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Biological variation of measured and estimated glomerular filtration rate (GFR) in patients with chronic kidney disease: the eGFR-C Study

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Significance statement of key findings

In this prospective study we have simultaneously, under controlled conditions, established the biological and analytical variability of glomerular filtration rate (GFR) and estimates of GFR in patients with moderate chronic kidney disease. Variability of estimates of GFR using the Modification of Diet in Renal Disease (MDRD) Study and Chronic Kidney Disease Epidemiology (CKD-EPI) equations were similar to each other, but slightly lower than that of GFR measured using iohexol clearance. Consequently estimated GFR would need to decline by approximately 14% for that change to be considered significant with 95% certainty, compared to an approximately 18% decline in measured GFR for the same degree of certainty. The data presented can be used to assist an objective understanding of GFR changes in clinical practice.

Abstract

When assessing changes in glomerular filtration rate (GFR) it is important to differentiate pathological change from intrinsic biological and analytical variation. GFR is measured

using complex reference methods (e.g. iohexol clearance). In clinical practice measurement of creatinine and cystatin C is used in equations (e.g. Modification of Diet in Renal Disease [MDRD] or Chronic Kidney Disease Epidemiology Collaboration [CKD-EPI]) to provide estimated GFR. We studied biological variability of measured and estimated GFR in twenty nephrology outpatients (10 male, 10 female; median age 71, range 50-80 years) with moderate CKD (GFR 30-59 mL/min/1.73 m^2). Patients underwent weekly GFR measurement by iohexol clearance over four consecutive weeks. Simultaneously GFR was estimated using the MDRD, CKD-$EPI_{creatinine}$, CKD-$EPI_{cystatinC}$ and CKD-$EPI_{creatinine+cystatinC}$ equations. Within-subject biological variation (CV_I) expressed as a percentage [95% CI] for the MDRD (5.0% [4.3-6.1]), CKD-$EPI_{creatinine}$ (5.3% [4.5-6.4]), CKD-$EPI_{cystatinC}$ (5.3% [4.5-6.5]), and CKD-$EPI_{creatinine+cystatinC}$ (5.0% [4.3-6.2]) equations were broadly equivalent. CV_I values for MDRD and CKD- $EPI_{creatinine+cystatinC}$ were lower (p=0.027 and p=0.022 respectively) than that of measured GFR (6.7% [5.6-8.2]). Reference change values (RCV), the point at which a true change in a biomarker in an individual can be inferred to have occurred with 95% probability were calculated: using the MDRD equation, positive and negative RCVs were 15.1% and 13.1% respectively. If an individual's baseline MDRD estimated GFR (mL/min/1.73 m^2) was 59, significant increases or decreases would be to values >68 or <51 respectively. Within-subject variability of estimated GFR is lower than measured GFR. RCVs can be used to understand GFR changes in clinical practice.

Keywords: biological variation, creatinine, cystatin C, glomerular filtration rate, iohexol, kidney disease, MDRD, CKD-EPI

Introduction

Chronic kidney disease (CKD) is prevalent in the general population[1-4] and is commonly identified using estimation of glomerular filtration rate (GFR). The aim of disease detection is to make decisions on therapeutic interventions, and to identify and manage those most likely to progress to kidney failure and/or those at high risk of morbidity and mortality. The ability of tests to identify which individuals with CKD are at high risk of progressive or fatal disease is a crucial issue. However, what constitutes progressive kidney disease has been variably defined. Furthermore, a significant problem has been the ability of GFR measurements and estimations to identify progression of kidney disease against background age-related change in GFR and the biological and measurement variability of both reference and estimated GFR.[5]

Ideally, for accuracy GFR would be measured using either inulin clearance or one of several surrogate 'reference methods' in specialist clinical use (e.g. plasma clearance of iothalamate, iohexol or ^{51}Cr ethylenediaminetetraacetic acid). However, these techniques are somewhat complex and time-consuming. Pragmatic estimates of GFR, based on serum creatinine or cystatin C measurement, or both, are widely used. As with any physiological measurement, GFR, whether measured or estimated, has an intrinsic within-subject biological variability (CV_I). Knowledge of this variability is critical to appreciation of disease-related change. Using a variety of reference markers, earlier studies have reported within-subject coefficients of variation (CV%) for the biological variation of GFR ranging between 5.5% and 12.1%.[6-12] Whilst forming a useful basis for comparison, many of these previous estimates did not follow an appropriate construct for a biological variation study and do not permit comparison of measured and estimated GFR.[13]

An understanding of biological variation of disease markers is essential to the interpretation of changes in response to disease events. Critical evaluation of the

significance of changes in results obtained on analysis of serial specimens can be performed only by consideration of CV_I and analytical (CV_A) variation.[14] These data enable the derivation of the reference change value (RCV), the point at which a true change in a biomarker in an individual can be inferred to have occurred with a stated degree of probability: typically 95% probability is chosen as this is conventionally regarded as significant.[14, 15]

The aim of the present study is to define under standardised conditions the normal biological variability of measured GFR and hence derive mathematically the RCV. A subsidiary question is whether the CV_I and RCV are the same if estimated instead of measured GFR is used.

Results

Characteristics of the study subjects are shown in Table 1. Medications were held constant during the four weeks of the study, except that two patients received a one week course of amoxicillin (500 mg tds) due to chest infection.

All 20 patients attended all four iohexol clearance procedures excepting one patient who missed one appointment. Results from five iohexol clearances (five separate patients) were excluded before analysis, as the dose given was not fully administered or it was given subcutaneously. Application of Cochran and Reed's tests led to the exclusion of between one and three duplicate measurements for measured or estimated GFR and to the exclusion of one outlying within-subject measurement for iohexol clearance (Supplementary Table S1). Overall, no patient was completely excluded and all calculations of biological variation for measured and estimated GFRs were based on a minimum of three weeks data in all individuals.

