

Comparison of Circulating Biomarkers in Predicting Diabetic Kidney Disease Progression With Autoantibodies to Erythropoietin Receptor



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Introduction: Several circulating markers, including autoantibodies to erythropoietin receptor (anti-EPOR antibodies), have been identified as useful biomarkers in predicting diabetic kidney disease progression. However, a direct comparison of their utility is lacking. We aimed to validate and to compare the prognostic value of anti-EPOR antibodies with that of other known biomarkers, using the ADVANCE trial and its long-term follow-up, ADVANCE-ON, cohorts.

Methods: In this nested case-control study from the ADVANCE trial cohort, we included 165 case participants who had the composite kidney outcome (renal replacement therapy, renal death, or doubling of serum creatinine to ≥ 200 $\mu\text{mol/l}$) and 330 matched controls. We compared the associations of baseline plasma levels of anti-EPOR antibodies, tumor necrosis factor receptor (TNFR)-1 and -2, and bone morphogenetic protein (BMP)-7 with kidney outcomes.

Results: Cases had higher baseline plasma levels of anti-EPOR antibodies than controls (median 1.7 vs. 0.6 enzyme-linked immunosorbent assay unit, $P < 0.001$). Higher levels of anti-EPOR antibodies were associated with an increased risk of kidney outcome (odds ratio 2.16 [95% confidence interval 1.51, 3.08], per 1 SD of log-transformed levels) after adjusting for conventional markers. Elevated circulating TNFR1 and TNFR2 levels, and lower BMP-7 levels at baseline, were associated with poor kidney outcome (odds ratios 2.06 [1.29, 3.30], 1.66 [1.13, 2.43], and 0.45 [0.32, 0.65], respectively). The addition of anti-EPOR antibodies into the model improved the prediction of kidney outcome, regardless of other biomarkers.

Conclusion: Anti-EPOR antibodies provide a promising biomarker, as with TNFR1, TNFR2, and BMP-7, in predicting kidney disease progression in people with type 2 diabetes mellitus.

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KEYWORDS: biomarker; end-stage kidney disease; type 2 diabetes

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Diabetic kidney disease (DKD) occurs in approximately 40% of patients with type 2 diabetes mellitus and can lead to devastating consequences

including end-stage kidney disease (ESKD), cardiovascular disease, and premature death.^{1,2} Early detection of progressive kidney disease in type 2 diabetes mellitus is critical for risk stratification and early intervention to prevent such adverse outcomes. Traditionally, estimated glomerular filtration rate (eGFR) and urine albumin/creatinine ratio (UACR) are the most commonly used clinical and biochemical parameters in predicting risks, but their predictive values in earlier stages of type 2 diabetes mellitus is modest at best, and highly

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variable.³ Furthermore, up to 30% of individuals with type 2 diabetes mellitus progress to impaired kidney function without microalbuminuria.⁴ Hence, a better biomarker in predicting the risk of kidney disease progression, above and beyond traditionally used eGFR and UACR, is urgently needed to target high-risk patients with earlier therapies prior to the onset of albuminuria or kidney function decline.

Anemia frequently occurs in individuals with DKD.⁵ The etiology of anemia in DKD is likely multifactorial, with erythropoietin (EPO) deficiency and EPO-hyporesponsiveness,^{6,7} commonly seen in individuals with type 2 diabetes mellitus. We have previously reported that the presence of autoantibodies against the EPO receptor, better known as anti-EPOR antibodies, is responsible for EPO-hyporesponsiveness observed in individuals with chronic kidney disease (CKD).^{8,9} In a cohort of 112 Japanese patients with DKD, higher levels of anti-EPOR antibodies at baseline were also associated with a higher risk of ESKD after adjustment for clinical covariates including baseline eGFR, proteinuria, and hemoglobin.¹⁰ However, because of the small sample size and limited ethnic diversity, validation in a larger and more diverse cohort is required to confirm the utility of anti-EPOR antibodies in predicting progressive kidney disease in people with type 2 diabetes mellitus.

In recent years, there has been a growing interest in identifying useful biomarkers for predicting DKD progression, both for early risk stratification and for therapeutic intervention.^{11,12} Several studies from the large multiethnic cohorts have confirmed that circulating tumor necrosis factor receptor (TNFR)-1 and -2 can independently predict risk of ESKD and kidney function decline in the early and advanced stages of type 2 diabetes mellitus.^{13–15} We have previously reported the inverse relationship between circulating bone morphogenetic protein (BMP)-7 and major kidney events.¹⁶ However, all of these studies had their own limitations, with inherent biases from different study cohorts with various stages of DKD. There is also a lack of consensus in comparing the predictive values of these biomarkers in progressive DKD. In addition, the cost implication of examining these biomarkers prospectively in large randomized controlled trials limits their clinical and experimental utility. Therefore, we believe that it is important to compare the predictive values of these biomarkers together for both utility and cost consideration.

In this study, we report the associations between baseline plasma levels of anti-EPOR antibodies and hard kidney outcome in patients with type 2 diabetes mellitus, using data from the Action in Diabetes and Vascular disease: Preterax and Diamicron MR Controlled Evaluation (ADVANCE) and its post-trial follow-up

(ADVANCE Post-Trial Observational Study¹⁷), which has a follow-up period of up to 10 years post randomization. We also compared the predictive value of anti-EPOR antibodies with that of other known circulating biomarkers, including TNFR1, TNFR2, and BMP-7.

