Item S1. Detailed description of Methods

Study design

In this cross-sectional study, participants were randomly selected from 10 communities in Karachi. Since few people were expected to have decreased kidney function among the general population, we enriched our sample with 40 participants with stage 3 chronic kidney disease (CKD) or worse (GFR ≤ 60 mL/min/1.73m²). The detailed description of recruitment and stratification have been published previously.¹ In brief, 581 participants (\geq 40 years) were enrolled in the current study, and 557 participants (men: 49.7%) with measurements of β 2-Microglobulin (B2M) and β-trace protein (BTP) were included in the current analysis. The flowchart of the study design is shown in **Figure S1**. The study approval for recruitment and measurement was obtained from the Ethics Review Committee, Aga Khan University, and consent was provided by all participants. The approval for analysis presented in this paper was obtained from the Institutional Review Board, National University of Singapore. *Assessment of levels of serum B2M, BTP, and other biomarkers*

In the morning, all participants visited the research laboratory following an overnight fast with the completion of 24-hour urine collections. The collection of blood samples was used for measuring serum β2-Microglobulin (B2M) (Roche COBAS), β-trace protein (BTP) (Simens ProSpec), cystatin C (Siemens Pro-spec instrument particle-enhanced immuno-nephelometric assay), serum creatinine (Beckman Coulter Inc), serum albumin (Beckman), and LDL cholesterol (Roche Diagnostics). The detailed measurement methods for serum cystatin C, serum creatinine, serum albumin and LDL cholesterol were described in detail previously.^{1, 2} The 24hour urine collection was used for measuring urine creatinine, urine albumin (nephelometry method) and urine urea nitrogen (enzymatic method). Urine albumin-to-creatinine ratio (ACR) was calculated by dividing urine albumin concentration in milligrams by urine creatinine concentration in grams. There was no missing data for any variable (age, sex, and serum creatinine levels) included in the CKD-EPI eGFR equations.

The measurement of serum B2M and BTP was conducted at the Advanced Research and Diagnostic Laboratory at the University of Minnesota. Stability of the assays over time was evaluated using pooled quality-control material and calibration panels.³ Serum cystatin C was traceable to the certified reference material ERM-DA471/IFCC from the Institute for Reference Materials and Measurements.⁴⁻⁶ Serum creatinine assays from Pakistan were calibrated using the

Roche enzymatic method (Hoffman-La Roche Ltd). Serum creatinine was traceable to the National Institute of Standards and Technology creatinine standard reference material 967 at Cleveland Clinic.^{7, 8} A calibration factor ($[-0.1256] + 0.9557x$) was applied to calibrate the serum creatinine assays from Pakistan.⁸

Measurement of GFR

The measurement of GFR has been described in detail previously.^{1, 8} In brief, urinary inulin clearance was used as the reference standard. Plasma and urinary inulin levels were assayed at the Renal Laboratories at the Saint-Etienne Hospital, France. GFR was calculated as the average of two or more measurements of urinary inulin clearance and multiplied by 1.73/BSA (body surface area; BSA = height^{0.725}[cm] \times weight^{0.425}[kg] \times 0.007184).⁹ The median coefficient of variation (CV) for participants with two and three urine collections during the inulin clearance measurement was 6.64% (95% confidence interval [CI]: 5.78% to 7.50%) and 7.06% (5.83% to 8.39%), respectively, which was consistent with a previous study reporting approximately 7% for repeated measures of inulin clearance.¹⁰ The CV of inulin clearance was relatively low compared to the smallest reported CVs of other mGFR methods (approximately 5%-15%).¹¹ The mGFR indexed for BSA served as the gold standard for comparison.⁸ *Assessment of demographic and clinical factors*