Estimates of components of biological variation are given in Table 2. The geometric exact CV_I value [95% CI] for measured GFR was 6.7% [5.6-8.2]. CV_I values for the estimated GFR equations were broadly equivalent: MDRD 5.0% [4.3-6.1], CKD-$EPI_{creatinine}$ 5.3% [4.5-6.4], CKD-$EPI_{cystatinC}$ 5.3% [4.5-6.5], and CKD-$EPI_{creatinine+cystatinC}$ 5.0% [4.3-6.2] to each other. Modelling to investigate differences showed the CV_I for MDRD and CKD-$EPI_{creatinine+cystatinC}$ estimated GFRs to be significantly (at 5% level) lower than for measured GFR (difference -1.8%, p=0.027 and difference -1.8%, p=0.022 respectively, see Supplementary Table S2). Using the MDRD equation, positive and negative RCVs were 15.1% and 13.1% respectively. For example, if baseline MDRD GFR (mL/min/1.73 m^2) in an individual is 59, significant increases or decreases would be to values >68 or <51 respectively.

Sensitivity analyses were carried out without outlier detection and deletion. Data were similar to those obtained following outlier removal, with analyses after outlier removal estimating slightly reduced CVs (Supplementary Table S3).

Modelling to identify any trends over time resulted in non-significant slopes (coef=-0.005; 95% CI (-0.020, 0.009); p=0.488), thus providing no evidence of a change in disease state (kidney function) over the duration of the study.

Discussion

To our knowledge, this is the first study to simultaneously establish the biological variation of measured and estimated GFR in patients with CKD. Following a recommended study design,[13] in a prospective study we observed the within-subject biological variation of measured GFR to be 6.7%, with similar, although in some cases significantly lower, biological variation of estimated GFR (5.0%, 5.3%, 5.3% and 5.0% for the MDRD, CKD-$EPI_{creatinine}$, CKD-$EPI_{cystatinC}$ and CKD-$EPI_{creatinine+cystatinC}$ equations respectively). Taking

analytical and within-subject biological variability into account produced RCVs (%, positive/negative) of 21.5/-17.7 (measured GFR), 15.1/-13.1 (MDRD), 15.9/-13.7 (CKD-EPI$_{creatinine}$), 15.9/-13.8 (CKD-EPI$_{cystatinC}$) and 15.1/-13.1 (CKD-EPI$_{creatinine+cystatinC}$).

Although there have been several previous studies of the biological variation of GFR, few have followed the rigour of design required of a biological variation study.[13, 14] Nevertheless, several of these earlier studies report biological variability of GFR of a similar magnitude to that observed here, despite a variety of techniques and study designs; 4.5% (healthy individuals, plasma iohexol clearance),[16] 5.7% (CKD patients, plasma iohexol clearance),[7] 6.3% (CKD patients, renal ^{125}I-Iothalamate clearance),[8] 5.5% (CKD patients with GFR >30 mL/min/1.73 m^2, plasma ^{51}Cr-ethylenediaminetetraacetic acid [EDTA] clearance),[6] with some authors reporting higher estimates; 9.8% (CKD patients, plasma ^{51}Cr-EDTA clearance)[10] and 8.0% (CKD patients, ^{99}mTe-DTPA clearance).[9] Some of the differences observed may reflect the underlying level of kidney function in the groups studied: both Levey et al[8] and Brochner-Mortensen et al[6] report higher variation estimates in individuals with GFR<30 mL/min/1.73 m^2. Other factors including length of time between repeat procedures (10 months) and total study duration (12 years),[10] inattention to hydration status, fasting and exercise before and during the test[9] may also have increased the variability reported in some studies.

When considering any change in a patient's results, healthcare practitioners need to be able to distinguish true change ('signal') from the 'noise' of variability. In clinical practice, biological variation is best considered in terms of the RCV, which takes both biological and analytical variation of measured GFR into account: the positive and negative RCVs of measured GFR were 21.5% and -17.7% respectively. Definitions of progressive kidney disease vary but it is important to consider whether, in the clinical context, the variability of measured GFR allows for detection of progressive kidney disease over a useful time frame. Reported 'normal' mean age-related decline in GFR of 1 mL/min/1.73 m^2/year,[17] or

reported rates of decline of 3.6 mL/min/1.73 m^2/year and 2.8 mL/min/1.73 m^2/year respectively in male and female community dwelling older adults with diabetes and moderate CKD[18] could not be detected in individuals by annual GFR measurement. It is possible that reported annual mean GFR declines of 7.0 mL/min/1.73 m^2/year amongst proteinuric (greater than 1 g/24 h) patients could be detected by annual monitoring of individual patient's GFR.[19]. Importantly, based on the data presented here, monitoring of GFR will permit detection of progressive kidney disease as defined by recent guideline recommendations from Kidney Disease Improving Global Outcomes (KDIGO) and the National Institute for Health and Care Excellence (NICE). Both guidelines define a certain drop in GFR as an increase in disease category (e.g. G3a [GFR 45-59 mL/min/1.73 m^2] to G3b [GFR 30-44 mL/min/1.73 m^2]) accompanied by a fall in GFR of greater than or equal to 25% between two serial results. Alternatively, they define a significant change as a decrease in GFR of 15 mL/min/1.73 m^2 or more per year.[5, 20] For example: if baseline measured GFR in an individual is 59 mL/min/1.73 m^2, significant increases or decreases would be to values >72 or <48 mL/min/1.73 m^2. Given the lower CV_I and CV_A of estimated GFR, slightly lower RCVs may be applied when monitoring patients using GFR estimating equations (e.g. if an individual's baseline MDRD estimated GFR was 59, significant increases or decreases would be to values >68 or <51 mL/min/1.73 m^2 respectively). However, it must be remembered that our biological variation estimates are obtained under idealised conditions, with optimisation of preanalytical variables and precise laboratory methods. In an uncontrolled operational clinical environment, it is likely that biological and analytical variation, and hence RCVs, would increase.

The within-subject biological variation of serum creatinine we have observed (4.4%) is in broad agreement with values reported in other studies in both healthy (4.1% to 7.6%,[16, 21-28]) and diseased (5.7% to 9.9%[23, 29-31]) cohorts. Enzymatic creatinine methods are less prone to interference than Jaffe methods and the use of an enzymatic assay in the present study improves confidence in the estimate of biological variation we have

reported. Whilst calculation of CV_I excludes any contribution due to CV_A, it cannot account for biological variability of non-creatinine chromogens (e.g. bilirubin, glucose, ketones, protein, and certain drugs) that are known to interfere in Jaffe methods of creatinine measurement. Similarly, our reported within-subject biological variation of cystatin C (4.0%) is similar to most (3.1%,[32] 4.1%,[25] 4.5%[16, 27] and 4.8%[29]) but not all (6.8%,[28] 8.6%[23] and 13.3%[24]) previous estimates. As for measured GFR, differences in study design and data analysis may account for differences in reported estimates of variation: for example, most of these studies did not report their approach to outlier detection; the time interval between repeat sampling was prolonged in some studies.[28]

Depending on the equation used, estimated GFR is based on the concentration of creatinine, cystatin C or both. Therefore estimated GFR will have a similar CV_I to creatinine or cystatin C, mathematically inflated by the power function in the respective equation. The point estimates for CV_I of the four studied equations lie between 5.0% and 5.3% and have overlapping confidence intervals.