MATERIALS AND METHODS

Study Design and Population

Our study used data and stored blood samples from the ADVANCE and ADVANCE-ON studies. The ADVANCE trial ([ClinicalTrials.gov](https://clinicaltrials.gov) registration no. NCT00145925) was a 2×2 factorial randomized controlled trial evaluating the effects of blood pressure (BP) and intensive blood glucose lowering treatment on vascular outcomes in patients with type 2 diabetes mellitus. A detailed description of the design has been published previously.^{18–20} In brief, a total of 11,140 individuals with type 2 diabetes mellitus who were ≥55 years of age and at high risk for cardiovascular events were recruited from 215 centers in 20 countries between June 2001 and March 2003. The median durations of follow-up for the BP- and glucose-lowering interventions were 4.4 and 5.0 years, respectively. Serum creatinine levels were measured as part of the study protocol at baseline, 4 months, and 1 year, and annually thereafter until completion of the study, with further tests at the discretion of clinicians. The eGFR was then estimated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula. A full blood count including hemoglobin was not collected during the trial.

The ADVANCE-ON study ([ClinicalTrials.gov](https://clinicaltrials.gov) registration no. NCT00949286) was a post-trial follow-up study, comprising 8494 of the 10,082 surviving participants at the end of the randomized treatment phase.¹⁷ The median total follow-up period (i.e., including both ADVANCE and ADVANCE-ON) was 9.9 years until the final visits, which occurred between January 2013 and February 2014. Approvals for the original trial and the post-trial follow-up phase were obtained from the institutional review board. At enrollment, all participants provided written informed consent for the future use of their samples for analyses relevant to the primary and secondary outcomes of the study. The ethical approval for this biomarker study was obtained from Royal North Shore Hospital (Sydney, Australia; HREC 2019/PID11668).

Outcomes

The primary outcome of this study was the composite of renal replacement therapy (in the absence of other medical causes requiring transient dialysis), death due to kidney disease, and doubling of serum creatinine to

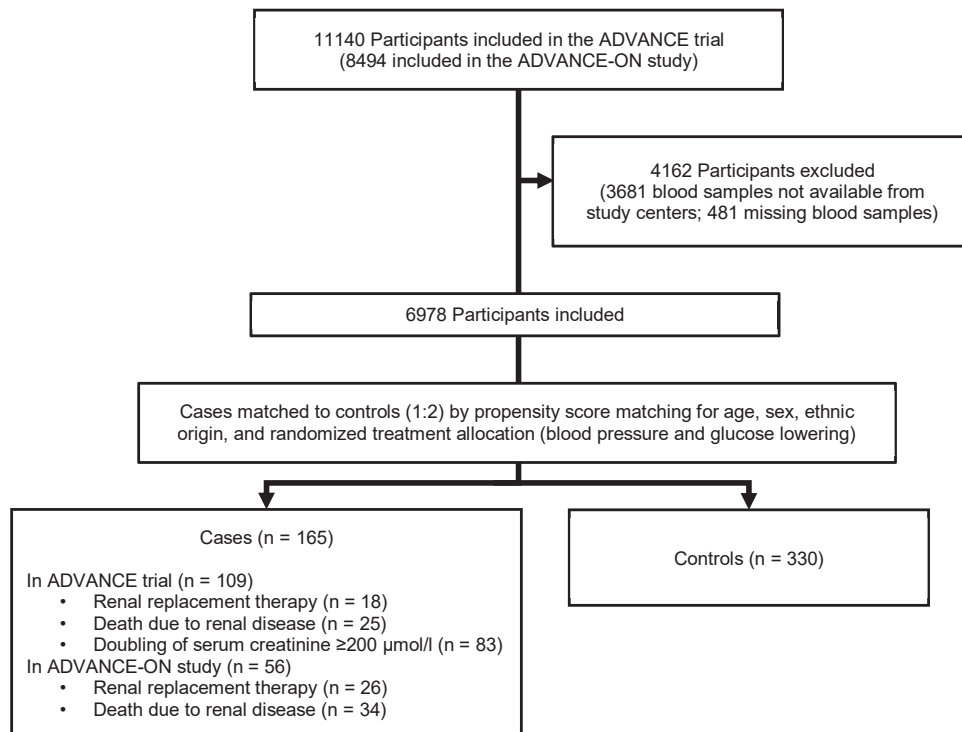


Figure 1. Flow chart of our nested case-control study. ADVANCE, Action in Diabetes and Vascular Disease: Preterax and Diamicon Modified Release Controlled Evaluation; ADVANCE-ON, ADVANCE Post-Trial Observational Study.

greater ≥ 200 $\mu\text{mol/l}$. Renal replacement therapy and death due to kidney disease recorded during the randomized treatment phase in ADVANCE were reviewed and validated by an independent endpoint adjudication committee. Outcomes occurring during post-trial follow-up in ADVANCE-ON included only renal replacement therapy and death due to kidney disease, and were reported by the study centers using the standardized definitions adopted during the trial, without central adjudication. As a secondary outcome, we evaluated eGFR slope, defined as an annual change in eGFR over time during the randomized treatment phase in ADVANCE.

Selection of Cases and Controls

To improve the efficacy of our biomarker study, we performed a nested case-control study to select cases and controls.²¹ Of 11,140 ADVANCE trial participants, 6978 participants (62.6%) had available blood samples at baseline (Figure 1). Among those with available blood samples, 165 participants had the kidney composite outcome and were all included as cases. We individually matched them to controls in a 1:2 fashion by using propensity score matching for key baseline characteristics (age, sex, ethnic origin [White or not White], and randomized treatment allocation [BP and glucose lowering]). The final sample size included 165 case participants and 330 controls.

Measurement of Circulating Biomarkers

This study measured baseline plasma levels of anti-EPOR antibodies, TNFR1, TNFR2, and BMP-7. We used heparin-anticoagulated plasma samples, which had been obtained at baseline from study participants and stored centrally at -80°C . Laboratory assay for biomarkers was done at Kolling Institute, Royal North Shore Hospital (Sydney, Australia) and was completed in November 2019.