Trained research staff visited homes of consented participants and conducted face-to-face interviews using a standardized questionnaire.¹ Information on demographic and lifestyle factors were collected and included age, sex, and smoking status (yes, no). History of heart disease was defined as self-reported physician-diagnosed heart disease (yes, no). A physical examination was performed, and anthropometry measurements (weight, height, and waist circumference) were taken. A bioimpedance device (QuadScan 4000, Bodyscan Ltd) was used to estimate total body fat and lean body mass.^{12, 13} Body mass index (BMI) was computed using weight (kg) divided by height (m) squared. Dietary protein intake (g/day) was calculated using urine urea nitrogen and weight (dietary protein intake = [urea nitrogen (g/day) + weight (kg) \times 0.031] \times 6.25)¹⁴. *Statistical analysis*

Analyses were conducted using STATA 14.0 (Stata Corp, Texas) and SAS 9.4 (SAS Institute, Inc., North Carolina), where the statistical significance was determined by a two-sided *P* value smaller than 0.05. Performance of eGFR equations and non-GFR determinants of serum BTP, B2M, cystatin C and creatinine were assessed for the entire dataset of 557 participants.

eGFR equations and metrics for equation performance. The current study calculated and compared eight CKD-EPI equations and two new eGFR equations: (1) 2009 creatinine equation (eGFRcr),¹⁵ (2) 2014 creatinine equation calibrated for Pakistan (eGFRcr-PK),⁸ (3) 2012 cystatin C equation (eGFRcys), 15 (4) 2012 creatinine-cystatin C equation (eGFRcr-cys), 15 (5) 2021 creatinine equation without the race term,¹⁶ (6) 2021 creatinine-cystatin C equation without the race term, ¹⁶ (7) 2020 Cystatin C-B2M-BTP equation (3-marker panel equation), ¹⁷ and (8) 2020 Creatinine-Cystatin C-B2M-BTP equation (4-marker panel equation). ¹⁷ The eGFRcr-PK was calibrated previously using this population $(n=581)$.⁸ Participants with missing values of serum B2M (*n*=23) and serum BTP (*n*=24) were excluded, leaving 557 participants for the current analysis. The metrics for comparing performance of estimating equations were bias, precision, accuracy, and root mean square logarithmic error (RMSLE) with corresponding 95% CI. Bias was expressed as the median difference in mGFR minus eGFR, with positive values suggesting an underestimation of mGFR.⁸ Precision was assessed using the interquartile range (IQR) of the differences.⁸ Accuracy (P_{30}) was defined as the percentage of participants with eGFR within 30% of mGFR.⁸ RMSLE was defined as the square root of the average squared difference of eGFR and mGFR on the logarithmic scale (base e).⁸ This is similar to the root mean squared error (RMSE) except that we took the difference between the logarithms instead of their actual values. Compared to RMSE, RMSLE is less affected by the outliers in the testing data.¹⁸ The 95% CIs for all these metrics were computed using the bootstrap method¹⁹ with 10,000 replications. Bias was expected to be near zero for eGFRcr-PK since the equation was developed in the study population. However, bias among the other seven CKD-EPI equations (2009 eGFRcr, 2012 eGFRcys, 2012 eGFRcr-cys, 2021 eGFRcr, 2021 eGFRcr-cys, the 3-marker panel equation [2020 Cystatin C-B2M-BTP equation], the 4-marker panel equation [2020 Creatinine-Cystatin C-B2M-BTP equation]) was compared. Improvement in bias, IQR and RMSLE was indicated by a smaller value, and improvement in P₃₀ was indicated by a larger value. The differences among equations were assessed using the Wilcoxon signed-rank test for bias, the McNemar test for P_{30} , and the bootstrap method for IQR and RMSLE with 10,000 replications.

Non-GFR determinants. mGFR, and serum levels of B2M, BTP, cystatin C and creatinine were log-transformed. Linear regression models were applied to evaluate association between each potential predictor after standardization using respective IQRs (age, sex, weight, height, BMI, waist circumference, total body fat, lean body mass, history of heart disease, serum albumin,

LDL cholesterol, urine creatinine, dietary protein intake) and log-transformed (base e) levels of serum B2M, BTP, cystatin C and creatinine to improve the normal distribution. Several models were established: Model 1 adjusted for mGFR to examine the residual association of each predictor (**Table S1**); Model 2 included mGFR, age, and sex as adjustments (**Table S1**); Model 3 included all non-GFR determinants (**Table 1 and Table S2**). For all three models, measurement error of mGFR was included in the adjustment. Measurement error of mGFR was account for by using error-in-variables regression models to assess associations between non-GFR determinants and log-transformed levels of B2M and BTP assuming log-transformed mGFR (base e) was measured with 98.5% reliability (**Table 1**).