It is uncertain why the CV_I of estimated GFR should be lower than that of measured GFR. Probably the complexity of the iohexol clearance procedure, involving multiple measurements and blood samplings, contributes to a higher CV_I for measured than estimated GFR. However, it is also possible that the variability of estimated GFR is somewhat attenuated compared to physiological fluctuations in measured GFR, as noted, in an extreme example, following renal insult in acute kidney injury where there is a delay between the fall in GFR and the consequent rise in blood creatinine concentration.

These data have implications for the use of measured versus estimated GFR in clinical practice and research. Within-subject biological variation of measured GFR was similar to that of estimated GFR, implying no disadvantage to the use of simple estimates of GFR when monitoring patients over time. The main priority for monitoring GFR is to detect

change and for this purpose estimated GFR is at least as reliable as measured GFR. This is important because measurement of GFR is time consuming and more costly than estimated GFR. However, this should not be interpreted as an indication that estimated GFR should replace measured GFR when an accurate assessment of GFR is required. Reference techniques are considered more accurate than estimated GFR primarily because they are not influenced by the non-GFR determinants of endogenous filtration markers. Reference GFR measurements will remain important as the benchmark in clinical research studies and to inform clinical situations in which more accurate knowledge of GFR is important. These situations include certain chemotherapies (e.g. carboplatin); the use of any drug that is nephrotoxic or renally-excreted and has a narrow therapeutic margin; the assessment of potential living related kidney donors; and the assessment of GFR in patients with muscle-wasting disorders, including spina bifida and paraplegia.

The strengths of this study include the use of an enzymatic creatinine assay and a three-point iohexol clearance procedure with the final sample being taken at 4 h postinjection, which is considered suitable for patients with GFR>30 mL/min/1.73 m^2.[33] The study was adequately powered[34] and followed a strict design to minimise preanalytical variation and investigator bias (Supplementary Table S4).[13] Outliers were excluded using a formal exclusion protocol: sensitivity testing was undertaken using excluded data to confirm that presented results were representative. Estimation of components of variation was derived using a nested ANOVA approach, which takes into account analytical variation for estimation of within-subject biological variation. The studied patient group represents a major population in which monitoring of kidney function to detect worsening disease is regularly undertaken and which is mandated in international guidance.[5, 20] Prescribed medication was unaltered during the study, with the exception of two patients who received a course of amoxicillin. No patients showed significant trends in GFR during the

study period, confirming that the variation we have reported is physiological and not pathological in nature.

Our study has some limitations. The cohort studied was recruited from a single centre and was exclusively Caucasian: biological variability estimates may not be transferable to other ethnic groups. Although the study was adequately powered to answer the primary question, we were unable to investigate whether variability is higher at differing levels of GFR or albuminuria. Although previous studies have observed statistically significant differences in CV_I when individuals are stratified for level of GFR/albuminuria[29] such effects are unlikely to be of practical importance.[25] Our measured GFR data was based on a plasma iohexol clearance procedure. Whilst constant infusion urinary inulin clearance would be considered the reference measure of GFR, single-bolus plasma clearance of iohexol demonstrates good agreement with this technique and is widely used in clinical practice.[35] In terms of CV_I, plasma clearance techniques are likely to produce lower values than urinary clearance techniques due to problems of inaccurate urine collection. We have chosen to calculate RCVs representing 95% probability, as is conventional. However, if a lower probability was considered clinically acceptable, then the RCV would be smaller.[22]

In clinical practice, in the setting of CKD identification of deterioration of kidney function tends to be based not upon two consecutive results but on multiple observations obtained over a period of time. Traditional RCV calculations only allow comparison between two consecutive measurements. When multiple measurements are available then use of RCV values as described herein will be susceptible to the effect of repeated testing, where the probability of a false-positive result increases with the number of results available. Because of this, in general terms RCV values increase with the number of observations available (i.e. a larger change is required compared to the baseline value to be deemed significant). Adjustments to the RCV calculation dependent on the number of results have been published but are relatively complex.[36, 37] Because of this, and also because our

patients were being studied within relatively controlled conditions as discussed above, the RCV values we have reported should be considered minimum values: in clinical practice, for the same certainty of change, larger RCVs may be required.

In conclusion we describe the biological variability of measured and estimated GFR in a carefully designed study. The data generated have implications for monitoring of patients with CKD and clinical ability to detect CKD progression, both in clinical practice and in clinical trials, whether using measured or estimated GFR. Within-subject biological variation of measured GFR is similar to that of estimated GFR and, in terms of variability, suggests no real advantage to the use of measured GFR when monitoring patients over time. Most importantly, the information presented provides an evidence-base allowing clinicians to have meaningful discussions with their patients about the implications of changes in their GFR results.

Methods

Chronic kidney disease patients (n=20) with MDRD estimated GFR between 30 and 59 mL/min/1.73 m^2 sustained over at least 90 days were recruited at the Kent Kidney Care Centre, UK between August 2014 and July 2015.[38] Patients with diabetes and proteinuria (ACR >30 mg/mmol) were included in the study. Patients who had a history of reaction to iodinated contrast media, who were pregnant, who had an episode of acute kidney injury within the last six months, amputees and those with an inability to consent due to cognitive impairment were excluded from the study. Patients provided written informed consent and the study had ethical approval (South-East Coast-Surrey Research Ethics Committee of the National Research Ethics Service reference number 13/LO/1349). The study conforms to the internationally agreed checklist for the reporting of studies of biological variation (Supplementary Table S4).[13]

The sample size was based on the precision of CV_I, which was estimated to be 10%. With twenty participants recruited, tested on four occasions and assayed in duplicate and assuming data are log-normally distributed, an approximate 95% confidence interval (CI) for CV_I has limits ±2% (absolute).

