Evaluation of a new immunoassay for cystatin C, based on a double monoclonal principle, in men with normal and impaired renal function

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Abstract

Background
Elevated cystatin C in blood reflects impaired glomerular filtration rate (GFR), but current cystatin C assays, based on polyclonal antibodies and immunoturbidimetric or nephelometric detection, have several limitations. We evaluated a new immunoassay based on monoclonal antibodies in samples from patients with and without chronic kidney disease (CKD).

Methods
The study enrolled 170 men without known CKD (group A) and 104 men with CKD (group B). All patients were assessed with iohexol clearance, plasma creatinine, and plasma cystatin C by a conventional PETIA turbidimetric assay and by the new double monoclonal assay. In group A, three serial blood draws were performed at median intervals of four hours and 12 days between samples, to also allow assessments of the variability in cystatin C values with the new assay. Concordance correlation coefficients and the 95% limits of agreement were used to estimate the agreement of reciprocal cystatin C and reciprocal creatinine with iohexol clearance.

Results
Median iohexol clearance (ml/min/1.73 m²) was 81 (IQR 70, 92) in group A and 23 (IQR 16, 34) in group B. The concordance correlation with GFR for the new cystatin C assay compared to the established assay was similar in group A (0.441 vs 0.465), but higher in group B (0.680 vs 0.593). Cystatin C measured by both assays exhibited closer agreement with GFR than creatinine. The agreement between the two cystatin C assays was high, with concordance correlations of 0.815 in group A and 0.935 in group B. Compared to the conventional assay, the new assay tended to yield lower values of cystatin C at the low end of the range in both cohorts.
**A new immunoassay for cystatin C**

The new cystatin C assay exhibited small intra-individual variability across serial samples (coefficient of variation ≤6%).

**Conclusions**

In this first clinical evaluation, the new cystatin C assay performed similarly to the established PETIA turbidimetric assay in patients with normal GFR and better in patients with CKD. The new assay may offer an alternative to current commercial assays to detect and monitor impaired kidney function.

**Keywords:** Biomarkers, creatinine, cystatin C, glomerular filtration rate, iohexol clearance, renal function

**Short summary:** A new assay for cystatin C, based on double monoclonal antibodies and fluorescent detection, was compared to an established cystatin C assay and to creatinine in men with or without known chronic kidney disease. In terms of correlation with glomerular filtration rate measured by iohexol clearance, both cystatin C assays were superior to creatinine, and the new cystatin C assay showed closer agreement with true GFR than the established assay for men with chronic kidney disease. The intra-individual variability for the new assay was low (coefficient of variation ≤6%). The new cystatin C assay therefore has promise for clinical monitoring of renal function.
Introduction

Assessment of kidney function is important in clinical practice for determining overall health, selecting correct dosages for drugs cleared by the kidneys, preparing for therapeutic procedures, and detecting acute and chronic kidney disease (CKD). Assessment of kidney function is also important because impaired kidney function is strongly associated with cardiac diseases [1-3]. Epidemiologic studies have shown that CKD in many countries has a prevalence of more than 10% [4] and that prevalence is rising [5]. However, the best index of kidney function, glomerular filtration rate (GFR), cannot be measured easily in clinical practice. Indirect estimation of GFR from serum creatinine has long been the only method practical for routine clinical testing, but serum creatinine has several limitations. Therefore, the recommended method for evaluating kidney function is to use the serum creatinine value in a formula that estimates GFR.

Cystatin C has many characteristics of an ideal endogenous GFR marker [6]. Several investigations have indicated that serum cystatin C is as good as or better than serum creatinine as a GFR marker in patients with native kidneys, especially in patients with mild or moderate reduction of GFR [7-10]. In a meta-analysis, serum cystatin C was superior to creatinine in measuring renal function both in children and adults [7]. However, there are conflicting results, and some investigators have not found cystatin C to be advantageous [11]. One possible reason for conflicting results on the relationship between GFR and cystatin C is that factors other than GFR may affect serum cystatin C levels [11-13]. Another possible reason is that some of the studies used samples that had been stored for a long period of time, which may have caused analytical problems. Nevertheless, the promising results on estimating renal function from cystatin C have led to the development of commercial assays, sold as reagent kits, which use
A new immunoassay for cystatin C

polyclonal antibodies against cystatin C. Signal detection is based on light scattering in particle-enhanced turbidimetric and nephelometric immunoassays (PETIA and PENIA, respectively). However, the use of polyclonal antibodies can cause variation from batch to batch. In addition, the use of light scattering can render the assay susceptible to interfering factors such as lipemia, hemolysis, and bilirubinemia. This interference is recognized on turbidimetric detection principles. Samples that have been stored may have other drawbacks in turbidimetric assays, such as opalescence from precipitated lipids.