Plasma levels of anti-EPOR antibodies were measured by enzyme-linked immunosorbent assay, as previously described.¹⁰ In brief, 96-well microplates (Corning Inc., Corning, NY) were coated with recombinant human EPOR protein (R&D Systems, Minneapolis, MN) at 5 $\mu\text{g/ml}$ diluted in 0.2 M sodium bicarbonate buffer at 4°C for 24 hours. The remaining free-binding sites were blocked with 1% bovine serum albumin in phosphate-buffered saline solution at 4°C . After the plates were washed with Tween 20-Tris-buffered saline, the plasma samples were added in duplicate at 1:1000 dilution to 1% bovine serum albumin in phosphate-buffered saline solution for 24 hours at 4°C . The plates were washed with the same buffer and incubated with goat anti-human Ig-conjugated with horseradish peroxidase (Sigma-Aldrich, Dorset, UK) at 1:5000 dilution for 1 hour at room temperature. The substrate tetramethylbenzidine (KPL, Gaithersburg, MD) was added, and the reaction was stopped by the addition of stop solutions (KPL, Gaithersburg, MD). The optical density (OD) at 620 nm

was determined by an automatic plate reader. The enzyme-linked immunosorbent assay unit (EU) of anti-EPOR antibodies was calculated from the 5-point linear approximation of control serum (set as 10 EU at 1:1000 dilution; linearity $r \geq 0.95$). Control serum was obtained from a patient with systemic lupus erythematosus in Japan, as previously used (Kanazawa, Japan; 2014-015).¹⁰

Plasma levels of TNFR1, TNFR2, and BMP-7 were measured using ultrasensitive enzyme-linked immunosorbent assays (Quantikine Human Immunoassay; R&D Systems, Minneapolis, MN) according to the manufacturers' protocols. For BMP-7, we used previously reported levels (269 participants, 81 case participants and 188 controls),¹⁶ in addition to newly identified specimens (226 participants, 84 case participants and 142 controls), giving a total of 495 participants. One case had missing values of TNFR1 and TNFR2 because of inadequate plasma for assays. All measurements were made in duplicate and random order. Values less than zero were considered to be zero. The average intra- and interassay coefficients of variation were <10% for all biomarkers.

Statistical Analysis

Continuous variables were reported as mean with SD for variables with approximately symmetric distributions. Results for variables with skewed distributions were presented as median and interquartile interval (IQI) and were transformed into natural logarithms before analysis. These distributions were assessed by histogram. Categorical variables were reported as numbers and percentages. Baseline characteristics were compared between cases and controls, by using *t* tests for data that were approximately symmetrically distributed, Wilcoxon rank sum tests for skewed continuous data, and χ^2 tests for categorical data. Correlations between baseline levels of biomarkers each other as well as each biomarker levels and eGFR or UACR were tested by using the Pearson correlation coefficient.

Conditional logistic regression models were used to estimate the odds ratios (ORs) and 95% confidence intervals (95% CIs) for the primary composite kidney outcome. The models were adjusted for baseline covariates including age, sex, ethnic origin (White or not White), duration of diabetes, history of macrovascular and microvascular disease, smoking habit, systolic BP, HbA1c, body mass index, eGFR, log-transformed UACR, and ADVANCE randomized BP and glucose lowering treatment allocation. Because the distributions of the measured biomarkers were rightward skewed, we applied the models to a natural log-transformed levels of biomarkers per 1-SD increase. The ORs for each biomarker were estimated with and

without the adjustment for other biomarkers. We also categorized levels of biomarkers based on tertile (defined as lowest third, middle, and highest third) and compared ORs across the categories, using the lowest third as reference. Linear trends across the categories were tested by including continuous values of categories into the models.

To determine the values of biomarkers based on the stages of CKD, we performed subgroup analyses by baseline eGFR (≥ 60 or < 60 ml/min per 1.73 m²) and albuminuria (< 30 or ≥ 30 mg/g) using logistic regression models to estimate the ORs and 95% CIs of log-transformed levels of the biomarkers per 1-SD increase.

The eGFR slope was calculated using linear mixed effects models with random slope and intercept, including the category of each biomarker, continuous time, and an interaction term of the category by continuous time, in addition to baseline covariates including age, sex, ethnic origin (White or not White), duration of diabetes, history of macrovascular and microvascular disease, smoking habit, systolic BP, HbA1c, body mass index, eGFR, log-transformed UACR, and ADVANCE randomized BP- and glucose-lowering treatment allocation. Linear trends across the categories were tested by including continuous values of categories into the models.

We also evaluated the predictive values of biomarkers for the primary composite kidney outcome, using the c-statistics, continuous net reclassification improvement, integrated discrimination improvement, Akaike information criterion, and Schwartz Bayesian information criterion. All analyses were conducted using Stata/MP, version 15 (StataCorp, Collage Station, TX). A 2-sided *P* value <0.05 was considered statistically significant.

RESULTS

Baseline Characteristics of Cases and Controls

Overall, the mean age of the participants was 68 (SD 7) years, and 73% were men. The mean eGFR was 64 (SD 18) ml/min per 1.73 m², and the median UACR was 20 (IQI 9–69) mg/g (Table 1). Baseline characteristics were well balanced between cases and controls with regard to age, sex, ethnic origin, duration of diabetes, history of macrovascular disease, smoking, systolic BP, HbA1c, body mass index, lipids, and randomized treatment (BP and glucose lowering) allocation. On the other hand, cases had lower levels of eGFR and higher levels of UACR and were likely to have a history of microvascular disease, compared with controls. No participant reported use of EPO-stimulating agents.