Measurement and estimation of GFR

Patients underwent four iohexol reference measures of GFR in four successive weeks, with standardisation for time of day and day of week. Participants were asked to follow a permitted food list from 22:00 the night before the procedure, being permitted a light breakfast with no high protein foods on the morning of the procedure. Demographic data, comorbidity information and prescription histories were recorded and blood pressure, weight and height documented. Blood samples were taken immediately prior to iohexol injection for serum creatinine and cystatin C measurement. Blood samples were collected using standard venepuncture procedures, including the use of a tourniquet, into gel-separator (for serum cystatin and creatinine) and lithium heparin- (for plasma iohexol) containing Vacuette™ tubes (Greiner Bio-One International) following manufacturer's recommended order of draw. Plasma/serum was separated by centrifugation within 4 h of venepuncture and sample aliquots were stored at -80°C pending analysis. All analyses were undertaken within 9 months of venepuncture at a central laboratory.

A 5 mL bolus of Omnipaque 240 (518 g/L iohexol corresponding to 240 g/L iodine, GE Healthcare www.gelifesciences.com) followed by 10 mL physiological saline was injected into the antecubital vein. A blood sample was taken at 5 minutes from the opposite arm to confirm that the iohexol had been administered intravenously. Further blood samples were collected at 120, 180 and 240 minutes after injection. Exact times of blood draws in relation to injection time were recorded. During the procedure individuals were allowed

free access to fluids (no carbonated drinks), but asked to refrain from protein intake and excessive exercise.

Detailed laboratory methods are available in the supplementary file. Briefly, iohexol was measured using electrospray isotope dilution tandem mass spectrometry. Iohexol concentrations were log transformed (natural log) and plotted as a function of time. GFR was calculated from the slope-intercept method using a single compartment model, GFR (mL/min) = 0.693 x iohexol volume of distribution (L) x 1000/half-life of iohexol (min). GFR was adjusted for body surface area (BSA)[39] and then corrected for the fast exponential.[40]

Serum creatinine was measured using an enzymatic assay standardised to the reference material, NIST SRM 967 and 914. Between-day imprecision (coefficient of variation, %) was 0.8%, 0.3% and 0.4% at concentrations of 75, 176 and 760 umol/L respectively. Cystatin C was measured by a turbidimetric immunoassay calibrated against the international certified reference material ERM-DA471/IFCC for cystatin C.[41] GFR was estimated using the simplified isotope dilution mass-spectrometric (ID-MS) traceable version of the MDRD equation[42] and the three CKD-EPI equations: CKD-EPI$_{creatinine}$, CKD-EPI$_{cystatinC}$ and CKD-EPI$_{creatinine\text{-}cystatinC}$.[43, 44]

Statistical analysis

Data were log-transformed and normality tests were performed using the Shapiro-Wilk test. Outliers between duplicate measurements and of within-subject variance were excluded using Cochran's test and outliers amongst mean values of subjects were excluded using Reed's test as advocated by Fraser and Harris.[14] Sensitivity analyses were also performed without exclusion of identified outliers. Log transformation was used to simplify calculation and because it improved the normality of the data as assessed by

an increase in Shapiro–Wilk W statistic and visual examination of the distributions (Supplementary Figure S1 and Table S5).

Terminology used was as proposed by Simundic et al.[45] Analytical (CV_A), CV_I and between-subject (CV_G) components of variation were calculated using standard approaches[14] of linear random effects modelling with restricted maximum likelihood estimation (allowing for the clustering of observations within time points and repeated observations per patient) (Stata version 15). Exact geometric CVs [$\sqrt{\exp(S^2) - 1} \times 100$,[46, 47]] were calculated. Confidence intervals for SDs and CVs were estimated as described by Burdick and Graybill.[48] Differences in measures of CV, comparing the estimated GFR measures to measured GFR were investigated using multilevel models accounting for the clustering of test observations within individuals, using unstructured covariance matrices, in addition to the clustering of test results (multiple results per person, observation points and assessments). The RCV for a change in GFR between two results with 95% probability was calculated using the approach for log-normal data giving a negative and positive limit.[49] The number of specimens (n) required to produce a precise estimate of the homeostatic set-point with 95% confidence within $\pm$10% was calculated as:

$$n = [1.96 \cdot (CV_I^2 + CV_A^2)^{1/2}/10]^2$$

For each biomarker the index of individuality (II) was calculated as:

$$II = (CV_I^2 + CV_A^2)^{1/2}/CV_G$$

To confirm kidney function was stable across the study period, the iohexol GFR measures were modelled to identify trend with time using a multilevel linear regression model (allowing for clustering of assessments within time points and observations within individuals).

Abbreviations:

ACR: Albumin to creatinine ratio; ANOVA; analysis of variance; BSA: body surface area; CI: confidence interval; CKD: chronic kidney disease; CKD-EPI: Chronic Kidney Disease-Epidemiology Consortium; CV: coefficient of variation; CV_A: analytical coefficient of variation; CV_G: between-subject biological variation; CV_I: within-subject biological variation; CV_T: total coefficient of variation; EDTA, ethylendiaminetetraacetic acid; eGFR: estimated glomerular filtration rate; GFR: glomerular filtration rate; ID-MS: isotope dilution mass spectrometry; KDIGO: Kidney Disease Improving Global Outcomes; MDRD: Modification of Diet in Renal Disease; mGFR: measured glomerular filtration rate; NICE: National Institute for Health and Care Excellence; RCV: reference change value

Disclosure:

All authors declare no competing interests.

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Author's contributions:

All authors contributed to the intellectual content and have met the following requirements: (a) significant contributions to the concept, (b) drafting or revising the article for intellectual content and (c) reading and approval of the final manuscript. Specific contribution: CR and ASi carried out the statistical analyses and reported the results.

Department of Health Disclaimer:

The views and opinions expressed herein are those of the authors and do not necessarily reflect those of the HTA, NIHR, NHS or the Department of Health.

Supplementary material:

Supplementary detailed laboratory methods

Figure S1. Effect of log transformations on distributions

Table S1. Identification of outliers by Cochran's and Reed's criterion.

Table S2. Differences between measures using each GFR estimate compared with measured GFR (calculated as eGFR-mGFR).

