increased the receiver operating characteristic AUC (ΔAUC = 0.027–0.054, $P < 0.0001$). We found a similar significant increase in AUC after the addition of the biomarkers to the clinical prediction model for eGFR trajectory (Table 2) (ΔAUC = 0.07, $P = 0.039$). We did not detect TNFR1, TNFR2, N-terminal pro B-type natriuretic peptide, or adrenomedullin during the earlier discovery phase of our study since these proteins were below the limit of detection in the mass spectrometry methodology used (19). A number of other studies have described additional biomarkers that may improve prediction of renal function decline in diabetes beyond traditional risk factors, but most were small or involved only individuals with impaired renal function at study entry (11,34,35,37).

A key finding in the current study was that the biomarkers predicted rapid eGFR decline even after adjustment for conventional indices of nephropathy (eGFR and ACR) as well as other known clinical predictors (sex, hypertension, HbA_{1c}, smoking, lipids, BMI, and RAAS inhibitor use). The observation that microalbuminuria did not enter any of our prediction models supports other studies that found TNFR1/TNFR2 predicted ESRD irrespective of albuminuria (9), although there is evidence that microalbuminuria occurs after the decline in eGFR has already started in individuals with type 2 diabetes (38). Therefore, the identification of individuals with type 2 diabetes who are at risk for future renal decline should not be limited to the assessment of albuminuria.

The major strengths of the current study are its longitudinal community-based design and the detailed phenotypic characterization of the cohort. It is the largest study to have used a targeted MRM approach for biomarker detection and validation. The current platform allows multiple proteins to be analyzed without an increase in cost even if a formal cost-benefit analysis is yet to be conducted. Recent improvements in the field show that the MRM approach has increased sensitivity compared with traditional antibody-based assays (19). The limitations of the current study include a relatively small sample size in the case of participants with rapid renal decline. In addition, the findings require external validation. Internal validation was performed by bootstrap resampling of 1,000 replicates that provided an estimate of external model performance (22), which supports further assessment of PromarkerD in other cohorts. The prediction models presented in this study were developed in people of mostly white origin (~80%), and whether the findings can be generalized to other ethnicities and to subjects with prediabetes or type 1 diabetes is as yet unknown. Nevertheless, the prediction models were adjusted for a large range of known risk factors to address confounding with the same result.

In conclusion, the current study has identified four plasma protein biomarkers (apoA4, CD5L, C1QB, and IBP3) that predict a rapid decline in eGFR in patients with type 2 diabetes independent of other clinical predictors including eGFR and ACR. The panel may be useful for risk stratification in future clinical trials, would enable earlier intervention of at-risk individuals and monitoring of disease progression, and would allow improvement in patient outcomes. Further analysis of these biomarkers via the PromarkerD test in diabetes and more generally in CKD is warranted.

Acknowledgments. The authors thank FDS2 participants for their involvement and FDS2 staff for help with collecting and recording clinical information. The authors also thank the staff at Proteomics International, who performed all of the biomarker assays.

Funding. The mass spectrometry analyses were performed in facilities provided by the Lotterywest State Biomedical Facility–Proteomics node and Bioplatforms Australia at the Harry Perkins Institute for Medical Research. Partial funding for the proteomics analysis was provided by the Federal Government of Australia under a Commercialization Australia grant (CAU05922). FDS2 has been supported by the National Health and Medical Research Council of Australia project grants 513781 and 1042231. T.M.E.D. is supported by a National Health and Medical Research Council of Australia Practitioner Fellowship (1058260).

Duality of Interest. Proteomics International and the University of Western Australia are owners of patent PCT/AU2011/001212, which relates to the biomarkers described in this article (20), that K.E.P., W.A.D., K.W., T.S., S.D.B., R.J.L., and T.M.E.D. are named inventors of. No other potential conflicts of interest relevant to this article were reported.