A newly designed assay for cystatin C based on two monoclonal antibodies and time-resolved fluorescent detection [14] is potentially less prone to these forms of interference. The use of monoclonal instead of polyclonal antibodies makes the assay more uniform and stable. The monoclonal antibody combination in the new assay gives fast binding and good correlation (R=0.949) with a PETIA assay from Roche. The new assay also demonstrates excellent linearity and a wide linear range in measuring cystatin C concentrations, covering all clinically relevant concentrations of cystatin C, and the assay measures highly similar concentrations of cystatin C in serum samples compared to anti-coagulated plasma samples [14]. The imprecision of the new assay is also suggested to be low with a total imprecision of less than 5.6% [14]. The new assay for cystatin C is sensitive and therefore uses extensive sample dilution, which diminishes possible interference from the sample, particularly interference caused by heterophilic antibodies. In addition, a wash step prior to detection in the new assay removes blood components that could potentially interfere with signal detection.
A new immunoassay for cystatin C

The aim of this study was to evaluate the performance of the new assay in patients with normal and reduced kidney function. Results of new and established cystatin C assays and creatinine assays were compared to plasma clearance of iohexol, which we considered the gold standard for assessment of GFR.
Subjects and Methods

Patients and blood collection

Two groups of patients were used in these analyses: those with normal renal function (group A) and those with slight to advanced renal dysfunction (group B). Group A consisted of 220 consecutive male patients without known renal disease seen at the Department of Urology, Skåne University Hospital, Sweden, during October 2001 and April 2004. Those missing data for iohexol clearance (n=7), cystatin C (n=9), or creatinine (n=34) were excluded, leaving 170 patients in group A available for analysis. In this group we collected three blood samples for analysis of variability. The median interval between Time I (before measurement of iohexol clearance) and Time II (immediately after iohexol clearance) was 4 hours (range: 3-7 hours); the median interval between Time I and Time III was 12 days (range: 6-38 days). The samples from times II and III were used only in the variability analysis.

Group B consisted of 108 patients with CKD enrolled at the Department of Nephrology and Transplantation, Skåne University Hospital, Sweden. During 2004 and 2006, at routine visits for GFR determination with iohexol clearance, consecutive men were invited to participate in the study, and all accepted the invitation. Those missing data for iohexol clearance (n=1) or cystatin C (n=3) were excluded, leaving 104 patients in group B available for analysis. The included men had been diagnosed with diabetic nephropathy (n=21), glomerulonephritis (n=22), non-specified renal disease (n=14), nephrosclerosis (n=15), or a group of miscellaneous renal diseases (n=10), or had renal transplants with stable but reduced renal function (n=22).
Blood was collected by venipuncture, centrifuged within less than one hour at 3,500 $g$ for 10 minutes and then immediately stored at –80°C pending analysis.

**Laboratory methods**

**Creatinine**

Plasma creatinine was determined by a creatininase-based procedure using the Hitachi Modular P analysis system (Roche, Basel, Switzerland, application 652). The total analytical (intra-assay + inter-assay) imprecision was 3.0% for a control sample at a concentration of 60 µmol/L and 1.4% for a control sample at 578 µmol/L. Reference range for men: 60–100 µmol/L.

**Cystatin C, conventional assay**

Plasma cystatin C was measured by a fully automated particle-enhanced immunoturbidimetric assay (PETIA) [15]. The reagents were obtained from DAKO (Dako A/S, Glostrup, Denmark) and the determination was performed on the Hitachi Modular P analysis system. The total analytical imprecision was 2.1% for a control sample at a concentration of 1.0 mg/L and 1.7% for a control sample at 4.0 mg/L. Reference range: 0.55–1.15 mg/L for age 1–50 years and 0.63–1.44 mg/L for age > 50 years [16].

**Cystatin C, new assay**

Cystatin C concentration of plasma samples was measured with an investigational immunoassay that uses monoclonal antibodies (HyTest, Turku, Finland) for both capture and detection and is based on time-resolved fluorometry [14]. The assay has a wash step prior to fluorescence measurement and it is performed in all-in-one dry-reagent wells, which contain all required assay
A new immunoassay for cystatin C

components, on an automated Aio! Immunoanalyzer (Innotrac Diagnostics, Turku, Finland).
One-hundred-fold dilution of plasma samples is used and the assay range covers undiluted
cystatin C concentrations from 0.1 to 20 mg/L. The bias compared to the conventional assay was
20% on average. The intra-assay imprecision percentages for the new method were 4.6 and 2.8,
the interassay imprecision percentages 1.9 and 3.2 and the total assay imprecision percentages
4.6 and 5.5 at 0.8 mg/L and 3.2 mg/L, respectively [14].