Table S3. Summary of components of variation for creatinine and cystatin C and measured and estimated glomerular filtration rate (GFR) *without outlier detection and removal*

Table S4. Critical appraisal checklist for studies of biological variation

Table S5. Shapiro-Wilk normality test p-values before and after log transformation.

Supplementary file references.

Supplementary information is available at *Kidney International's* website

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Table 1. Characteristics of the study population. Values for continuous data are shown as median (range). Anthropometric data is based on baseline measurements. Estimated and measured* GFR, creatinine and cystatin C data are calculated using all values over the four weeks.

n	20
Age, y	71 (50-80)
M:F	10:10
Caucasian (n)	20
Height, cm	170.5 (154-194)
Weight, kg	79.5 (47.1-118.1)
Body surface area, m^2	1.99 (1.42-2.47)
Body mass index, kg/m^2	28.2 (19.6-40.9)
Medication record (n)	Thiazide diuretic (3), loop diuretic (3), potassium sparing diuretic (2), beta-blocker (7), calcium antagonist (4), ACE inhibitor (8), angiotensin 2 receptor blocker (6), alpha-blocker (1), isosorbide mononitrate (1), HMG CoA reductase inhibitor (13), allopurinol (4), antiplatelet drugs (7)
Comorbidity (n)	Type 2 diabetes mellitus (3), ischaemic heart disease (7), angina (1), heart failure (2)
Smoker – current/former (n)	1/10
Urine albumin concentration <3 mg/mmol (n)	9
Urine albumin concentration 3-30 mg/mmol (n)	7
Urine albumin concentration >30 mg/mmol (n)	4
Serum creatinine, µmol/L	124 (79-182)
Serum cystatin C, mg/L	1.67 (1.01-2.30)
Measured GFR, mL/min/1.73 m^2	49.0 (30.8-71.6)*
MDRD, mL/min/1.73 m^2	42.2 (31.5-61.4)
$CKD\text{-}EPI_{creatinine}$, mL/min/1.73 m^2	43.0 (30.8-62.8)

CKD-EPI$_{cystatinC}$, mL/min/1.73 m^2	36.8 (23.5-67.1)
CKD-EPI$_{creatinine+cystatinC}$, mL/min/1.73 m^2	38.2 (27.2-65.4)

Abbreviations: ACE, angiotensin converting enzyme; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; HMG, hydroxymethyl glutaryl; MDRD, Modification of Diet in Renal Disease

*Excludes data from five failed iohexol procedures (five separate patients).

Table 2. Summary of components of variation for creatinine and cystatin C and measured and estimated glomerular filtration rate (GFR)

				Estimated GFR			
	Measured GFR	Creatinine	Cystatin C	MDRD	CKD-EPI$_{creatinine}$	CKD-EPI$_{CystatinC}$	CKD-EPI$_{creatinine+CystatinC}$
Geometric exact							
CV_A (%)	2.3 (1.9, 2.7)	0.7 (0.6, 0.8)	0.6 (0.5, 0.7)	0.8 (0.7, 0.9)	0.8 (0.7, 1.0)	0.7 (0.6, 0.9)	0.6 (0.5, 0.7)
CV_I (%)	6.7 (5.6, 8.2)	4.4 (3.7, 5.3)	4.0 (3.4, 4.9)	5.0 (4.3, 6.1)	5.3 (4.5, 6.4)	5.3 (4.5, 6.5)	5.0 (4.3, 6.2)
CV_G (%)	16.7 (12.5, 24.9)	20.0 (15.0, 29.6)	19.0 (14.4, 28.2)	17.8 (13.4, 26.0)	19.3 (15.5, 29.2)	25.2(18.9, 37.5)	20.2 (15.2, 30.0)
Positive RCV (%)	21.5	13.0	11.8	15.1	15.9	15.9	15.1
Negative RCV (%)	-17.7	-11.5	-10.6	-13.1	-13.7	-13.8	-13.1
Homeostatic set point	2	1	1	1	1	1	1
Index of Individuality	0.4	0.2	0.2	0.3	0.3	0.2	0.3

All CV values expressed as percentages. 95% confidence intervals were calculated using methods of Burdick and Graybill.[48]

Abbreviations: CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; CV_A, analytical variation; CV_G, between-subject variation; CV_I, within-subject biological variation; MDRD, Modification of Diet in Renal Disease; RCV, reference change value

Supplementary File

Biological variation of measured and estimated glomerular filtration rate (GFR) in patients with chronic kidney disease: the eGFR-C Study

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Detailed laboratory methods

Iohexol was measured using electrospray isotope dilution tandem mass spectrometry on an ABSCIEX API6500 Q-trap (ABSCIEX, Warrington, UK). Iohexol stock standard, 10 mmol/L, was prepared by diluting Omnipaque 300 solution (647 g/L) in deionised water and stored in 1 mL aliquots at -80^{o}C. Aqueous iohexol calibrators (0, 10, 100 and 500 µmol/L) were prepared from the stock iohexol standard by dilution and stored in 0.5 mL aliquots at -80^{o}C. Iohexol stable isotope, d5-iohexol (Toronto Research Chemicals), was obtained from 2BScientific Ltd, Upper Heyford, UK, dissolved in deionised water at circa 10 mmol/L, and stored at -80^{o}C. Plasma control samples were prepared by spiking a plasma pool with iohexol stock standard at 10, 100, and 400 µmol/L. Calibrators, controls, patient samples and stable isotope stock solutions were thawed from frozen on a roller mixer at room temperature for no more than 60 minutes, and then centrifuged for 4 minutes at 1,500 g at 4°C (Eppendorf 5810R centrifuge, VWR International Ltd, Lutterworth, UK). Working iohexol stable isotope was prepared by diluting the circa 10 mmol/L solution 1:200 with deionised water. Calibrators, controls, and samples were pipetted (20 µL) into 2 mL microcentrifuge tubes (000-MICR-200, Elkay Laboratory Products (UK) Ltd, Basingstoke, UK) and 50 µL working iohexol stable isotope, followed by 200 µL acetonitrile (Rathburn Chemicals Ltd, Walkerburn, UK), were added to each tube. Samples were capped, vortex mixed for 5 seconds and centrifuged for 5 minutes at 20,800 g at 4°C (Eppendorf 5417R centrifuge, VWR International Ltd, Lutterworth, UK). Supernatants (200 µL) were then transferred into a 96 deep well plate and loaded onto the autosampler. Sample (2 µL) was automatically injected into a mobile phase stream of acetonitrile:water (1:1) with 0.025% formic acid using a Hewlett-Packard 1100 Series

autosampler and pump (Applied Biosystems, Warrington, UK) at 250 µL/min. Chromatography was performed on a Chirobiotic T 100 x 2.1 mm column with a 2 cm x 4.0 mm guard column (Sigma-Aldrich Company Ltd, Poole, UK).

