The Impact of Interlaboratory Differences in Cystatin C Assay Measurement on Glomerular Filtration Rate Estimation

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Summary

Background and objectives Cystatin C (CysC) is a promising marker of GFR. Several equations have been derived to estimate GFR from its serum concentration. Heterogeneity in the performance of these equations exists in validation studies even when the same CysC assay from the same manufacturer is utilized. This study was designed to examine the differences in CysC and GFR estimation (eGFR) using Siemens' nephelometric immunoassay and the Mayo Clinic equation. The ability of the eGFRs to predict measured GFR was also examined.

Design, setting, participants, & measurements Ninety-seven split samples were sent to laboratories at Children's Hospital of Eastern Ontario (CHEO) in Ottawa, Canada, and at the Mayo Clinic in Rochester, Minnesota.

Results The mean CHEO CysC was 0.17 mg/L (10%) lower than the mean Mayo Clinic CysC. Using the Mayo Clinic equation, the mean eGFR difference was 7.2 ml/min per 1.73 m^2 (15%). Approximately 36% of the results agreed within 10%, while 13% were discordant by greater than 30%. Larger absolute differences in mean eGFR between the two laboratories were found in the subgroup with CysC less than 1.41 mg/L as compared with the subgroup greater than 1.41 mg/L (9.5 *versus* 5.0 ml/min per 1.73 m^2). Correction of CHEO values to the Mayo Clinic did not improve GFR estimation.

Conclusions Significant differences in CysC measurement exist between laboratories using the same assay by the same manufacturer and these lead to clinically relevant differences in GFR estimation. This interlaboratory variability needs to be recognized when interpreting and comparing CysC and eGFR results.

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Introduction

Cystatin C (CysC) is a low molecular weight protein (1) that has generated much interest as a marker of the GFR. It possesses many properties of an ideal filtration marker and its serum concentration is significantly less affected by biometric and demographic variables than serum creatinine (2,3). Many equations have been developed to translate its serum concentrations into an estimate of GFR (eGFR) (4).

CysC is measured by immunoassay using nephelometric (PENIA), turbidimetric (PETIA), or spectrophotometric (ELISA) methodologies. Several different manufacturers produce the assay kits, which can be run on a number of different platforms (the machine used to measure the analyte). As for most analytes, the array of measurement procedures and manufacturers leads to difficulties comparing quantitative values between laboratories (5–7). Interlaboratory differences result, in a large part, from variation in the calibrators (standards) produced by different assay manufacturers. Development and use of higher order reference materials and reference methods allows for harmonization of calibrators across manufacturers and should address much of the manufacturer-to-manufacturer bias. The lack of higher order reference materials for CysC has repeatedly been identified as a source of concern (8–10). In 2008, The International Federation of Clinical Chemistry (IFCC) announced the successful production of primary and secondary reference materials for CysC, but these have not yet been globally implemented (11).

It has been hypothesized that interlaboratory calibration variation accounts for much of the heterogeneity in findings among studies that have compared the diagnostic performance of the different CysC eGFR equations (2,4,9,12). Differences between PENIA, PETIA, and ELISA methodologies have been explored by a number of groups, with notable differences demonstrated (5,6). However, these studies do not address the variability in equation performance

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seen in studies that utilized the same methodology and manufacturer (13–16). The purpose of this study was to examine the differences in CysC measured using Siemens' PENIA assay in two different laboratories experienced in measuring CysC and the effect any differences would have on GFR estimation.

Methods and Materials

Patients and Samples

Serum was obtained and frozen (−80°C) and GFR (plasma clearance of ^{99m}Tc-DTPA) was measured on the same day as part of the ACE-Inhibition for the Preservation of Renal Function and Patient Survival in Kidney Transplantation Trial (17). CysC was measured using the N Latex cystatin C kit (Dade Behring, now Siemens) on a Behring BN ProSpec analyzer in the laboratory at the Children's Hospital of Eastern Ontario (CHEO), Ottawa, Canada, between 2006 and 2009. The between-day precision (coefficient of variation; CV) for the serum CysC daily internal controls was 3.1% at 1.06 mg/L, 3.5% at 2.04 mg/L, and 6.7% at 5.26 mg/L. At the Mayo Clinic (Rochester, MN), CysC was also measured using the N Latex cystatin C kit (Dade Behring, now Siemens) on a Dade Behring BN II Nephelometer on a single day in 2009.