Measured glomerular filtration rate (iohexol clearance)

GFR was determined by measuring the plasma clearance of iohexol according to a one-
compartment model, where samples were taken after the distribution phase, in the assumed
monoexponential part of the plasma decay curve. Iohexol was analyzed by HPLC technique
[17,18].

Statistical methods

To facilitate comparisons between cystatin C, creatinine, and iohexol measurements, we used
1/cystatin C (L/10^{-1} g) and 1/creatinine (L/10^{-2} mol). These conversions were used for all
analyses and in all figures. Iohexol clearance (ml/min/1.73 m^2) was considered the true GFR
(gold standard). To quantify the amount by which values of 1/cystatin C and 1/creatinine differed
from GFR, we calculated the concordance correlation coefficient and the 95% limits of
agreement. The concordance correlation coefficient combines measures of both precision and
accuracy to determine how far the observed data deviate from the line of perfect concordance
(i.e. the line at 45 degrees on a square scatterplot). The 95% limits of agreement represent the
region within which 95% of the differences between measurements are expected to lie. For illustrative purposes, we created scatter plots of all patients’ reciprocal cystatin C or creatinine and GFR measurements. To describe the variability of cystatin C across the three time points, we calculated the intra-individual coefficients of variation. These coefficients of variation were calculated as the ratio of the standard deviation to the mean within each individual and are expressed as percentages. All analyses were conducted separately by cohort because group A and B represent distinct patient populations. All statistical analyses were conducted using Stata 10.0 (Stata Corp, College Station, TX).

Ethical considerations

The study was approved by the regional ethics committee at Lund University, Sweden, LU 587-00, and all subjects provided written consent to participate in the study and to allow retrieval of information from medical records.
Results

Patient characteristics and renal function measurements for the two groups are shown in Table 1. Median age was 65 years in group A (patients without known CKD) and 57 years in group B (patients with CKD). Overall, cystatin C measurements were lower and more homogeneous in group A than in group B. Iohexol clearance measurement showed a median GFR of 81 (range of 29-119, IQR 70-92) ml/min/1.73 m$^2$ in group A. This “normal renal function” group included 49 participants (29%) with no CKD or stage 1 CKD, 104 (61%) with stage 2, 16 (9%) with stage 3, and 1 (0.6%) with stage 4. In group B, median GFR measured from iohexol clearance was significantly lower at 23 (range 8-83, IQR 16-34) ml/min/1.73 m$^2$. In this group, 4 patients (4%) had stage 2 CKD, 29 (28%) had stage 3 CKD, 51 (49%) had stage 4, and 20 (19%) had stage 5.

Table 2 summarizes the concordance correlation coefficients and limits of agreement for 1/cystatin C and 1/creatinine compared to iohexol clearance. Overall, the agreement between cystatin C and the true GFR was higher in patients with renal disease (group B) than in those with normal renal function (group A). When the two cystatin C assays were compared with iohexol clearance in group A, the conventional assay had a slightly higher concordance correlation than the new assay, whereas in group B the new assay had the higher concordance correlation. Both cystatin C assays tended to overestimate true GFR in both cohorts (Figures 1 and 2, Table 2), but both cystatin C assays exhibited better agreement with iohexol clearance than did creatinine in group A and B (Figures 1-3). The lowest concordance correlation was shown between 1/creatinine and iohexol clearance. However, at low levels of renal function (GFR<30), creatinine appeared to have better agreement with GFR (Figure 3).
Figure 4 shows the correlation plots for reciprocal plasma cystatin C values determined with the conventional and new cystatin C assays. The agreement between the two assays for cystatin C was high, with concordance correlation 0.815 in group A and 0.935 in group B. The new assay, compared to the conventional assay, tended to yield slightly higher values at the high end of 1/cystatin C (Figure 4). The increasing difference between the two methods for cystatin C at higher GFR is also seen in Bland Altman plots for groups A and B (Figures 5a and 5b, respectively).