Author Contributions. K.E.P. collected data, performed the statistical analyses, and wrote the manuscript. W.A.D. performed the statistical analyses. J.I. performed MRM method development and analysis on the FDS2 cohort. K.W. carried out iTRAQ and MRM experiments and sample processing. T.S. designed and performed iTRAQ and MRM experiments in the discovery phase. S.D.B. and R.J.L. were involved in experimental design, data analysis, and interpretation. T.M.E.D. is the principal investigator of the FDS2, provided the FDS2 cohort plasma samples, wrote the manuscript, and was responsible for clinical interpretation. All authors reviewed and edited the manuscript. K.E.P. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. Parts of this work were presented in abstract form at the 11th Australian Peptide Conference, Kingscliff, New South Wales, Australia, 25–30 October 2015; the World Diabetes Congress 2015, 30 November to 4 December 2015, Vancouver, British Columbia, Canada; the Australian Diabetes Society–Australian Diabetes Educators Association Annual Scientific Meeting 2016, Gold Coast, Queensland, Australia, 24–26 August 2016; International BioFest 2016, 24–27 October 2016, Melbourne, Victoria, Australia; the International Conference on Functional & Interaction Proteomics: Application in Food & Health, 14–17 December 2016, New Delhi, India; and the 77th Scientific Sessions of the American Diabetes Association, San Diego, CA, 9–13 June 2017.

References

1. United States Renal Data System. 2015 annual data report [article online], 2015. Available from https://www.usrds.org/2015/view/. Accessed March 2017
2. Centers for Disease Control and Prevention. National Chronic Kidney Disease Fact Sheet, 2017. Available from https://www.cdc.gov/diabetes/pubs/pdf/kidney_factsheet.pdf. Accessed March 2017
3. Naresh CN, Hayen A, Weening A, Craig JC, Chadban SJ. Day-to-day variability in spot urine albumin-creatinine ratio. Am J Kidney Dis 2013;62:1095–1101
4. Mottl AK, Kwon KS, Mauer M, Mayer-Davis EJ, Hogan SL, Kshirsagar AV. Normoalbuminuric diabetic kidney disease in the U.S. population. J Diabetes Complications 2013;27:123–127
5. Dunkler D, Gao P, Lee SF, et al.; ONTARGET and ORIGIN Investigators. Risk prediction for early CKD in type 2 diabetes. Clin J Am Soc Nephrol 2015;10:1371–1379
6. Lin CH, Chang YC, Chuang LM. Early detection of diabetic kidney disease: present limitations and future perspectives. World J Diabetes 2016;7:290–301
7. Bjornstad P, Cherney DZ, Maahs DM, Nadeau KJ. Diabetic kidney disease in adolescents with type 2 diabetes: new insights and potential therapies. Curr Diab Rep 2016;16:11
8. Looker HC, Colombo M, Hess S, et al.; SUMMIT Investigators. Biomarkers of rapid chronic kidney disease progression in type 2 diabetes. Kidney Int 2015;88:888–896
9. Niewczas MA, Gohda T, Skupien J, et al. Circulating TNF receptors 1 and 2 predict ESRD in type 2 diabetes. J Am Soc Nephrol 2012;23:507–515
10. Pena MJ, Lambers Heerspink HJ, Hellemons ME, et al. Urine and plasma metabolites predict the development of diabetic nephropathy in