Previous validation data revealed between-day precision was 3.2% at 0.76 mg/L and 3.8% at 1.44 mg/L for this assay.

Interlaboratory Differences

Absolute and percent differences between CysC values were calculated, and the corresponding SD and 95% confidence intervals (CI) were determined. Paired *t* tests were used to compare mean differences. The percentage of samples with paired values within 10%, 20%, and 30% of each other was calculated. This analysis was repeated after stratifying patients by CysC less than and greater than 1.41 mg/L (median of the average of CHEO and Mayo Clinic CysC values). The relationship between CHEO and Mayo Clinic CysC values was examined using Deming regression, which accounts for measurement error in both assays and by Bland–Altman analysis.

Impact of Assay on eGFR

The Mayo Clinic equation developed in transplant recipients was used to determine the eGFR from CysC measured in each laboratory (2). The absolute mean and percent differences in these estimates were calculated and compared using paired *t* tests. The percent of samples with paired estimates within 10%, 20%, and 30% of each other was calculated. The analysis was repeated after stratifying patients by CHEO CysC less than and greater than 1.41 mg/L. Similar calculations were performed using the Filler equation, a CysC-based estimation equation derived in a nontransplant population (18). Classification of patients into chronic kidney disease (CKD) stage 3 using both CHEO and Mayo Clinic values and the Mayo Clinic equation was examined.

Equation Performance

The bias, precision, and accuracy of the Mayo Clinic equation were calculated as recommended in the National Kidney Foundation guidelines on CKD (19). For this analysis we used CysC values measured at the Mayo Clinic and CHEO, as well as CHEO values "corrected" using the regression analysis between paired CysC values. Performance measures were also determined for the Filler equation. Differences in equation bias were assessed using paired *t* tests or the Wilcoxon test, as appropriate. Accuracy was compared using the McNemar test.

Results

A total of 97 patients had both a CysC (CHEO) and a measured GFR completed and qualified for enrollment in this study. Three results differing by more than 4 SD of the mean difference between sites were considered outliers and thus omitted from any further analysis (CHEO/Mayo Clinic: 3.55/2.57, 0.55/1.76, 1.43/3.41 mg/L), as they were likely different due to handling issues rather than differences between assays.

Interlaboratory Differences

Interlaboratory differences are shown in Table 1. The mean CHEO CysC was 0.17 mg/L (9.9%) lower than the mean Mayo Clinic CysC ($P < 0.0001$). The greatest positive difference of a CHEO result compared with a Mayo Clinic result was + 0.44 mg/L, and the greatest negative difference was −0.88 mg/L. Eighty-seven percent of CHEO results were lower than Mayo Clinic results. Forty-seven percent of results were within 10% of each other, 84% were within 20%, and 4% demonstrated a >30% difference. Subanalysis for results below or above 1.41 mg/L are

Table 1. Interlaboratory CysC Differences[a]

	Mean Mayo Clinic CysC ± SD (range)	Mean CHEO CysC ± SD (range)	Mean Absolute Difference (95% CI)[b]	Mean % Difference (95% CI)[c]
Whole cohort (n = 94)	1.58 ± 0.61 (0.65 to 3.82)	1.41 ± 0.53 (0.63–3.15)	−0.17 (−0.21, −0.13)[d]	−9.9 (7.6, 12.2)[d]
CysC <1.41 mg/L (n = 47)	1.14 ± 0.24	1.01 ± 0.19	−0.12 (−0.17, −0.07)[d]	−9.7 (−6.2, −13.1)[d]
CysC ≥1.41 mg/L (n = 47)	2.03 ± 0.53	1.81 ± 0.44	−0.22 (−0.29, −0.16)[d]	−10.1 (−7.0, −13.2)[d]

[a] Expressed as mg/L.
[b] Absolute difference = CHEO CysC − Mayo Clinic CysC.
[c] % difference = (CHEO CysC − Mayo Clinic CysC)/Mayo Clinic CysC × 100.
[d] $P < 0.0001$.

shown in Table 1. The mean CHEO values were lower the Mayo Clinic values by a similar percentage in both groups.