Tandem mass spectrometry was performed in positive ion multiple reaction monitoring (MRM) mode: iohexol 821.849/602.8, d5-iohexol 826.849/607.8. Data acquisition time was 6 minutes with a pause time of 5.0070 msec between transitions and a scan speed of 10 Da/s. Iohexol concentrations were calculated in Analyst 1.6 (ABSCIEX, Warrington, UK) using the ratio of sample peak area to stable isotope peak area. Between-day imprecision (coefficient of variation, %) was 1.0%, 0.8% and 1.5% at 10, 100 and 400 umol/L respectively. The laboratory participated in an international proficiency testing scheme (EQUALIS, https://www.equalis.se/en/start/) for iohexol measurement with satisfactory performance.

Iohexol concentrations were log transformed (natural log) and plotted as a function of time. GFR was calculated from the slope-intercept method using a single compartment model,
GFR (mL/min) = 0.693 x iohexol volume of distribution (L) x 1000/half-life of iohexol (min). GFR was adjusted for body surface area (BSA)[1] and corrected for the fast exponential.[2]

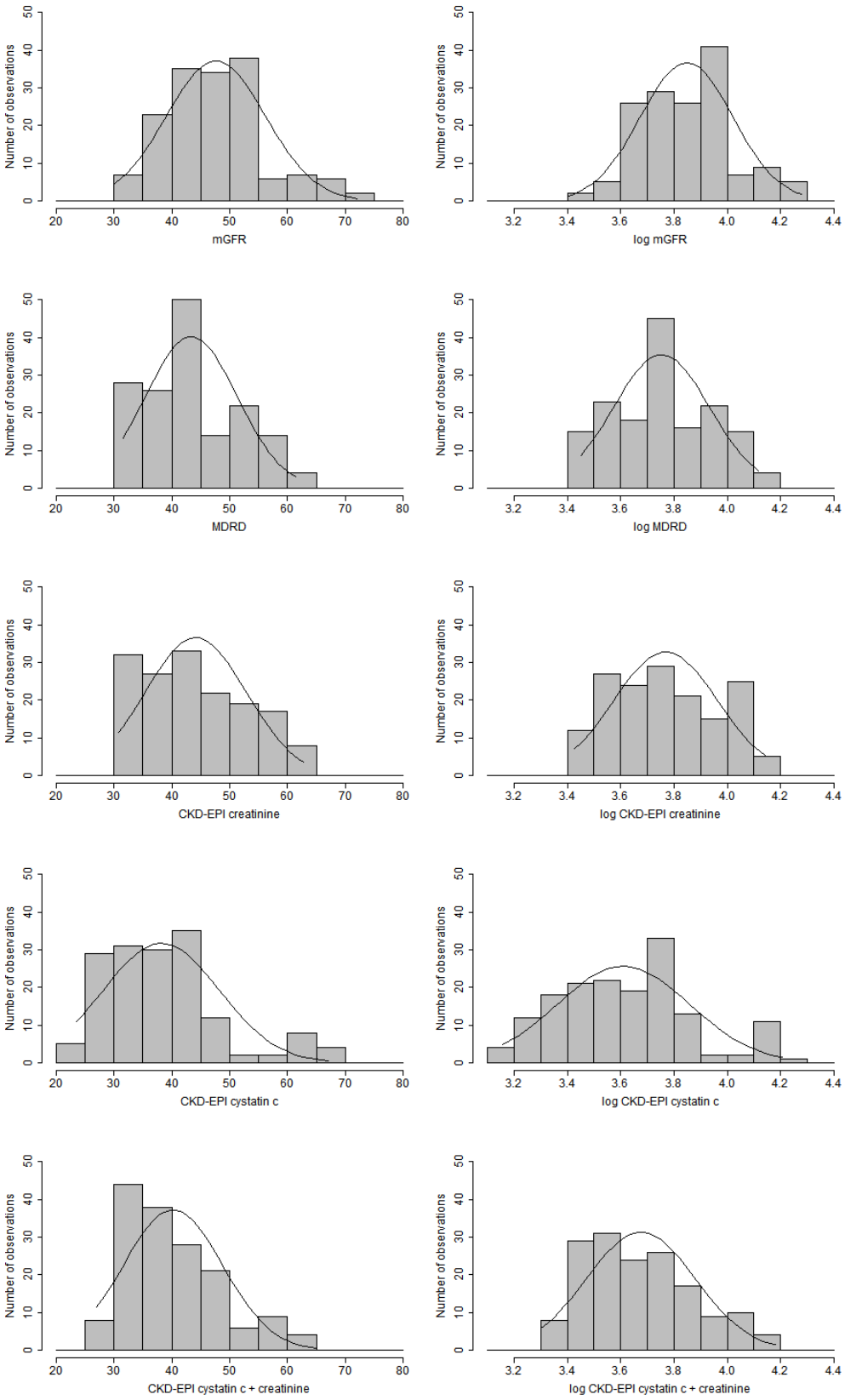
Serum creatinine was measured using an enzymatic assay on an Abbott Architect analyser (Abbott Diagnostics Ltd, www.international.abbottdiagnostics.com) standardised to the reference material, NIST SRM 967 and 914. Between-day imprecision (coefficient of variation, %) was 0.8%, 0.3% and 0.4% at concentrations of 75, 176 and 760 umol/L respectively. The laboratory participated in an international proficiency testing scheme (UKNEQAS, https://birminghamquality.org.uk/) for creatinine measurement and GFR estimation with satisfactory performance. Cystatin C was measured by a turbidimetric immunoassay on an Abbott Architect analyser. The assay was calibrated against the international certified reference material ERM-DA471/IFCC for cystatin C.[3] Between-day imprecision was 2.3% and 1.6% at concentrations of 0.9 and 4.0 mg/L respectively. The laboratory participated in an international proficiency testing scheme (EQUALIS, https://www.equalis.se/en/start/) for cystatin C measurement and GFR estimation with satisfactory performance.