Regression coefficients relating serum B2M, BTP, cystatin C or creatinine levels to all potential predictors from linear regression models were transformed as $100 \times (e^{coefficient} - 1)$ so that they could be interpreted as the average percent difference in serum B2M, BTP, cystatin C or creatinine levels for an IQR difference in continuous predictors or a difference between categories for dichotomous predictors.^{20, 21} The strength of association for results with statistical significance (95% CI excludes zero) was defined as strong, intermediate or weak if the absolute percent difference in serum B2M, BTP, cystatin C or creatinine levels was >10%, 5%-10% and $\langle 5\%,$ respectively.²⁰

*****Participants were excluded per protocol due symptoms suggestive of medical illness, such as shortness of breath, tachycardia (heart rate \geq 120 beats/min) at rest, joint pains, jaundice, and fever.

******Participants were excluded due to febrile illness, detection of concurrence of hypertension and diabetes, or symptoms suggestive of minor allergic reaction following inulin infusion (*n* =5). **Abbreviation:** B2M, β2-Microglobulin; BTP, β-trace protein; GFR, glomerular filtration rate.

^aBias was expressed as the median difference in measured GFR minus estimated GFR (95% bootstrapped confidence interval). Negative bias indicates eGFR overestimation of measured GFR, and positive bias indicates eGFR underestimation of measured GFR. NA, not applicable because bias was expected to be zero (the equation was developed in the study population). A larger absolute value indicates greater bias.

bPrecision was expressed as the interquartile range (IQR) of differences in measured GFR minus estimated GFR (95% bootstrapped confidence interval). A larger absolute value indicates poorer precision.

 $c_{P_{30}}$ was defined as the percentage of individuals with estimated GFR within 30% of measured GFR (95% bootstrapped confidence interval). The 95% CI on P_{30} was calculated using the Clopper-Pearson (exact) method. A smaller P_{30} indicates poorer accuracy. ^dRMSLE was defined as the square root of the mean squared difference of measured GFR and estimated GFR on the logarithmic scale. A larger RMSLE indicates poorer accuracy.

^eThe sample size for eGFR subgroups were: 2009 CKD-EPI eGFRcr <60 ml/min/1.73 m² ($n=58$), 2014 CKD-EPI eGFRcr-PK <60 ml/min/1.73 m²(*n*=73), 2021 CKD-EPI eGFRcr <60 ml/min/1.73 m²(*n*=55), 2012 CKD-EPI eGFRcys <60 ml/min/1.73 m²(*n*=127), 2012 CKD-EPI eGFRcr-cys <60 ml/min/1.73 m²(*n*=83), 2021 CKD-EPI eGFRcr-cys <60 ml/min/1.73 m²(*n*=77), 2020 Cystatin C-B2M-BTP equation (3-marker panel) <60 ml/min/1.73 m² (n=121), 2020 Creatinine-Cystatin C-B2M-BTP equation (4-marker panel) <60 ml/min/1.73 m² (n=85), 2009 CKD-EPI eGFRcr 60-90 ml/min/1.73 m² (n=84), 2014 CKD-EPI eGFRcr-PK 60-90 ml/min/1.73 m² (*n*=145), 2021 CKD-EPI eGFRcr 60-90 ml/min/1.73 m²(*n*=67), 2012 CKD-EPI eGFRcys 60-90 ml/min/1.73 m²(*n*=255), 2012 CKD-EPI eGFRcr-cys 60-90 ml/min/1.73 m²(*n*=180), 2021 CKD-EPI eGFRcr-cys 60-90 ml/min/1.73 m²(*n*=151), 2020 Cystatin C-B2M-BTP equation (3-marker panel) 60-90 ml/min/1.73 m² (n=339), 2020 Creatinine-Cystatin C-B2M-BTP equation (4-marker panel) 60-90 ml/min/1.73 m² (n=222), 2009 CKD-EPI eGFRcr >90 ml/min/1.73 m² (n=415), 2014 CKD-EPI eGFRcr-PK >90 ml/min/1.73 m²