Deming linear regression yielded the following equation: CysC CHEO = 0.058 + 0.855 × CysC Mayo Clinic (R^2 = 0.83; Figure 1), indicating that the CHEO results demonstrated a proportional bias of approximately −15% across entire data range. Bland–Altman analysis, shown in Figure 2, again demonstrates that the mean difference between the two assays is −0.17 mg/L, that higher cystatin results tend to have poorer precision, and that the percent bias is stable across the range of CysC values.

Impact of Assay on eGFR

As expected, the average eGFR was higher with CysC values from CHEO as compared with the Mayo Clinic (Table 2). Using the Mayo Clinic equation, the mean eGFR difference was 7.2 ml/min per 1.73 m^2 or 15.3% ($P <$ 0.0001). Approximately 36% of the results agreed within 10%, 76% within 20%, and 87% within 30%.

Subanalysis for results below or above 1.41 mg/L are shown in Table 2 and demonstrate a larger absolute difference in eGFR between the two values in the lower CysC subgroup (9.5 ml/min per 1.73 m^2) as compared with the higher CysC subgroup (5.0 ml/min per 1.73 m^2). The percent differences, however, were similar.

Similar findings were seen for the Filler equation, with mean absolute and percentage differences between the two laboratories of 8.4% and 14.8% ml/min per 1.73 m^2 (n = 94). These were 11.0% and 5.7% ml/min per 1.73 m^2 for the lower CysC subgroup, and 5.8% and 14.4% ml/min per 1.73 m^2 for the higher CysC subgroup.

In the 50 patients with stage 3 CKD (GFR between 30 and 60 ml/min per 1.73 m^2), as determined by eGFR calculated using Mayo Clinic values, 9 (20%) had eGFR greater than 60 ml/min per 1.73 m^2, when calculated using CHEO values. In comparison, of the 54 patients classified as stage 3 CKD using CHEO results, four (8%) would be classified as stage 4 CKD (GFR between 15 and 30 ml/min per 1.73 m^2) using Mayo Clinic values.

Performance of eGFR Compared with Measured GFR

The performance of the Mayo Clinic eGFR using CysC values from Mayo Clinic, CHEO, and CHEO "corrected" values are shown in Table 3. On average, the Mayo clinic equation marginally overestimated the measured GFR (mGFR) by 1.8 ml/min per 1.73 m^2 when CysC was assayed at CHEO, whereas it underestimated the mGFR by −5.4 ml/min per 1.73 m^2 when CysC was assayed at the Mayo Clinic ($P <$ 0.0001). The bias was −4.9 ml/min per 1.73 m^2 using CHEO-"corrected" values, which was not statistically different from Mayo Clinic values (P = 0.57) but was from CHEO values ($P <$ 0.0001). Precision was poor for all, with no statistically significant differences between the different assays. Overall accuracy was modest (78% to 80% of estimates within 30% of mGFR), with no significant differences between estimates calculated from the three CysC values.

Similar trends were seen in both CysC subgroups, with significant differences in bias between the Mayo Clinic and CHEO values, and CHEO and CHEO-"corrected" values ($P <$ 0.0001 for all), and, again, there were no significant differences in precision or 30% accuracy. The between-