Table 1. Summary baseline characteristics of participants

Characteristic	Total (n = 495)	Cases (n = 165)	Controls (n = 330)	P value
Age, yr	68 (7)	68 (7)	68 (7)	0.95
Men, %	361 (73)	120 (73)	241 (73)	0.94
White, %	379 (77)	124 (75)	255 (77)	0.60
Duration of diabetes, y	9.4 (7.3)	9.9 (6.9)	9.2 (7.4)	0.32
History of macrovascular disease, %	181 (37)	66 (40)	115 (35)	0.26
History of microvascular disease, %	1106 (21)	45 (27)	61 (18)	0.03
Current smoking, %	49 (10)	18 (11)	31 (9)	0.60
Current alcohol drinking, %	215 (43)	66 (40)	149 (45)	0.28
Participation in moderate or vigorous activity, %	223 (45)	68 (41)	155 (47)	0.23
Systolic blood pressure, mm Hg	153 (22)	154 (23)	153 (22)	0.50
Diastolic blood pressure, mm Hg	82 (11)	82 (12)	82 (11)	0.46
HbA _{1c} , mmol/l	60 (17)	61 (16)	60 (17)	0.62
HbA _{1c} , %	7.6 (1.6)	7.7 (1.5)	7.6 (1.6)	0.62
eGFR, ml/min per 1.73 m ²	64 (18)	58 (20)	67 (16)	<0.001
UACR, µg/mg	20 (9–69)	53 (15–210)	15 (7–37)	<0.001
HDL cholesterol, mmol/l	1.2 (0.3)	1.2 (0.3)	1.2 (0.3)	0.36
LDL cholesterol, mmol/l	3.0 (1.1)	3.0 (1.2)	3.0 (1.1)	0.41
Triglycerides, mmol/l	1.6 (1.2–2.2)	1.6 (1.2–2.3)	1.6 (1.2–2.2)	0.37
Body mass index, kg/m ²	29 (5)	29 (6)	29 (5)	0.81
Randomized blood pressure treatment, %	274 (55)	91 (55)	183 (55)	0.95
Randomized intensive blood glucose control, %	234 (47)	81 (49)	153 (46)	0.57
Circulating biomarkers				
Anti-EPOR antibodies, EU	0.96 (0–2.36)	1.66 (0.78–3.66)	0.63 (0–1.74)	<0.001
TNFR1, pg/ml [†]	1553 (1217–2021)	1960 (1516–2552)	1429 (1150–1760)	<0.001
TNFR2, pg/ml [†]	3002 (2472–3738)	3615 (2878–4389)	2825 (2364–3459)	<0.001
BMP-7, pg/ml	12.4 (4.3–29.1)	7.4 (2.3–14.4)	15.4 (6.8–42.5)	<0.001

Values are presented as mean (SD) for approximately symmetrically distributed continuous values, as median (interquartile interval) for skewed continuous values, and as n (%) for categorical values. BMP, bone morphogenetic protein; eGFR, estimated glomerular filtration rate; EPOR, erythropoietin receptor; EU, enzyme-linked immunosorbent assay unit; HDL, high-density lipoprotein; IQR, interquartile interval; LDL, low-density lipoprotein; TNFR, tumor necrosis factor receptor; UACR, urinary albumin/creatinine ratio.

[†]One case had missing values of TNFR1 and TNFR2 because of inadequate plasma for assays.

Cases had significant higher baseline plasma levels of anti-EPOR antibodies, TNFR1, and TNFR2, and lower levels of BMP-7, compared with controls (all $P < 0.001$). There was a strong correlation between TNFR1 and TNFR2 ($r = 0.80$, $P < 0.001$), suggesting that these biomarkers are dependent on each other, which is not surprising considering the structural and mechanistic similarity of these 2 biomarkers; whereas weak or no correlations were observed across other biomarkers (all $|r| < 0.20$) (Supplementary Figure S1). Furthermore, we found no correlations of anti-EPOR antibodies and BMP-7 with baseline eGFR, whereas moderate negative correlations were observed between TNFR1 and TNFR2 with eGFR (Supplementary Figure S2). There was a weak linear relationship between each biomarker and baseline albuminuria (all $|r| < 0.40$) (Supplementary Figure S3).

Comparison of Circulating Biomarkers With Hard Kidney Outcomes

The mean follow-up period was 8.0 (SD 2.9) years. As shown in Figure 2, higher baseline levels of log-transformed anti-EPOR antibodies (per 1-SD increase) were associated with an increased risk of the composite kidney outcome (OR 2.16 [95% CI 1.51, 3.08]) after

adjusting for conventional risk factors. In addition, higher levels of TNFR1 and TNFR2, and lower levels of BMP-7 were associated with a higher risk of the composite kidney outcome (2.06 [1.29, 3.30], 1.66 [1.13, 2.43], and 0.45 [0.32, 0.65], respectively). The strong association between anti-EPOR antibodies and the composite kidney outcome remained unchanged following further adjustment for TNFR1, TNFR2, and BMP-7 (Table 2). The associations with the risk of the composite kidney outcome were positive linear with each tertile of anti-EPOR antibodies, TNFR1, and TNFR2, and the reverse was observed for BMP-7 (Figure 3).

Circulating Biomarkers in eGFR and UACR Subgroups

The relationship of anti-EPOR antibodies and BMP-7 with the composite kidney outcome remain unchanged across eGFR and albuminuria subgroups (Supplementary Figure S4). The associations of TNFR1 and TNFR2 with the composite kidney outcome were directionally concordant across these subgroups, although TNFR1 demonstrated a higher risk in participants with eGFR < 60 than in those with eGFR ≥ 60 ml/min/1.73 m², and TNFR2 demonstrated a higher risk in