We summarized the variation of reciprocal cystatin C levels across different time points as the coefficient of variation measured with the new assay using serial blood samples obtained at three separate time points from the men in group A (Table 3). The intra-individual coefficients of variation between the time points were low and relatively consistent. The mean (SD) variation in cystatin C as well as in reciprocal cystatin C was 6% (4%) between Time 1 and 3 and was 5% (4%) both between Time 1 and Time 2 and between Time 2 and Time 3.
Discussion

Many studies have shown advantages of cystatin C over creatinine as a GFR marker. Although some studies have not shown such an advantage, almost none have shown better diagnostic performance for creatinine than for cystatin C. However, the established cystatin C assays have some methodologic weaknesses, such as susceptibility to interference from substances in blood, especially in samples that have been stored. Some of these methodologic weaknesses of the standard cystatin C assays are addressed by a recently developed, highly sensitive assay for plasma cystatin C that uses two monoclonal antibodies [14], and the current report is the first clinical evaluation of this new assay. Here, we demonstrate that this new assay performs well compared to the conventional immunoturbidimetric assay (PETIA). In particular, we have demonstrated that the new assay performs better than the old assay in male patients with chronic kidney disease. Cystatin C, with both assays, is superior to creatinine in correlation with iohexol clearance.

From our findings, that reciprocal cystatin C levels exhibited much better agreement with true GFR than reciprocal creatinine levels, we anticipate that subsequent development and use of an eGFR formula that is based on cystatin C, or possibly could incorporate both creatinine and cystatin C, would substantially improve the agreement with measured GFR. However, we did not use the published cystatin C-based eGFR-formula, which was developed for use with the conventional assay [19], as the accuracy of the cystatin C-based eGFR formula would be biased in favor of the conventional assay and would not permit a fair comparison with the new cystatin C assay.
The concordance correlation coefficient with measured GFR was higher in the group of men with impaired renal function for all assessments, both cystatin C assays and creatinine. The reason for this is not completely clear, but formulas for eGFR also perform better in decreased GFR ranges than in the normal range [20]. It is well known that MDRD eGFR underestimates GFR in the normal range by up to 30%, but only by 6% in patients with chronic kidney disease.

With the new cystatin C assay, intra-individual variability between time points, described as coefficients of variation was found to be small and clinically insignificant. This low short-term variability reflects not only the stability of the new assay as a diagnostic test and strengthens the precision, reliability, and clinical utility of cystatin C as a GFR marker, but also suggests strongly that the intra-individual variation in cystatin C levels in blood among subjects with normal or only moderately impaired GFR is very small. Although the analytical imprecision of the new method is higher than that of the conventional method, the measured intra-individual variation, which sets the actual variability of the test results, was higher than the imprecision of the new cystatin C method.

In this study, the established cystatin C assay used for comparison was a PETIA. A recent investigation has suggested that PENIA may be more reliable than PETIA [21], which could limit the conclusion of our study. However, numerous studies with PETIA have shown a good correlation with GFR measured by standard methods [7,22], and we also had true GFR values included in this study. Nevertheless, our results need to be confirmed in relation to a PENIA assay. Another possible limitation in comparative studies is that the conventional assays for cystatin C are not standardized. The lack of an international calibrator complicates the estimation
of agreement of cystatin C values with true GFR since systematic differences in cystatin C levels between different assay technologies are known to exist and were seen in this study, especially at high levels of cystatin C. The difference between the conventional and the new method is interesting, and it actually seems that compared to the iohexol GFR values, 1/cystatin C measured with the new assay shows a higher parallelism to the line of identity than does 1/cystatin C measured with the conventional assay. The non-linear association between the cystatin C assays could be related to the fact that the linear range of the conventional method is narrower compared to other commercial cystatin C methods.

A strength of our study is the wide range of GFR encompassed by the study groups with and without known CKD. It is both a strength and limitation in our study that we assessed male patients only. The advantage of examining renal function according to sex is that we do not have to account for known differences in creatinine between males and females. However, as a limitation, these results need to be confirmed in a female population, which is a future project of ours. If similar data showing high concordance correlation between the new cystatin C assay and iohexol clearance can be replicated also in women, it would be appropriate to develop a formula for eGFR based on this new cystatin C assay. The ongoing development of an international calibrator will further strengthen cystatin C as a GFR marker.

**Conclusion**

In this first clinical evaluation, the new assay for cystatin C shows good agreement with the established conventional PETIA turbidimetric assay. In patients with CKD it showed better
A new immunoassay for cystatin C

agreement with true GFR than PETIA. These results imply that the new assay may be an alternative to the present commercial assays.
Acknowledgements

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Transparency declaration

None to be declared.

Conflict of Interest Statement

We declare that the results presented in this paper have not been published previously in whole or part, except in abstract format.
References


A new immunoassay for cystatin C


Table 1. Summary of patient characteristics. All values are median (IQR).