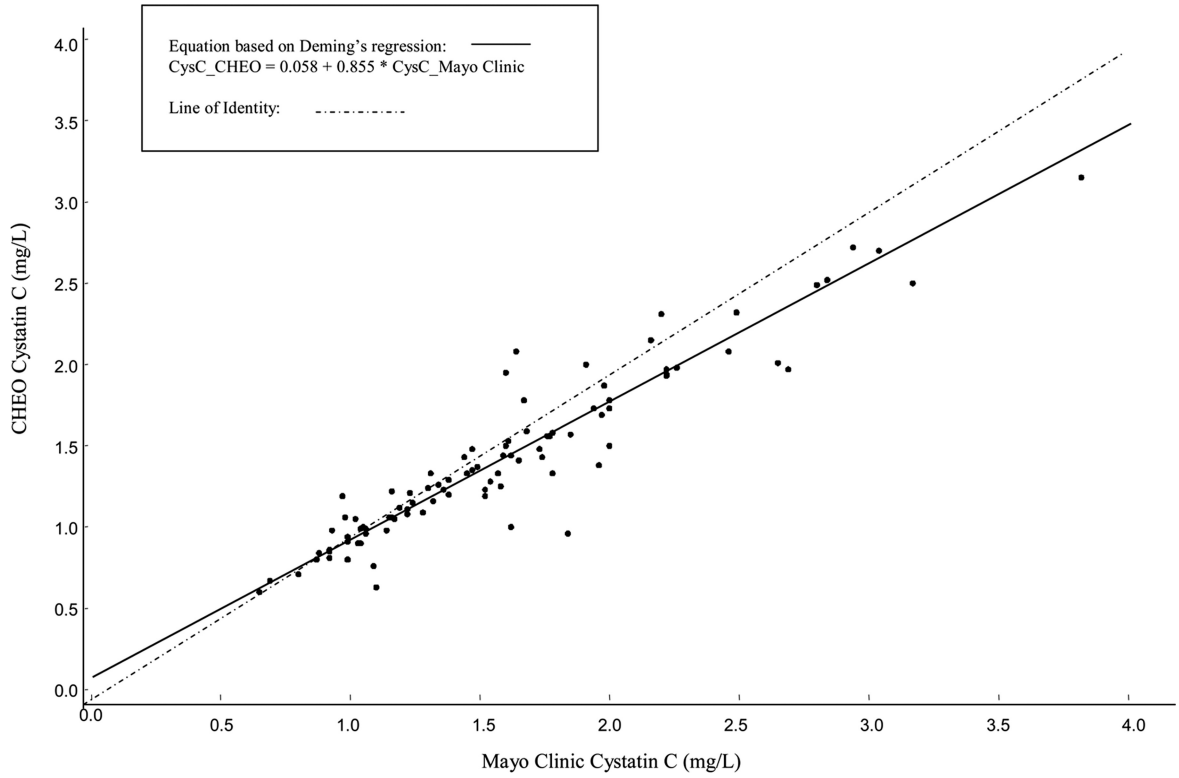

Figure 1. | **Linear regression analysis between paired CysC values.** CysC, cystatin C; CHEO, Children's Hospital of Eastern Ontario.