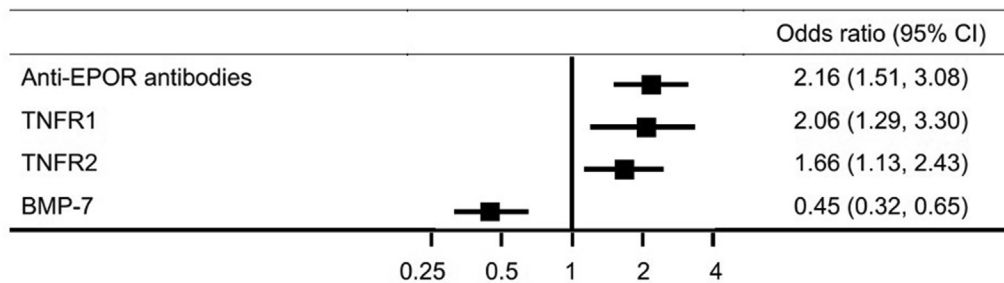


Figure 2. Adjusted odds ratios (95% confidence intervals [CIs]) for the composite kidney outcome associated with log-transformed circulating biomarkers. Odds ratios were estimated per 1-SD increase of log-transformed circulating biomarkers. Increase in 1 SD is 0.65 log(EU) for anti-EPOR antibodies, 0.39 log(pg/ml) for TNFR1, 0.33 log(pg/ml) for TNFR2, and 1.42 log(pg/ml) for BMP-7. Adjusted for age, sex, race, duration of diabetes, history of macrovascular and microvascular disease, smoking habit, systolic blood pressure, HbA1c, estimated glomerular filtration rate, log-transformed urine albumin/creatinine ratio, body mass index, and randomized treatment (blood pressure and glucose lowering) allocation. BMP, bone morphogenetic protein; EPOR, erythropoietin receptor; TNFR, tumor necrosis factor receptor.

participants with UACR ≥ 30 than in those with UACR < 30 mg/g.

Associations of Circulating Biomarkers With eGFR Slope

Participants with the highest third levels of anti-EPOR antibodies had a greater decline in eGFR than those with lowest third; the mean annual change in eGFR was -3.23 (standard error [SE] 0.32) vs. -1.68 (0.31) ml/min per 1.73 m²/year; difference was -1.55 (95% CI -2.43 , -0.67) (Figure 4). Participants with the highest third levels of TNFR1 and TNFR2 also demonstrated a greater eGFR decline than those with the lowest third (difference -1.94 [95% CI -2.84 , -1.05] and -2.26 [-3.15 , -1.37], respectively), whereas participants with the highest third levels of BMP-7 had a slower eGFR decline than those with the lowest third (1.09 [0.20, 1.98]).

Prediction of Circulating Biomarkers for Hard Kidney Outcomes

Using eGFR and UACR combined, the c-statistic value was 0.673 (95% CI 0.618, 0.728). With additional conventional clinical and biochemical factors, the c-statistic value increased to 0.711 (0.659, 0.762). The addition of anti-EPOR antibodies to the base model consisting of conventional risk factors increased the c-statistic value to 0.746 (0.698, 0.795) ($P < 0.001$) and net reclassification

improvement ($+0.596$ [95% CI 0.400, 0.792], $P < 0.001$) (Table 3). The addition of TNFR1, TNFR2, and BMP-7 individually also improved the c-statistics (to 0.733 [0.684, 0.783], 0.722 [0.672, 0.773], and 0.742 [0.694, 0.790], respectively; all < 0.001) and net reclassification improvement ($+0.504$ [0.100], $+0.411$ [0.100], $+0.628$ [0.100], respectively; all $P < 0.001$). Similar findings were observed for integrated discrimination improvement, Akaike information criterion, and Schwartz Bayesian information criterion. The addition of anti-EPOR antibodies to the base model resulted in a similar c-statistic compared with TNFR1 and BMP-7 ($P = 0.14$ and 0.63 , respectively), whereas this c-statistic was higher than that after adding TNFR2 to the base model ($P = 0.003$). Further improvements in the c-statistic values were observed when anti-EPOR antibodies were added to each biomarker (all $P < 0.001$) (Supplementary Table S1). When anti-EPOR antibodies, TNFR1, and BMP-7 were included in the base model, the model indicated the greatest c-statistics (to 0.810 [0.769, 0.851], $P < 0.001$).

DISCUSSION

In this study, we found that elevated plasma levels of anti-EPOR antibodies, TNFR1, and TNFR2, along with low plasma levels of BMP-7 at baseline, were associated

Table 2. Adjusted odds ratios (95% confidence intervals) for the composite kidney outcome after adjustment for other circulating biomarkers

Model	Odds ratio (95% CI)	Odds ratio (95% CI)	Odds ratio (95% CI)
Anti-EPOR antibodies	2.50 (1.63, 3.84) ^a	2.50 (1.63, 3.84) ^a	2.49 (1.63, 3.79) ^a
TNFR1	1.98 (0.82, 4.78)	2.20 (1.22, 3.98)	–
TNFR2	1.13 (0.54, 2.36)	–	1.76 (1.08, 2.85)
BMP-7	0.37 (0.24, 0.57) ^a	0.37 (0.24, 0.57) ^a	0.37 (0.24, 0.57) ^a

Odds ratios were estimated per 1-SD increase in log-transformed circulating biomarkers. Increase in 1 SD is 0.65 log(EU) for anti-EPOR antibodies, 0.39 log(pg/ml) for TNFR1, 0.33 log(pg/ml) for TNFR2, and 1.42 log(pg/ml) for BMP-7.

Adjusted for log-transformed circulating biomarkers as well as age, sex, race, duration of diabetes, history of macrovascular and microvascular disease, systolic blood pressure, HbA1c, estimated glomerular filtration rate, log-transformed urinary albumin/creatinine ratio, body mass index, and randomized treatment (blood pressure and glucose lowering) allocation. EPOR, erythropoietin receptor; TNFR, tumor necrosis factor receptor; BMP, bone morphogenetic protein.

^a $P < 0.001$.