individuals with type 2 diabetes mellitus. Diabet Med 2014;31:1138–1147
11. Agarwal R, Duffin KL, Laska DA, Voelker JR, Breyer MD, Mitchell PG. A prospective study of multiple protein biomarkers to predict progression in diabetic chronic kidney disease. Nephrol Dial Transplant 2014;29:2293–2302
12. Saulnier PJ, Gand E, Velho G, et al.; SURDIAGENE Study Group. Association of circulating biomarkers (adrenomedullin, TNFR1, and NT-proBNP) with renal function decline in patients with type 2 diabetes: a French prospective cohort. Diabetes Care 2017;40:367–374
13. Levey AS, Inker LA, Matsushita K, et al. GFR decline as an end point for clinical trials in CKD: a scientific workshop sponsored by the National Kidney Foundation and the US Food and Drug Administration. Am J Kidney Dis 2014;64:821–835
14. Kidney Disease: Improving Global Outcomes Chronic Kidney Disease Guideline Development Work Group Members. KDIGO 2012 clinical practice guideline for the evaluation and management of chronic kidney disease. Kidney Int Suppl 2013; 3:1–150
15. Davis TM, Chubb SA, Davis WA. The relationship between estimated glomerular filtration rate trajectory and all-cause mortality in type 2 diabetes: the Fremantle Diabetes Study. Eur J Endocrinol 2016;175:273–285
16. Ragot S, Saulnier PJ, Velho G, et al.; SURDIAGENE and DIABHYCAR Study groups. Dynamic changes in renal function are associated with major cardiovascular events in patients with type 2 diabetes. Diabetes Care 2016;39:1259–1266
17. Davis TM, Bruce DG, Davis WA. Cohort profile: the Fremantle Diabetes Study. Int J Epidemiol 2013;42:412–421
18. Levey AS, Stevens LA, Schmid CH, et al.; CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration). A new equation to estimate glomerular filtration rate. Ann Intern Med 2009;150: 604–612
19. Bringans SD, Ito J, Stoll T, et al. Comprehensive mass spectrometry based biomarker discovery and validation platform as applied to diabetic kidney disease. EuPA Open Proteom 2017;14:1–10
20. Stoll T, Bringans S, Winfield K, et al. Method of assessing diabetic nephropathy using CD5 antigen-like. U.S. patent 9146243 B2. 29 September 2015
21. DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. Biometrics 1988;44: 837–845
22. Moons KG, Kengne AP, Woodward M, et al. Risk prediction models: I. Development, internal validation, and assessing the incremental value of a new (bio)marker. Heart 2012;98:683–690
23. Pencina MJ, D'Agostino RB Sr, Steyerberg EW. Extensions of net reclassification improvement calculations to measure usefulness of new biomarkers. Stat Med 2011;30:11–21
24. Pickering JW, Endre ZH. New metrics for assessing diagnostic potential of candidate biomarkers. Clin J Am Soc Nephrol 2012;7:1355–1364
25. Kronenberg F, Kuen E, Ritz E, et al. Apolipoprotein A-IV serum concentrations are elevated in patients with mild and moderate renal failure. J Am Soc Nephrol 2002;13:461–469
26. Stangl S, Kollerits B, Lamina C, et al. Association between apolipoprotein A-IV concentrations and chronic kidney disease in two large population-based cohorts: results from the KORA studies. J Intern Med 2015;278:410–423
27. Guo Z, Liu X, Li M, et al. Differential urinary glycoproteome analysis of type 2 diabetic nephropathy using 2D-LC-MS/MS and iTRAQ quantification. J Transl Med 2015;13:371
28. Boes E, Fliser D, Ritz E, et al.; MMKD Study Group. Apolipoprotein A-IV predicts progression of chronic kidney disease: the mild to moderate kidney disease study. J Am Soc Nephrol 2006;17: 528–536
29. Kelly KJ, Liu Y, Zhang J, Dominguez JH. Renal C3 complement component: feed forward to diabetic kidney disease. Am J Nephrol 2015;41: 48–56
30. Sanjurjo L, Aran G, Roher N, Valledor AF, Sarrias MR. AIM/CD5L: a key protein in the control of immune homeostasis and inflammatory disease. J Leukoc Biol 2015;98:173–184
31. Arai S, Kitada K, Yamazaki T, et al. Apoptosis inhibitor of macrophage protein enhances intraluminal debris clearance and ameliorates acute kidney injury in mice. Nat Med 2016;22:183–193
32. Narayanan RP, Fu B, Heald AH, et al. IGFBP2 is a biomarker for predicting longitudinal deterioration in renal function in type 2 diabetes. Endocr Connect 2012;1:95–102
33. Frystyk J, Ivarsen P, Skjaerbaek C, Flyvbjerg A, Pedersen EB, Orskov H. Serum-free insulin-like growth factor I correlates with clearance in patients with chronic renal failure. Kidney Int 1999;56:2076–2084
34. Pena MJ, Heinzel A, Heinze G, et al. A panel of novel biomarkers representing different disease pathways improves prediction of renal function decline in type 2 diabetes. PLoS One 2015;10: e0120995
35. Pena MJ, Jankowski J, Heinze G, et al. Plasma proteomics classifiers improve risk prediction for renal disease in patients with hypertension or type 2 diabetes. J Hypertens 2015;33:2123–2132
36. Yamanouchi M, Skupien J, Niewczas MA, et al. Improved clinical trial enrollment criterion to identify patients with diabetes at risk of end-stage renal disease. Kidney Int 2017;92:258–266
37. Carlsson AC, Östgren CJ, Länne T, Larsson A, Nystrom FH, Ärnlöv J. The association between endostatin and kidney disease and mortality in patients with type 2 diabetes. Diabetes Metab 2016;42:351–357
38. Viazzi F, Piscitelli P, Giorda C, et al.; AMD-Annals Study Group. Association of kidney disease measures with risk of renal function worsening in patients with hypertension and type 2 diabetes. J Diabetes Complications 2017;31:419–426
39. Levey AS, de Jong PE, Coresh J, et al. The definition, classification, and prognosis of chronic kidney disease: a KDIGO Controversies Conference report. Kidney Int 2011;80:17–28