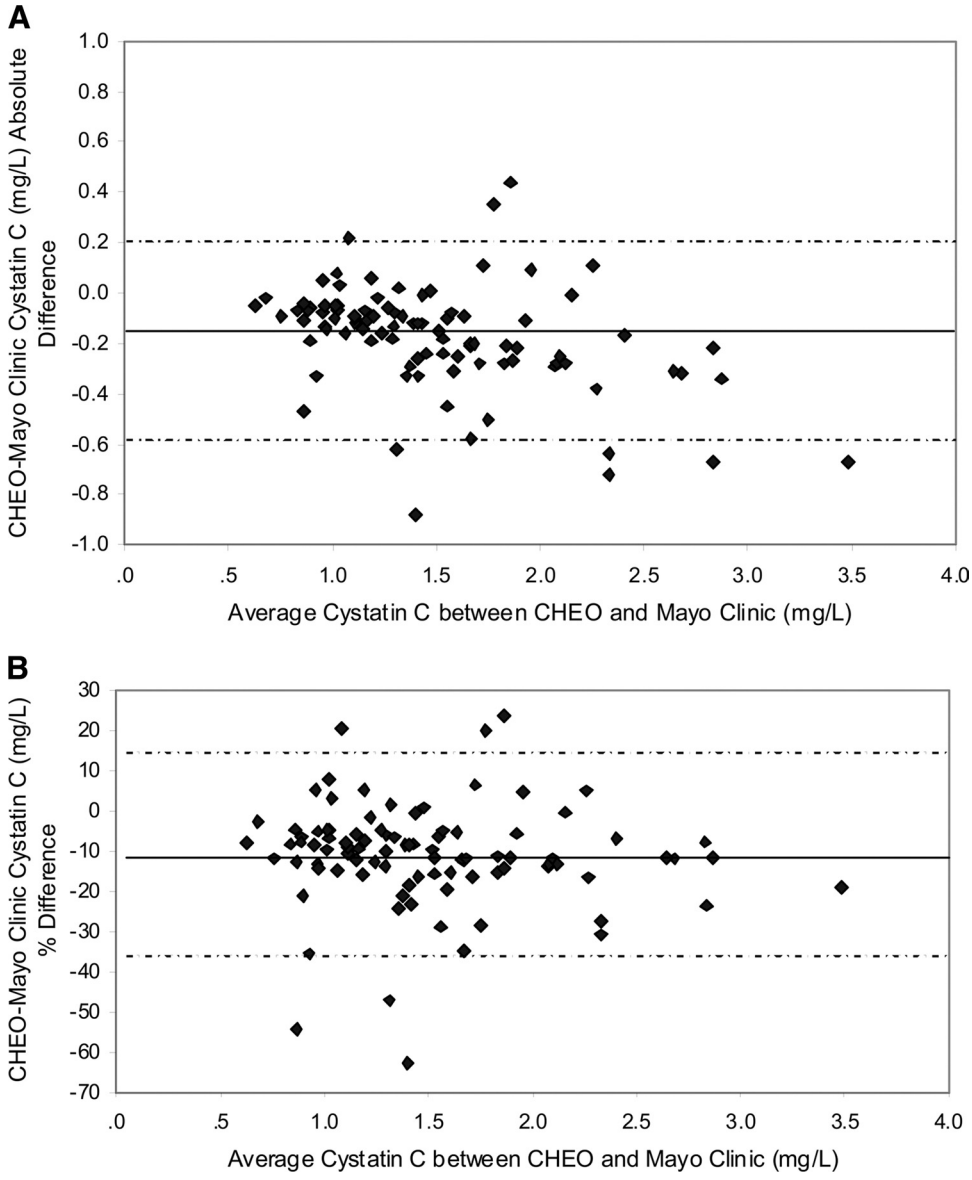

Figure 2. | **Bland–Altman analysis with (A) absolute and (B) percent bias; solid middle line represents mean bias and two flanking hatched lines the 95% confidence intervals.** CHEO, Children's Hospital of Eastern Ontario.

Table 2. GFR Estimates and Difference in Estimates[a]

	Mean eGFR (Mayo Clinic CysC) ± SD	Mean eGFR (CHEO CysC) ± SD	Mean Absolute Difference (95% CI)[b]	Mean % Difference (95% CI)[c]
Whole cohort (n = 94)	52.9 ± 21.9	60.1 ± 24.7	7.2 (5.2, 9.3)[d]	15.3 (11.3, 19.4)[d]
CysC <1.41 mg/L (n = 47)	69.8 ± 17.6	79.2 ± 19.7	9.5 (5.8, 13.1)[d]	15.7 (8.9, 22.4)[d]
CysC ≥1.41 mg/L (n = 47)	36.0 ± 8.9	41.0 ± 10.1	5.0 (3.5, 6.5)[d]	15.0 (10.8, 19.2)[d]

CysC, cystatin C; CHEO, Children's Hospital of Eastern Ontario; CI, confidence interval.
[a]Expressed as ml/min per 1.73 m^2.
[b]Absolute difference = CHEO eGFR − Mayo Clinic eGFR.
[c]% difference = (CHEO eGFR − Mayo Clinic eGFR)/Mayo Clinic eGFR × 100.
[d]$P < 0.0001$.

Table 3. Mayo Clinic eGFR Clinic Equation Performance[a]

	Mean Absolute Bias	Precision (SD)	Mean % Bias	Precision (SD)	Median Absolute Bias	Precision (IQR)	Accuracy % Within 30%
Full cohort (n = 94)							
eGFR (Mayo Clinic)	−5.4	12.2	−7.0	22.7	−4.9	16.6	80
eGFR (CHEO)	1.8	14.7	6.4	28.1	1.0	19.9	79
eGFR (CHEO corrected)	−4.9	14.3	−6.0	25.4	−4.7	19.3	78
CysC <1.41 (n = 47)							
eGFR (Mayo Clinic)	−4.1	14.5	−2.1	24.7	−3.7	23.5	82
eGFR (CHEO)	5.3	17.7	12.0	30.3	4.4	28.7	76
eGFR (CHEO corrected)	−2.6	17.5	−0.4	27.5	−3.8	28.2	80
CysC ≥1.41 (n = 47)							
eGFR (Mayo Clinic)	−6.7	9.3	−11.9	19.6	−5.2	11.4	74
eGFR (CHEO)	−1.7	9.8	0.9	24.8	−0.80	13.6	74
eGFR (CHEO corrected)	−7.1	9.9	−12.4	21.7	−5.5	13.2	72

[a]Bias, percent bias, and precision expressed in ml/min per 1.73 m^2. Bias was defined as the absolute difference between the eGFR and the mGFR (eGFR − mGFR), and as the percentage difference ([eGFR − mGFR]/ mGFR × 100). A negative bias indicates that the GFR is underestimated by the prediction equation. Precision was described by both the interquartile range of the median bias and the standard deviation (SD) of the mean bias. Accuracy was defined as the percentage of eGFR *estimates* lying within 30% of the mGFR. eGFR, estimated GFR; IQR, interquartile range; CysC, cystatin C; CHEO, Children's Hospital of Eastern Ontario; mGFR, measured GFR.

laboratory effect on bias and precision for the Filler equation was similar (results not shown).