Prior to analysis, samples were thawed at room temperature, mixed by inversion and centrifuged. All samples from each individual subject were measured in duplicate in random order in a single assay. Each of the biomarker analyses was undertaken by a single operator blinded to participant data using a single instrument. Creatinine and

cystatin C measurements were undertaken in an accredited laboratory by scientists registered with the Health and Care Professions Council.

GFR was estimated using the simplified isotope dilution mass-spectrometric (ID-MS) traceable version of the MDRD equation[4] and the three CKD-EPI equations: CKD-$EPI_{creatinine}$, CKD-$EPI_{cystatinC}$ and CKD-$EPI_{creatinine\text{-}cystatinC}$.[5,6]

Supplementary Figure 1 – effect of log transformations on distributions



Supplementary Table S1. Identification of outliers by Cochran's and Reed's criterion. Outliers removed shown as patient ID (week of sample, A to D)

Marker	Outlier amongst duplicate measurements	Outlier in within-subject measurements	Outlier in measurements between subjects
Serum creatinine (enzymatic)	B10561 (B), B14944 (C),	N/A	N/A
Serum cystatin C	B10561 (B), B12450 (C), B14944 (D)	N/A	N/A
MDRD	B10561 (B), B14944 (C)	N/A	N/A
$CKD\text{-}EPI_{creatinine}$	B10561 (B), B14944 (C)	N/A	N/A
$CKD\text{-}EPI_{cystatinC}$	B10561 (B), B12450 (C), B14944 (D)	N/A	N/A
$CKD\text{-}EPI_{creatinine+cystatinC}$	B10561 (B)	N/A	N/A
Plasma iohexol clearance	B10561 (A), B14605 (C), B14944 (C)	B15222 (C)	N/A

Abbreviations: CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; MDRD, Modification of Diet in Renal Disease

Supplementary Table S2: Differences between measures using each GFR estimate compared with measured GFR (calculated as eGFR-mGFR). Differences estimated using further modelling which was a paired analysis to tests differences in CVs between tests.

	MDRD			$CKD\text{-}EPI_{creatinine}$			$CKD\text{-}EPI_{CystatinC}$			$CKD\text{-}EPI_{creatinine+CystatinC}$		
	diff	95% CI*	p	diff	95% CI*	p	diff	95% CI*	p	diff	95% CI*	p
σ_G	0.013	(-0.047, 0.073)	0.665	0.027	(-0.030, 0.085)	0.351	0.084	(0.028, 0.140)	0.003	0.039	(-0.003, 0.081)	0.072
σ_I	-0.018	(-0.033, -0.002)	0.027	-0.015	(-0.031, 0.001)	0.058	-0.015	(-0.031, 0.001)	0.063	-0.018	(-0.034, -0.003)	0.021
σ_A	-0.015	(-0.018, -0.011)	<0.001	-0.014	(-0.018, -0.010)	<0.001	-0.015	(-0.019, -0.011)	<0.001	-0.017	(-0.020, -0.013)	<0.001
Geometric exact												
CV_G (%)	1.35	(-4.76, 7.46)	0.665	2.79	(-3.08, 8.67)	0.351	8.64	(2.79, 14.49)	0.004	3.97	(-0.37, 8.32)	0.073
CV_I (%)	-1.77	(-3.33, -0.20)	0.027	-1.54	(-3.13, 0.05)	0.058	-1.49	(-3.06, 0.08)	0.063	-1.82	(-3.37, -0.27)	0.022
CV_A (%)	-1.46	(-1.84, -1.07)	<0.001	-1.42	(-1.81, -1.03)	<0.001	-1.48	(-1.86, -1.10)	<0.001	-1.65	(-2.03, -1.27)	<0.001

*95% confidence intervals (in brackets) were calculated using the delta method and may be conservative. Note that differences will not be the same as differences between estimates for each test individually as differences here have been calculated using a paired analysis.

Abbreviations: CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; CV_A, analytical variation; CV_G, between-subject variation; CV_I, within-subject biological variation; MDRD, Modification of Diet in Renal Disease; RCV, reference change value

Supplementary Table S3. Summary of components of variation for creatinine and cystatin C and measured and estimated glomerular filtration rate (GFR) *without outlier detection and removal*

				Estimated GFR			
	GFR	Creatinine	Cystatin C	MDRD	CKD-EPI$_{creatinine}$	CKD-EPI$_{CystatinC}$	CKD-EPI$_{creatinine+CystatinC}$
Geometric exact							
CV_A (%)	2.4 (2.1, 2.8)	0.7 (0.6, 0.8)	0.60 (0.52, 0.71)	0.8 (0.7, 0.9)	0.8 (0.7, 0.9)	0.8 (0.7, 0.9)	0.6 (0.5, 0.7)
CV_I (%)	6.9 (5.8, 8.5)	4.3 (3.7, 5.3)	4.0 (3.4, 4.9)	5.0 (4.2, 6.1)	5.2 (4.4, 6.4)	5.3 (4.5, 6.5)	5.0 (4.3, 6.1)
CV_G (%)	16.4 (12.2, 25.6)	20.1 (15.2, 29.8)	19.0 (14.3, 28.1)	17.8 (13.5, 26.6)	19.4 (14.6, 28.8)	25.1 (18.9, 37.4)	20.2 (15.2, 30.0)
Positive RCV (%)	22.5	12.9	11.8	15.0	15.7	16.0	15.0
Negative RCV (%)	-18.3	-11.4	-10.6	-13.0	-13.6	-13.8	-13.1
Homeostatic set point	2	1	1	1	1	1	1
Index of Individuality	0.5	0.2	0.2	0.3	0.3	0.2	0.3

All CV values as expressed as percentages. 95% confidence intervals (in brackets) were calculated using methods of Burdick and Graybill. [7]

Abbreviations: CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; CV_A, analytical variation; CV_G, between-subject variation; CV_I, within-subject biological variation; MDRD, Modification of Diet in Renal Disease; RCV, reference change value

Supplementary Table S4: Critical appraisal checklist for studies of biological variation[8]