(*n*=339), 2021 CKD-EPI eGFRcr >90 ml/min/1.73 m²(*n*=435), 2012 CKD-EPI eGFRcys >90 ml/min/1.73 m²(*n*=175), 2012 CKD-EPI eGFRcr-cys >90 ml/min/1.73 m²(*n*=294), 2021 CKD-EPI eGFRcr-cys >90 ml/min/1.73 m²(*n*=329), 2020 Cystatin C-B2M-BTP equation (3-marker panel) >90 ml/min/1.73 m² ($n=97$), 2020 Creatinine-Cystatin C-B2M-BTP equation (4-marker panel) >90 ml/min/1.73 m² (*n*=250).

Abbreviations: BTP, β-trace protein; B2M, β2-microglobulin; GFR, glomerular filtration rate; eGFR, estimated glomerular filtration rate; CI, confidence interval; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; RMSLE, Root Mean Squared Logarithmic Error.

Table S2. Linear regression between baseline characteristics and log-transformed BTP and B2M adjusting for age, sex and measured GFR (N=557).

^aAverage percent difference in serum B2M and BTP levels for an IQR (difference between the $25th$ and $75th$ percentiles) higher level in continuous variables was calculated as $100 \times (e^{beta-coefficient} - 1)$ using error-in-variables regression models assuming log-transformed mGFR was measured with 98.5% reliability. Strength of association for statistically significant results is indicated by color: red, strong (absolute average percent difference in B2M/BTP levels >10%); blue, intermediate (absolute average percent difference in B2M/BTP levels 5%-10% inclusive); and yellow, weak (absolute average percent difference in B2M/BTP levels <5%). ^bOne missing value ($n = 556$).

Three missing values $(n = 554)$.

Abbreviations: BTP, β-trace protein; B2M, β2-microglobulin; GFR, glomerular filtration rate; LDL, low-density lipoprotein.

Table S3. Linear regression between baseline characteristics and log-transformed B2M and BTP adjusting for all non-GFR

determinants and measured GFR (N=557).

^aAverage percent difference in serum B2M and BTP levels for an IQR (difference between the 25th and 75th percentiles) higher level in continuous variables was calculated as $100 \times (e^{beta-coefficient} - 1)$ using error-in-variables regression models assuming log-transformed mGFR was measured with 98.5% reliability. Strength of association for statistically significant results is indicated by color: red, strong (absolute average percent difference in B2M/BTP levels >10%); blue, intermediate (absolute average percent difference in B2M/BTP levels 5%-10% inclusive); and yellow, weak (absolute average percent difference in B2M/BTP levels <5%). bMultivariable model 1 included all variables, mGFR, measurement error of mGFR except for total body fat and lean body mass. ^cMultivariable model 2 included all variables, mGFR, measurement error of mGFR except for body mass index and waist circumference.

^dOne missing value ($n = 556$).

^eThree missing values ($n = 554$).

 $fR²$ was based on variables included in each model.

Abbreviations: BTP, β-trace protein; B2M, β2-microglobulin; GFR, glomerular filtration rate; LDL, low-density lipoprotein.