Discussion

This study is the first to report interlaboratory CysC assay differences and their impact on eGFR using Siemens' PENIA assay. An overall 9.9% difference in CysC concentration was observed between the CHEO and Mayo Clinic laboratories, leading to a 15.3% (7.2 ml/min per 1.73 m^2) difference in eGFR. The absolute difference was more pronounced in the subgroup with lower CysC values (9.9 ml/min per 1.73 m^2) *versus* higher CysC values (4.6 ml/min per 1.73 m^2), as can be expected given the exponential relationship between CysC and GFR. Twenty percent of patients with stage 3 CKD using Mayo Clinic values had an eGFR greater than 60 ml/min per 1.73 m^2 using CHEO values. These results support the suggestion that interlaboratory differences contribute to some of the heterogeneity in equation performance observed in validation studies using Siemens' PENIA assay (13–16).

The variation in this study is consistent with 2010 data from the College of American Pathologists' (CAP) external quality assurance program (20). The CAP coefficient of variation (%CV) for the Siemens' assays (n = 39) was 6%, revealing that CysC results from any Siemens laboratory should be expected to be within ± 11.8% (95% CI) of each other. In our current study, the interlaboratory average bias was 9.9%. Across all laboratories and assay manufacturers, CAP data reveals a much higher between-laboratory CV of 20% for CysC. Both are significantly above the IFCC recommended total allowable error "optimal" goal of 5.2% and "desirable" goal of 7.2%, which are based on CysC biologic variation specifications (21). It should be noted that the modified material used for proficiency testing samples may not be perfectly commutable across methods, and thus may cause extra bias or imprecision than would be seen with patient samples.

Interlaboratory assay biases have been extensively discussed with respect to serum creatinine and GFR estimation, and these have largely been attributed to differences in creatinine assay calibration (22). Assay calibration relates the signal observed in a sample to that expected when known concentrations are analyzed (22). For automated assays, calibrators are provided by the manufacturer, along with the reagents. Purchased control samples are then used to monitor the performance of the assay over time. Ideally, values assigned to calibrators by manufacturers are traceable to primary or secondary reference materials that have been assigned by reference methods. For analytes where these do not exist or have not yet been implemented, the concentration of a given calibrator is determined by the manufacturer. This is a common reason why there can be significant differences in a given concentration when measured by two different assays. International harmonization of creatinine calibration using assays traceable to the gold-standard isotope dilution mass spectrometry (IDMS) is now well underway (7), although it remains to be demonstrated to what extent this will resolve interlaboratory differences. The global implementation of IFCC reference procedures and standards for CysC is eagerly awaited (8–10). To date, the Siemens assay has not adopted the new IFCC standards, which, it should be noted, are not IDMS traceable. The IFCC standards have now been adopted in Europe.

In this study, notable differences with an impact on GFR estimation were demonstrated despite the utilization of the same assay produced by the same manufacturer. There are several possible explanations for these findings. First, the bias might be calibrator or reagent lot related. Evidence for differences or variations in calibrators or reagent lots over time is suggested by the differences in the Mayo Clinic equation bias noted between the current study (2 ml/min per 1.73 m^2 overestimation of mGFR) and our previous report (10 ml/min per 1.73 m^2 underestimation of mGFR), despite similar patient populations, the use of the same

mGFR protocol and cystatin measurement, occurring in the same laboratory (CHEO), using the same platform (15). This theory is also supported by recently reported data by Larsson *et al.* indicating a downward shift in Siemens assay calibration between 2006 and 2007 (23). International harmonization of CysC using reference standards should therefore, theoretically, lead to a reduction in interlaboratory bias.

