

# Clinical Usefulness of Cystatin C for the Estimation of Glomerular Filtration Rate in Type 1 Diabetes

Reproducibility and accuracy compared with standard measures and iothexol clearance

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**OBJECTIVE** — Assessment and follow-up of early renal dysfunction is important in diabetic nephropathy. Plasma creatinine is insensitive for a glomerular filtration rate (GFR) >50 ml/min and creatinine clearance is unwieldy and subject to collection inaccuracies. We aimed to assess the reproducibility, reliability, and accuracy of plasma cystatin C as a measure of GFR ranging from normal to moderate impairment due to type 1 diabetes in the presence of a normal plasma creatinine concentration.

**RESEARCH DESIGN AND METHODS** — A sensitive immunoturbidimetric cystatin C assay was examined in 29 subjects with type 1 diabetes and 11 nondiabetic subjects. Duplicate measurements of the following were collected from each subject, 2 weeks apart: cystatin C, enzymatic plasma creatinine, 24-h creatinine clearance, GFR estimated from plasma creatinine by the Cockcroft-Gault equation, and iothexol clearance as a gold standard.

**RESULTS** — Iothexol clearance ranged from 35 to 132 ml · min<sup>-1</sup> · 1.73 m<sup>-2</sup>. Plasma cystatin C compared well with the other clinically used tests. The reliability of cystatin C, as assessed by the discriminant ratio, was superior to creatinine clearance (3.4 vs. 1.5,  $P < 0.001$ ) and the correlation of cystatin C with iothexol clearance ( $R_s -0.80$ ) was similar to that of creatinine clearance ( $R_s -0.74$ ) and superior to that of plasma creatinine and the Cockcroft-Gault estimate ( $R_s -0.54$  and 0.66, respectively). Duplicate estimations were used to provide an unbiased equation to convert plasma cystatin C to GFR.

**CONCLUSIONS** — Based on this study, cystatin C is a more reliable measure of GFR than creatinine clearance, is more highly correlated with iothexol clearance than plasma creatinine, and is worthy of further investigation as a clinical measure of GFR in type 1 diabetes.

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**R**enal failure develops in  $\leq 30\%$  of people with type 1 diabetes; however, our ability to assess renal function is poor in early diabetic nephropathy, when active management is important. Serum creatinine level, the most commonly used surrogate measure of glomerular filtration rate (GFR), does

not increase until renal function decreases to  $\sim 50\%$  of its normal value; its excretion rate varies with age, sex, physical exercise, and lean body mass (1,2). The population variance of serum creatinine level is large, making it a poor measure for comparison with a reference range. Creatinine clearance, measured from a 24-h urine collection and a concurrent plasma creatinine concentration, is unwieldy and often inaccurate but is widely used in clinical practice (3). ‘Gold standard’ tests such as clearance methods using radioisotopes (such as <sup>51</sup>Cr-labeled EDTA, <sup>99m</sup>Tc-labeled DTPA, and <sup>125</sup>I-labeled iothalamate) or iothexol (4,5) are too cumbersome to use in the clinic setting (3). A more precise and accurate marker of GFR as an assessment of renal function would be clinically useful.

Cystatin C has been proposed as a good marker of GFR (6,7), particularly in patients with moderate to severe renal impairment. It is a nonglycosylated protein belonging to the superfamily of cysteine protease inhibitors (8). Plasma cystatin C fulfills a number of the criteria that would make it suitable as a marker of GFR (3); it has a low molecular weight ( $M_r = 13,359$ ) (8) and is therefore freely filtered at the glomerular membrane. It is reabsorbed and catabolized by renal tubular cells (9). Cystatin C is produced by all nucleated cells; its rate of production is constant and the gene is of the housekeeping type (10). The production of cystatin C is not altered by inflammatory conditions (11), is not related to lean muscle mass (12), and does not have a circadian rhythm (13). The function of cystatin C seems to be to protect connective tissue from destruction by intracellular enzymes (14). It may also have an antibacterial or antiviral function (14). In stored plasma, its concentration is stable, probably due to the high concentration of other proteinase inhibitors (14).