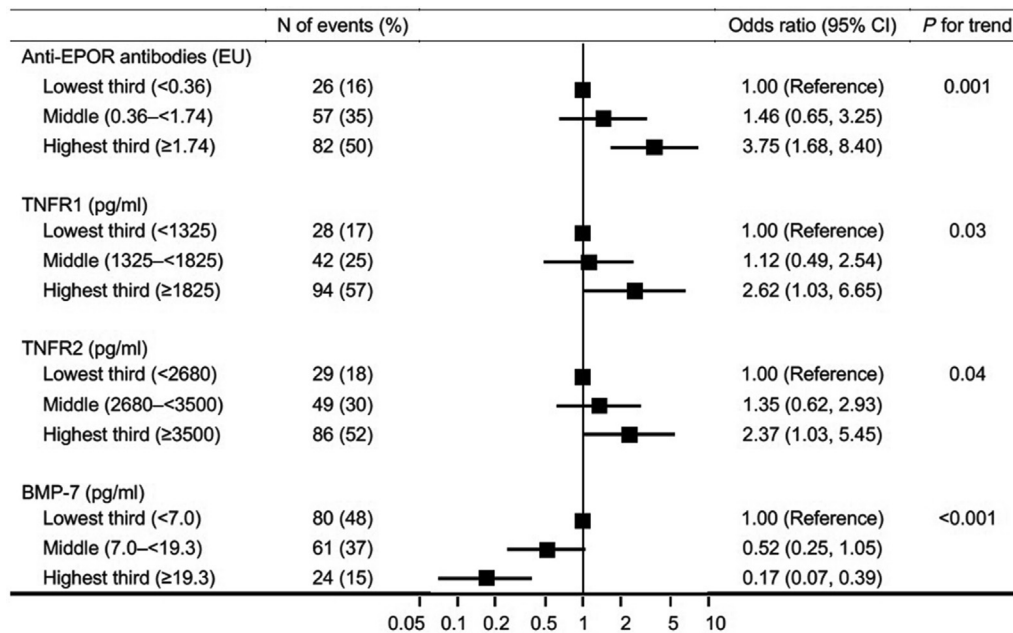


Figure 3. Adjusted odds ratios (95% confidence intervals) for the composite kidney outcome associated with thirds of circulating biomarkers. Adjusted for age, sex, race, duration of diabetes, history of macrovascular and microvascular disease, smoking habit, systolic blood pressure, HbA1c, estimated glomerular filtration rate, log-transformed urine albumin/creatinine ratio, body mass index, and randomized treatment allocation (blood pressure and glucose lowering). BMP, bone morphogenetic protein; EPOR, erythropoietin receptor; EU, enzyme-linked immunosorbent assay unit; TNFR, tumor necrosis factor receptor.

with an increased risk of the composite kidney outcome in participants with type 2 diabetes mellitus. The associations of these biomarkers remained strong and independent even after adjustment for conventional risk factors including demographics, comorbidities, eGFR, and albuminuria, as well as other biomarkers except for TNFR1 and TNFR2, which showed a strong correlation. In addition, the levels of these biomarkers at baseline strongly correlated with eGFR slope, which has been increasingly recognised as a useful surrogate endpoint for CKD.²² Finally, various prediction statistics confirmed that the addition of any of these biomarkers independently improved the risk prediction for poor kidney outcome, with anti-EPOR antibodies giving higher modest c-statistic value either individually or in combination with other biomarkers. These results suggest that anti-EPOR antibodies are comparable, if not adding further value, to other currently available circulating biomarkers in predicting the progression of kidney disease in people with type 2 diabetes mellitus.

We have previously reported, using a homogenous Japanese cohort with DKD (mean eGFR was 42 ml/min per 1.73 m² and mean urinary protein was 2.7 g/d), that elevated anti-EPOR antibodies levels carried the highest risk of ESKD compared to that in individuals with undetectable or low anti-EPOR antibodies levels (hazard ratio 2.78 [95% CI 1.20, 6.43]).¹⁰ We validated these findings in the current study using a more diverse

cohort of the ADVANCE trial cohort that consists of individuals with earlier stages of DKD (mean eGFR was 74 ml/min per 1.73 m² and median UACR was 15 mg/g). Our findings further suggest that anti-EPOR antibodies can be a useful circulating biomarker for predicting the risk of kidney disease progression even in earlier stages of DKD.

Unlike TNFR1, TNFR2, and BMP-7, the mechanistic link of anti-EPOR antibodies with kidney disease progression remains unclear. Anti-EPOR antibodies are known to inhibit EPO activity by blocking the EPO-EPOR binding pathway, which results in EPO-hyporesponsiveness leading to anemia.^{8,9} *In vitro* experiments showed that addition of anti-EPOR antibodies, which bind to EPOR on the surface of human erythroid progenitor cells, prevented EPO-dependent proliferation of erythroid progenitor cells.⁸ EPORs are also expressed in other tissues including pro-inflammatory cells,²³ tubular epithelial cells,²⁴ and endothelial cells,²⁵ and are highly expressed in diabetic animals,²⁶ suggestive of a yet-undefined role of EPOR in DKD. This may explain the clinical observation that individuals with diabetes mellitus are more likely to be anemic than individuals without.^{5,6} We also found that anti-EPOR antibodies upregulated the expression of monocyte chemoattractant protein-1 (MCP-1) mRNA on human tubular epithelial cells under high glucose conditions,¹⁰ suggesting a mechanistic link of anti-EPOR antibodies with the inflammatory cascade.