Second, if it is assumed that the calibration set points for assays from the same manufacturer are the same over time, the noted differences could be attributed, to some extent, to the process and repetitive nature of assay calibration. Manufacturers produce calibrators in batches or lots that have a shelf life of 1 to 2 years. Since the size of each lot is limited, there are usually many lots in use around the world at the same time. A laboratory may go through three to six reagent lots and one to three calibration lots in a year in various combinations. Assay calibration is performed whenever there is a new reagent lot or when daily internal quality control monitoring indicates a trend or shift in assay performance. There is a small allowable SD in the manufacturing of each calibrator lot (1% to 2%) and each reagent lot (5%). This, combined with the small allowable variation in the performance of every instrument (5%), means that there is inherent variation associated with each calibration effort. Given this background, it is not surprising that a split sample may yield a different result when measured at different times or in different laboratories, despite the utilization of the same instrumentation, methods, or calibrators. The impact, if any, of the availability of higher order reference materials on errors inherent with calibration efforts is not clear.

Although the platforms used to measure CysC were different, both were nephelometers. It is possible that some of the observed interlaboratory differences could be explained by this factor (20). It is also possible that sample stability was affected during freezing and shipping, although evidence, to date, does not suggest that this is a significant issue (24).

This study reveals that correcting CHEO CysC values using the regression analysis between paired CysC values did not improve the accuracy of eGFR estimation using the Mayo Clinic eGFR equation. It should be emphasized that the correction factor is only relevant to this particular study and is not applicable to other centers wishing to "calibrate" their CysC values to the Mayo Clinic. GFR was measured using urinary iothalamate clearance for equation derivation at the Mayo Clinic (2) and by plasma ^{99m}Tc-DTPA clearance in the current study. Mounting evidence suggests that the various mGFR measurement techniques are not interchangeable and that they, too, have an inherent measurement uncertainty associated with their performance (25–27). A lack of improvement in performance of the creatinine-based MDRD study equation with calibration was also reported by Stevens *et al.* in over half of the studies included in an analysis of 10 different research and clinical populations (28), which could be attributed to either or a combination of differences in measurement technique or of residual bias inherent to calibration efforts.

That the bias of the Mayo equation was greater for the Mayo Clinic CysC as compared with CHEO CysC is somewhat unexpected and may be explained by drift in the CysC assay at the Mayo Clinic, differences in GFR measurement techniques between centers, and differences in characteristics of the transplant populations that could affect the non-GFR determinants of CysC.

An important clinical implication of this study is that the use of universal global decision (cutoff) concentrations without consideration of current method performance capabilities (standardization, imprecision at the specific concentrations, and potential endogenous interferences) and biologic variation factors has the potential to adversely affect patient care on an individual level. This is relevant as new cut offs for diagnosing and classifying chronic kidney disease are being developed by the Kidney Disease: Improving Global Outcomes (KDIGO) (29). It is also relevant when considering results of studies examining cardiovascular risk or renal risk based on specific CysC/estimated GFR values (30,31). Clinical and laboratory experts need to work collaboratively to provide and understand method performance capabilities and measurement-uncertainty information necessary to meet clinical requirements. In addition, clinicians should endeavor to follow laboratory values at a single laboratory over time to reduce the impact of interlaboratory variation.

Limitations of this study should be noted. The study cannot investigate whether the observed variation is due calibrator and reagent lot differences, as these are no longer available. In addition, sample analysis occurred over a 4-year period at CHEO, while analysis at the Mayo Clinic was done in batch mode on a single day. CAP data did not reveal significant drift in the CHEO assay over time (2008 to 2009; 2007 data were not available) compared with the peer mean (20). This, however, does not completely rule out the potential for drift over time. Also, the CHEO results would have a larger overall variation due to both random (between day) variation and systematic variation (calibrator and reagent lot variations), as compared with Mayo results, again because of the timetable of the measurements.

Conclusions

This study has demonstrated significant interlaboratory variations in CysC when measured in different clinical laboratories using reagents from the same manufacturer (Siemens). These differences impact on GFR estimation, particularly when this is well preserved. Correction of CHEO values the Mayo Clinic did not improve GFR estimation using a Mayo Clinic–derived estimation equation. While widespread implementation of international reference standards should minimize the manufacturer-to-manufacturer bias (systematic error), it will not eliminate variation due to imprecision (random error) and errors associated with the process and repetitive nature of calibration itself. Clinicians and researchers need to be mindful of the "measurement uncertainty" and biologic variation associated with quantitative results when interpreting a result against global "cut off" recommendations and against values assayed in different laboratories.

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Disclosures

None.

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