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**Abbreviations:** CV, coefficient of variation; DR, discriminant ratio; GFR, glomerular filtration rate; GFR-CG, GFR calculated by the Cockcroft-Gault method; GFR-IO, GFR as measured by iothexol clearance; SDU, underlying between-subject SD; SDW, within-subject SD.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

Therefore, cystatin C would seem to be a promising candidate as a marker of GFR in type 1 diabetes. However, its role has not been fully examined in this context. However, widespread clinical use of cystatin C as a marker of GFR is limited by an absence of data on its inter- and intra-individual variation. Therefore, we set out to assess the reproducibility and reliability of plasma cystatin C in patients with type 1 diabetes in whom GFR ranged from normal to moderate impairment. Participants were selected to include healthy nondiabetic subjects and subjects with type 1 diabetes with normal plasma creatinine concentrations and a range of urinary protein concentrations. Plasma cystatin C was compared with commonly used clinical measures of GFR (creatinine clearance, serum creatinine level, and GFR estimated by the Cockcroft-Gault formula) (15) and a reference method, iohexol clearance, by assessing the inter- and intra-individual variation and the intercorrelation of the tests.

## RESEARCH DESIGN AND METHODS

### Subjects

A total of 40 volunteers with plasma creatinine concentrations within the local normal reference range (70–150  $\mu\text{mol/l}$ ) were recruited for the study: 11 nondiabetic subjects and 29 healthy type 1 diabetic subjects with varying degrees of albuminuria (<20 to >200 mg/l) from the Oxford Diabetes Clinic. The study was approved by the Central Oxfordshire Research Ethics Committee. All subjects gave their written informed consent.

### Protocol

Subjects were studied on two occasions over a 4-week period. We have used GFR as measured by iohexol clearance (GFR-IO) as a 'gold standard' measure of GFR, as previously recommended (4,5). At each visit, a 5-ml intravenous bolus of iohexol (omnipaque, 300 mg I/ml) was administered. Venous blood was drawn at 0, 120, 150, 180, 240, and 300 min after iohexol injection. Iohexol clearance, plasma cystatin C level, enzymatic plasma creatinine concentration, and 24-h urinary creatinine clearance (expressed per  $1.73 \text{ m}^2$ ) were assessed.

Plasma cystatin C was measured by an optimized immunoturbidimetric method (Dako, High Wycombe, U.K.)

and evaluated using a Cobas FARA centrifugal analyzer (Roche Diagnostics, Lewes, U.K.) (16). Assay coefficient of variation (CV) was 3.9% at 0.72 mg/l and 1.3% at 5.29 mg/l, with a sensitivity of 0.4 mg/l. Plasma and urine creatinine were measured using a specific enzymatic assay on a Bayer Axon analyzer (Bayer Diagnostics, Newbury, U.K.) (17). Assay CV was 6.7% at 69  $\mu\text{mol/l}$  and 2.3% at 478  $\mu\text{mol/l}$ , with a sensitivity of 10  $\mu\text{mol/l}$ . Iohexol level was determined using high-performance liquid chromatography (Waters autosampler, pump, and UV detector set at 254 nm and a  $250 \times 4.6 \text{ mm}$  column of 5- $\mu\text{m}$  Phenosphere ODS2 from Phenomenex, Macclesfield, U.K.) with an established method (4). Assay CV was 5.8% at 13.4  $\mu\text{mol/l}$  and 1.6% at 64.9  $\mu\text{mol/l}$ , with a sensitivity of 3  $\mu\text{mol/l}$ . Specimens for creatinine and cystatin C were stored at  $-20^\circ\text{C}$  and analyzed as a batch on a single occasion. Iohexol assays were analyzed in a series of batches.

### Calculations and statistics

GFR-IO was calculated using the rate constant derived from the linear portion of the graph of the natural logarithm of the iohexol concentration against time (120, 180, 240, and 300 min). The formula of Bröchner-Mortensen was used to calculate the GFR and was then expressed per  $1.73 \text{ m}^2$ . GFR was also estimated using the Cockcroft-Gault formula (GFR-CG) from plasma creatinine, age, weight, and body surface area (15) and expressed per  $1.73 \text{ m}^2$ . In the literature, the units of measurement for cystatin C have uniformly been mg/l, and for this reason, these units have been used in the present study. Cystatin C has a molecular weight of 13,359 Da; therefore, 1 mg/l is equivalent to 74.9 nmol/l, in SI units.

To reflect the clinical situation, but to include all available data, Spearman rank correlations were calculated on single tests, using the first replicate of test 1 versus the second replicate of test 2 and vice versa. However, for scattergrams and for determining underlying relationships between variables, the means of the two determinations were used, because these represent the most precise estimation of variables for each individual subject.

The within-subject SD (SDW) for the group as a whole was calculated as the square root of the mean of the individual subject variances for each measure, after

checking for homoscedasticity using Bland-Altman plots (18).