REFERENCES

- **1.** Jafar TH, Islam M, Jessani S, et al. Level and determinants of kidney function in a South Asian population in Pakistan. *Am J Kidney Dis.* 2011;58(5): 764-772.
- **2.** Wang Y, Levey AS, Inker LA, et al. Performance and Determinants of Serum Creatinine and Cystatin C–Based GFR Estimating Equations in South Asians. *Kidney Int Rep.* 2021;6(4): 962-975.
- **3.** Karger AB, Eckfeldt JH, Rynders GP, et al. Long-Term Longitudinal Stability of Kidney Filtration Marker Measurements: Implications for Epidemiological Studies and Clinical Care. *Clin Chem.* 2021;67(2): 425-433.
- **4.** Grubb A, Blirup-Jensen S, Lindström V, Schmidt C, Althaus H, Zegers I. First certified reference material for cystatin C in human serum ERM-DA471/IFCC. *Clin Chem Lab Med.* 2010;48(11): 1619-1621.
- **5.** Blirup-Jensen S, Grubb A, Lindstrom V, Schmidt C, Althaus H. Standardization of Cystatin C: development of primary and secondary reference preparations. *Scand J Clin Lab Invest Suppl.* 2008;241: 67-70.
- **6.** Inker LA, Eckfeldt J, Levey AS, et al. Expressing the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) cystatin C equations for estimating GFR with standardized serum cystatin C values. *Am J Kidney Dis.* 2011;58(4): 682-684.
- **7.** Levey AS, Coresh J, Greene T, et al. Expressing the Modification of Diet in Renal Disease Study equation for estimating glomerular filtration rate with standardized serum creatinine values. *Clin Chem.* 2007;53(4): 766-772.
- **8.** Jessani S, Levey AS, Bux R, et al. Estimation of GFR in South Asians: a study from the general population in Pakistan. *Am J Kidney Dis.* 2014;63(1): 49-58.
- **9.** Rolin HA, 3rd, Hall PM, Wei R. Inaccuracy of estimated creatinine clearance for prediction of iothalamate glomerular filtration rate. *Am J Kidney Dis.* 1984;4(1): 48-54.
- **10.** Davies DF, Shock NW. The variability of measurement of insulin and diodrast tests of kidney function. *J Clin Invest.* 1950;29(5): 491-495.
- **11.** Levey AS, Coresh J, Tighiouart H, Greene T, Inker LA. Strengths and limitations of estimated and measured GFR. *Nat Rev Nephrol.* 2019;15(12): 784-784.
- **12.** Houtkooper LB, Lohman TG, Going SB, Howell WH. Why bioelectrical impedance analysis should be used for estimating adiposity. *Am J Clin Nutr.* 1996;64: 436s-448s.
- **13.** Segal KR, Van Loan M, Fitzgerald PI, Hodgdon JA, Van Itallie TB. Lean body mass estimation by bioelectrical impedance analysis: a four-site cross-validation study. *Am J Clin Nutr.* 1988;47(1): 7-14.
- **14.** Fadem SZR, B. Protein Intake Calculator*.* http://nephron.org/nephsites/nic/protein_intake, Accessed November 1 2019.
- **15.** Inker LA, Schmid CH, Tighiouart H, et al. Estimating glomerular filtration rate from serum creatinine and cystatin C. *N Engl J Med.* 2012;367(1): 20-29.
- **16.** Inker LA, Eneanya ND, Coresh J, et al. New Creatinine- and Cystatin C–Based Equations to Estimate GFR without Race. *N Engl J Med.* 2021. doi: 10.1056/NEJMoa2102953.
- **17.** Inker LA, Couture SJ, Tighiouart H, et al. A New Panel-Estimated GFR, Including β(2)- Microglobulin and β-Trace Protein and Not Including Race, Developed in a Diverse Population. *Am J Kidney Dis.* 2021;77(5): 673-683.e671.
- **18.** Peng X, Wu WT, Xu J. Will You Be in Hospital Next Year: Leveraging Machine Learning in Improving Healthcare. Vol 2021.
- **19.** Martinez WL, Martinez AR. Computational Statistics Handbook with MATLAB, Second Edition (Chapman & Hall/Crc Computer Science & Data Analysis): Chapman & Hall/CRC; 2007.
- **20.** Liu X, Foster MC, Tighiouart H, et al. Non-GFR Determinants of Low-Molecular-Weight Serum Protein Filtration Markers in CKD. *Am J Kidney Dis.* 2016;68(6): 892- 900.
- **21.** Stevens LA, Schmid CH, Greene T, et al. Factors other than glomerular filtration rate affect serum cystatin C levels. *Kidney Int.* 2009;75(6): 652-660.