Section and Topic	Item #		Evidenced
Title/abstract/ keywords	1	The title should indicate that the content relates to a study of biological variation, the subject of the study, the sample matrix, and the population studied. Analyte (component being measured), the measurand/s (the quantity or quantities to be measured), and state of well-being of the subjects under study should be clearly and unambiguously identified. *Relevant coding systems might be employed*, (e.g., LOINC, SNOMED, C-NPU)	Title: Biological variation of measured and estimated glomerular filtration rate (GFR) in patients with chronic kidney disease: the eGFR-C Study.
Abstract	1.1	As a minimum it should contain the headline biological variation data, the major characteristics of the population studied (numbers of subjects with demographics), clearly identify the analyte and measurand/s studied [the analyte quantities studied in a particular sample matrix, (e.g., concentration of glucose in plasma)], the statistical approach taken, the duration of the study and the geographical location of the study.	The abstract includes all required information, except that geographical setting is given in methods section due to space constraints in abstract.
Introduction	2	Introduction should clearly identify the context and aims of the study and cite any previous relevant studies of biological variability of the target analyte. Recommended terminology to be adopted re description of variability.[9]	The introduction addresses these points. Previous relevant studies are cited. Appropriate recommended terminology is used.[9]

Methods	3	Described in enough detail to facilitate transportability of the derived data across populations and health care systems. The biological variation data produced are effectively reference data and their applicability requires delivery of appropriately described metadata to enable their use as such.	Methods are given within the main manuscript with more detailed laboratory methods provided within the supplementary file.
Analyte/ measurand	3.1	The described study should clearly identify the target analyte and measurand/s. Where available internationally agreed terminology and codings should be utilised.	Detailed descriptions of the methods used to measure iohexol, creatinine and cystatin C, and of the use of these measurements in estimating and measuring GFR are given.
Subjects	3.2	The description of the subjects and population studied should be detailed enough to enable transportability of the biological variation data. Minimum data set should be present. This should include number of subjects studied, age, gender, and state of well-being.	The subjects and population are described in the methods section and in table 1.
Measurement procedure	3.3	A clear description of the analytical methodology used should form part of the metadata. This may be made available via an appropriate reference or be presented within the publication. Deviation from standard operating procedures, use of adaptations of published methods, and deviation from manufacturers recommended methods in the case of commercially available systems should be documented. Standardisation and traceability should be clearly identified.	See Methods section in main paper and detailed analytical methods in supplementary file. Details of standardisation are given.

Length of study	3.4	Length of the study periods should be clearly identified.	Stated in methods section.
Sampling	3.5	Sampling protocols (e.g., subject preparation, sampling conditions) that minimise pre-analytical variation should be adequately described to enable transportability of the data.[10] Numbers of samples taken should be sufficient to deliver the required power to the study.[10,11]	Minimisation of preanalytical variables is clearly described in methods section. Power of study was adequate and is addressed in the discussion.
Samples	3.6	Recorded details should include the beginning and end date of the study and timings of sampling. Sampling conditions and sample type should be described in detail. Pre-analytical storage conditions of samples should be described.	See methods section.
Conditions for analysis of samples	3.7	A description of conditions under which the samples were analysed. Analytical protocols should be designed to minimise sources of analytical variation (Optimal Conditions Precision).	See methods section and detailed methods in supplementary file. Analytical imprecision is presented and was <2.4% in all cases.
Data analysis	4	Data analysis techniques should be described. The power of the study to identify indices of biological variation should be calculated and presented.[11]	See methods/data analysis section.
Outlier analysis	4.1	Outliers should be excluded from the final analysis of the data. Test for outliers should be applied to all levels of data (between replicate analysis, between samples within subject, between subjects).[10] The numbers of outliers and reasons for their	Outlier exclusion is clearly described in the methods section and in more detail in supplementary table S1. An analysis without outlier

		exclusion must be given.	removal is given in supplementary table S3.
Heterogeneity of variance	4.2	Subjects with outlying within subject variance should be rejected from calculations used to determine an estimate of common true variance. The numbers of outliers and reasons for their exclusion must be given.	One outlier for within subject variance for iohexol clearance was removed (supplementary table S1).
Statistical methods described and appropriate	4.3	Statistical methods used should be appropriately identified, fit for purpose and referenced. Data that do not conform to a normal distribution should be appropriately transformed.[10]	Statistical methods are described in the methods section. Data were not normally distributed. Log transformation was used to simplify calculation and because it improved the normality of the data as assessed by an increase in Shapiro–Wilk W statistic and visual examination of the distributions (supplementary figure S1 and table S5).
Results	5	Unified terminology[9] should be used and appropriately defined metadata clearly presented to enable understanding and transportation of the data through time and across health care systems.	Recommended terminology used throughout.
Terminology	5.1	Terms and symbols should be used to describe biological variation should conform to standards identified by Simundic et al.[9]	Recommended terms and symbols used throughout.

Results clearly presented and managed	5.2	Biological variation data, with derived indices, should be tabulated in a format that enables extraction of the key data unambiguously associated with a minimum data set to enable transportability of the data. Power of the study and confidence limits around estimates of biological variation should be presented.[11] The results section should clearly identify the results of outlier analysis undertaken and confirm homogeneity of the data sets. If data are stratified the variables used to enable this should be clearly characterised.	Table 2 summarises components of variation. Power of the study was confirmed using the recommended approach. [11] Confidence limits for data are given in Table 2. Outlier analysis is clearly described in the results section and in more detail in supplementary table S1. An analysis without outlier removal is given in supplementary table S3.
Discussion	6	The discussion of the data should clearly include a focus on factors that impact on the transportability of the data to other settings. Limitations and strengths of the study should be addressed. If the data are used to set analytical performance specifications, derive reference change values and study individuality, the recommendations of Simundic et al. should be followed.[9]	Strengths and limitations of the study are addressed in the discussion. The focus of the study was not primarily to derive analytical performance specifications.

Supplementary Table S5: Shapiro-Wilk normality test p-values before and after log transformation

	Measured GFR	MDRD	CKD-EPI$_{creatinine}$	CKD-EPI$_{cystatin\ C}$	CKD-EPI$_{creatinine+cystatinC}$
Untransformed	0.00038	0.00002	0.00002	<0.00001	<0.00001
Log transformed	0.11279	0.00037	0.00026	0.00106	0.00045

References for supplementary file

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