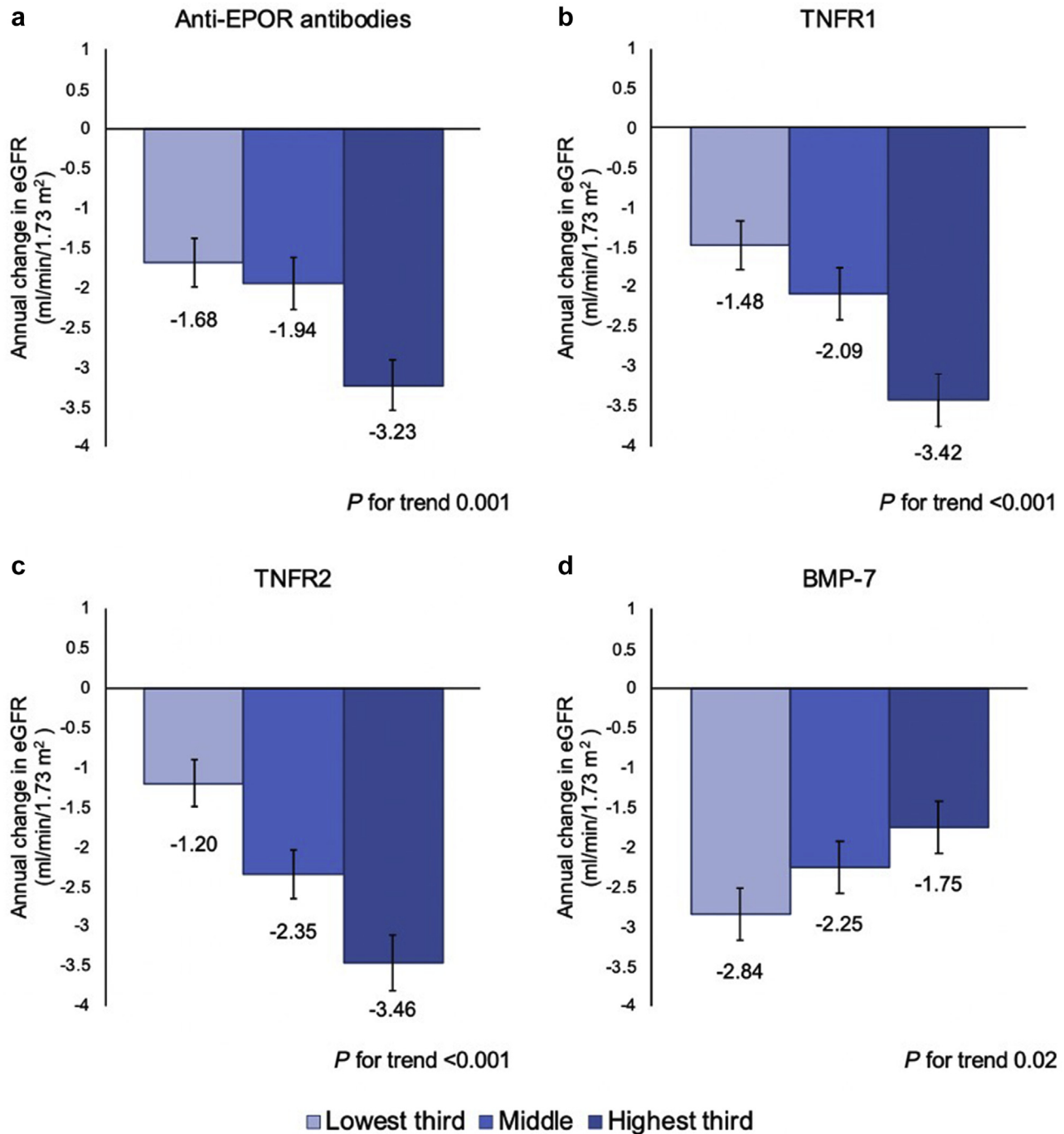


Figure 4. Annual change in estimated glomerular filtration rate (eGFR) by thirds of circulating biomarkers. Adjusted for age, sex, race, duration of diabetes, history of macrovascular and microvascular disease, smoking habit, systolic blood pressure, HbA1c, eGFR, log-transformed urine albumin/creatinine ratio, body mass index, and randomized treatment (blood pressure and glucose lowering) allocation. BMP, bone morphogenetic protein; CI, confidence interval; EPOR, erythropoietin receptor; EU, enzyme-linked immunosorbent assay unit; SE, standard error; TNFR, tumor necrosis factor receptor.

Furthermore, both *in vivo* and *in vitro* studies have reported that EPO had renoprotective effects through the suppression of inflammation,²³ oxidative stress,^{24,25} apoptosis,²⁷ and podocyte injury,²⁸ which were mainly mediated by the EPO-EPOR interaction.²⁹ Further studies are required to investigate the pathomechanistic link of anti-EPOR antibodies with progressive kidney disease.

Our results are consistent with previous reports of TNFR1, TNFR2, and BMP-7 as circulating biomarkers in predicting kidney outcomes in DKD.^{13–16, 30} The TNFRs, which are present in both membrane-bound forms and soluble forms in blood, have been reported to be involved in kidney inflammation, through the tumor necrosis factor- α pathway.^{31,32} In our subgroup analysis, baseline levels of TNFR2 were not significantly

Table 3. Discrimination statistics for models including circulating biomarkers

Model	C-statistic (95% CI)	NRI (95% CI)	IDI (95% CI)	AIC	BIC
eGFR and UACR	0.673 (0.618, 0.728)			209.0	217.1
Base model	0.711 (0.659, 0.762)			200.7	261.7
Additional markers into base model					
+Anti-EPOR antibodies	0.746 (0.698, 0.795) ^a	+0.596 (0.400, 0.792) ^a	+0.105 (0.074, 0.136) ^a	180.7	245.8
+TNFR1	0.733 (0.684, 0.783) ^a	+0.504 (0.308, 0.698) ^a	+0.073 (0.025, 0.076) ^a	191.5	256.5
+TNFR2	0.722 (0.672, 0.773) ^a	+0.411 (0.215, 0.607) ^a	+0.041 (0.022, 0.060) ^a	194.4	259.4
+BMP-7	0.742 (0.694, 0.790) ^a	+0.628 (0.432, 0.824) ^a	+0.074 (0.049, 0.100) ^a	179.1	244.2

Base model included age, sex, duration of diabetes, history of macrovascular and microvascular disease, smoking habit, systolic blood pressure, HbA1c, eGFR, log-transformed UACR, body mass index, and randomized treatment allocation (blood pressure and glucose lowering). AIC, Akaike information criterion; BIC, Schwarz Bayesian information criterion; BMP, bone morphogenetic protein; eGFR, estimated glomerular filtration rate; EPOR, erythropoietin receptor; IDI, integrated discrimination improvement; NRI, net reclassification improvement; TNFR, tumor necrosis factor receptor; UACR, urinary albumin/creatinine ratio.