<table>
<thead>
<tr>
<th></th>
<th>Group A (Normal Renal Function)</th>
<th>Group B (Chronic Kidney Disease)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=170</td>
<td>N=104</td>
</tr>
<tr>
<td>Age (years)</td>
<td>65 (60, 71)</td>
<td>57 (47, 70)</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>78 (70, 85)</td>
<td>224 (160, 295)</td>
</tr>
<tr>
<td>Conventional cystatin C (mg/L)</td>
<td>1.08 (1.01, 1.16)</td>
<td>2.63 (2.11, 3.40)</td>
</tr>
<tr>
<td>New cystatin C (mg/L)</td>
<td>1.03 (0.910, 1.14)</td>
<td>2.42 (1.91, 3.02)</td>
</tr>
<tr>
<td>Iohexol clearance (mL/min/1.73m²)</td>
<td>81 (70, 92)</td>
<td>23 (16, 34)</td>
</tr>
</tbody>
</table>
### Table 2. Agreement of reciprocal creatinine and cystatin C values with measured GFR. (Average difference is the estimated GFR – true GFR [iohexol clearance].)

<table>
<thead>
<tr>
<th></th>
<th>Group A (Normal Renal Function)</th>
<th>Group B (Renal Disease)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (IQR)</td>
<td>Concordance Correlation Coefficient</td>
</tr>
<tr>
<td>1/Creatinine (L/10⁻² mol)</td>
<td>128 (118, 143)</td>
<td>0.161</td>
</tr>
<tr>
<td>1/Conventional cystatin C assay (L/10⁻¹ g)</td>
<td>93 (86, 99)</td>
<td>0.465</td>
</tr>
<tr>
<td>1/New cystatin C assay (L/10⁻¹ g)</td>
<td>97 (88, 110)</td>
<td>0.441</td>
</tr>
</tbody>
</table>
Table 3. Variation of cystatin C levels between three time points. All values were measured with the new assay in group A.

<table>
<thead>
<tr>
<th>Time Points</th>
<th>Mean Intra-individual Coefficient of variation, % (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time 1, Time 2</td>
<td>5 (4)</td>
</tr>
<tr>
<td>Time 1, Time 3</td>
<td>6 (4)</td>
</tr>
<tr>
<td>Time 2, Time 3</td>
<td>5 (4)</td>
</tr>
</tbody>
</table>
Legends to Figures

Figure 1. Calibration plot of 1/cystatin C values from the conventional assay as compared to iohexol clearance (gold standard). The black line represents perfect agreement. Patients with chronic kidney disease (group B) are represented by solid circles; patients without diagnosis of kidney disease (group A) are represented by hollow circles.

Figure 2. Calibration plot of 1/cystatin C values from the new assay as compared to iohexol clearance (gold standard). The black line represents perfect agreement. Patients with chronic kidney disease (group B) are represented by solid circles; patients without diagnosis of kidney disease (group A) are represented by hollow circles.

Figure 3. Calibration plot of 1/creatinine as compared to iohexol clearance (gold standard). The black line represents perfect agreement. Patients with chronic kidney disease (group B) are represented by solid circles; patients without diagnosis of kidney disease (group A) are represented by hollow circles.

Figure 4. Calibration plot of conventional cystatin C assay as compared to the new cystatin C assay. The black line at 45° represents perfect agreement. Patients with chronic kidney disease (group B) are represented by solid circles; patients without diagnosis of kidney disease (group A) are represented by hollow circles.

Figure 5. Bland Altman plots for the two measures of cystatin C, (a) for group A and (b) for group B. The x-axis is the average of the two 1/cystatin C measures and the y-axis is the
difference (conventional method – new method). The horizontal lines represent the average difference and the 95% limits of agreement.
A new immunoassay for cystatin C

Picture 2

[Graph showing the relationship between iohexol clearance (mL/min/m²) and 1/cystatin C in a new assay (L/10⁸-1 g).]
A new immunoassay for cystatin C

Picture 3

![Graph showing the relationship between iohexol clearance (mL/min/1.73m²) and 1/creatinine (L/10⁻² mol).]
A new immunoassay for cystatin C

Picture 4
Picture 5a

A new immunoassay for cystatin C

The scatter plot shows the difference (Conventional - New Cystatin C Assay) against the average of the Conventional and New 1/Cystatin C Assay. The y=0 line represents the line of perfect average agreement.
A new immunoassay for cystatin C

Picture 5b

The graph shows the difference between the conventional and new cystatin C assays plotted against the average of the two assays. The horizontal line at y=0 represents the line of perfect average agreement. The distribution of points suggests that there is variability in the agreement between the two methods, with some deviations from the line of perfect agreement.