^aValues indicate significant improvement ($P < 0.001$) compared with base model.

associated with the risk of adverse kidney outcome in the participants without albuminuria (OR 1.41 [95% CI 0.97, 2.03]). However, the relatively small sample size may not have adequate statistical power to detect a difference. On the other hand, BMP-7, a member of the transforming growth factor (TGF)- β superfamily, has been reported to have anti-fibrotic effects by the inhibition of TGF- β signaling in DKD.^{33,34} We previously reported the positive and negative associations of TGF- β 1 and BMP-7 with kidney outcome, using data of 282 participants from the ADVANCE trial cohort.¹⁶ In this study, the prognostic value of BMP-7 remained robust after adding newly identified specimens in the ADVANCE-ON cohort. We tried but were unable to validate TGF- β 1 in this study due to discontinuation of the previously used enzyme-linked immunosorbent assay kit.¹⁶

Clinical trials in type 2 diabetes mellitus are traditionally hindered by the requirement of large sample sizes or long follow-up periods to ensure an adequate number of established kidney endpoints (doubling of serum creatinine and ESKD). To date, most renal outcome trials use eGFR and albuminuria for risk stratification, but not without their limitations.³⁵ Various strategies including enrichment designs have been recommended to ensure the feasibility and success of renal outcome trials.³⁶ However, enrichment using validated biomarkers is not commonly used because of the paucity of evidence as well as cost implications. Our findings suggest that all circulating biomarkers in this study add value in predicting risk of kidney outcome above and beyond the conventional markers (eGFR, albuminuria, and clinical parameters), and the addition of any of these biomarkers to inclusion criteria may enhance selection of patients who will most likely benefit from an intervention. Such a targeted approach is likely to reduce the sample size or to shorten the follow-up period, which will have significant cost implication. To demonstrate the utility of these biomarkers as enrichment tools and the potential impact on sample size estimation in a clinical trial, we

used a simulation model (available at <http://prognosticenrichment.com/>).³⁷ Assuming an event rate similar to that in the long-term follow up of the ADVANCE-ON study,³⁸ 1.3% of individuals with type 2 diabetes mellitus receiving standard of care (control group) will develop ESKD. The estimated sample size required is >11,000. If anti-EPOR antibodies (c-statistic of 0.746) are used, and assuming that 3.0% of the “high risk” (anti-EPOR antibodies–positive) individuals are predicted to develop ESKD and a similar efficacy of the intervention (intensive glucose lowering), the sample size required is effectively halved to 5872 participants (Supplementary Table S2). Although cost-effectiveness analysis is beyond the scope of this paper, the significant reduction in sample size is likely to translate to a much lower trial running cost.

The strengths of this study include the use of a well-characterized clinical trial with a large and diverse population and a long duration of follow-up. This study also includes the comparison of prediction abilities of circulating biomarkers after adjustment for multiple covariates, including eGFR and albuminuria. However, the present study has several limitations. As it was a nested case-control study design in which controls were not randomly selected from the entire cohort, the estimated association and discrimination statistics are likely to be susceptible to bias, which may limit generalizability to the general population. Because circulating biomarkers were measured in pragmatically collected blood samples in a randomized trial, we cannot rule out the potential for differential pre-analytical sample handling or sample degradation during storage, which may have biased our result.³⁹ In addition, we did not have measurements of hemoglobin and could not adjust for anemia. However, we previously reported that the association between anti-EPOR antibodies and kidney outcome remained significant even after adjusting for hemoglobin.¹⁰ Finally, at the current stage, the cost for measuring circulating biomarkers may limit clinical use, especially in low-income economies.

In conclusion, anti-EPOR antibodies, TNFR1, TNFR2, and BMP-7 are useful biomarkers for predicting progressive kidney disease in people with type 2 diabetes mellitus, above and beyond eGFR and albuminuria. Use of these circulating biomarkers enables us to identify patients at the highest risk for progressive DKD, who would most likely benefit from early targeted intervention with currently recommended standard of care. Validating the utility of these biomarkers is likely to have significant implications for the feasibility and cost of future clinical trials.

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AUTHOR CONTRIBUTIONS

MO, AH, TT, MW, JC, VP, MGW, and TW designed the study. MO performed the assays and statistical analyses, with advice from AH, TT, M Jun, M Jardine, MW, JC, VP, MGW, and TW. All authors interpreted the data. MO, AH, and MGW drafted the article; all authors revised it. All authors approved the final version of the manuscript. All authors agreed with the work.

SUPPLEMENTARY MATERIAL

[Supplementary File \(PDF\)](#)

Figure S1. Scatter plots of circulating biomarkers.

Figure S2. Scatter plots of circulating biomarkers and baseline eGFR.

Figure S3. Scatter plots of circulating biomarkers and baseline albuminuria.

Figure S4. Adjusted odds ratios (95% confidence intervals) for the composite kidney outcome associated with log-transformed circulating biomarkers by baseline eGFR and albuminuria.

Table S1. Discrimination statistics for models including multiple circulating biomarkers.

Table S2. Required sample size using the Biomarker Prognostic Enrichment Tool (BioPET).

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