The CV was calculated as the ratio of the SDW to the mean value and expressed as a percentage, but this measure has inherent limitations (19). Test reliability (i.e., the relationship of the between-subject to the within-subject variation) was compared using the discriminant ratio (DR). The DR was calculated as the ratio of the underlying between-subject SD (SDU) divided by the SDW. SDU was estimated as the square root of ( $\text{SDB}^2 - \text{SDW}^2$ ), where SDB was the measured between-subject SD. Differences between DRs may be compared statistically (20).

Because linear regression underestimates the relationship between two imprecise variables by assuming the independent variable to be precise, the underlying line of equivalence between measures taking the imprecision of both into account was calculated using the 'PW' method (perpendicular least-squares method, weighted for imprecision in the variables) (21).

The study size (40 subjects each with duplicate tests) had a 90% power to detect a 1.5-fold difference between the DRs of two tests at a two-tailed significance of  $P = 0.05$ . This was based on the standard error of  $\ln(\text{DR})$  having a near-normal distribution and approximate constant value of 0.13 for values of DR between 2.5 and 6.0 (20).

**RESULTS**— Subject characteristics are presented in Table 1.

Mean of duplicate plasma creatinine concentrations, 24-h creatinine clearance values, plasma cystatin C concentrations, and GFR-IO from the two visits are presented in Table 2 for the nondiabetic and diabetic subjects. Diabetic subjects had creatinine concentrations within the local reference range with creatinine clearance and GFR-IO ranging from normal to moderate impairment.

Plasma creatinine and cystatin C were inversely related to the direct measure of GFR using iohexol. The Spearman rank correlation of cystatin C with iohexol clearance ( $-0.80, P < 0.001$ ) was similar ( $P = 0.19$ ) to that of creatinine clearance ( $0.74, P < 0.001$ ) and superior to that of plasma creatinine ( $-0.54, P < 0.001$ ) and the Cockcroft-Gault estimate ( $0.68, P < 0.001$ ) ( $P < 0.001$  and  $P = 0.03$  versus Rs for iohexol clearance, respectively).

Assessment of reproducibility and

Table 1—Baseline characteristics of patients

	No diabetes	Type 1 diabetes		
		No proteinuria	Microalbuminuria	Proteinuria
Number of subjects	11	10	10	9
Male:female	6:5	5:5	5:5	5:4
Age (years)	43 ± 11	46 ± 13	60 ± 10	54 ± 15
Duration of diabetes (years)	—	24 ± 14	28 ± 13	26 ± 13
BMI (kg/m <sup>2</sup> )	24.3 ± 2.9	25 ± 3	29.4 ± 5.3	27.7 ± 3.5
HbA <sub>1c</sub> (%)	—	7.9 ± 1.2	9.1 ± 1.6	10.3 ± 1.6

Data are means ± SD.

discrimination were made on the combined nondiabetic and diabetic groups. Bland-Altman plots (difference between two determinations versus mean) for the various measures are shown in Fig. 1. The within-subject variation of cystatin C was homoscedastic (uniform over the range). Means, within- and between-subject SDs, CVs, and DRs are illustrated in Table 2. GFR-IO had the lowest CV and the greatest DR. The simple estimates of renal function, plasma creatinine, GFR-CG, and cystatin C all had similar CVs and DRs and were superior in both respects to creatinine clearance (Table 2).

Analysis of the data of the type 1 diabetic subjects alone changed estimates of CVs and DRs by <1 and 12%, respectively, for all tests except 24-h creatinine clearance, for which reproducibility was relatively poor in the nondiabetic subjects. However, this had no material effect on the significance of the differences between the tests.

Because indirect measures of GFR are inversely related to clearance rate, the reciprocal of cystatin C was plotted against GFR-IO; the relationship was found to be

linear (Fig. 2). To allow the calculation of GFR-IO (ml · min<sup>-1</sup> · 1.73 m<sup>-2</sup>) from cystatin C concentrations (mg/l), the linear equation of equivalent values, calculated by the PW method (21) was as follows:

$$\text{GFR-IO} = (87.1/\text{plasma cystatin C}) - 6.87$$

The relationship between cystatin C and iohexol clearance in the nondiabetic subjects was similar to that in the diabetic subjects (Fig. 2).

**CONCLUSIONS**— As far as the authors are aware, this is the first published study simultaneously examining the relative precision and the correlation of plasma cystatin C with routine clinical measures and a reference method (GFR-IO). It is also the first to address the specific context of type 1 diabetes, in which current methods are insensitive at detecting early nephropathy.

Cystatin C proved more reliable than the 24-h creatinine clearance and was comparable to plasma creatinine and the Cockcroft-Gault estimation. It had a

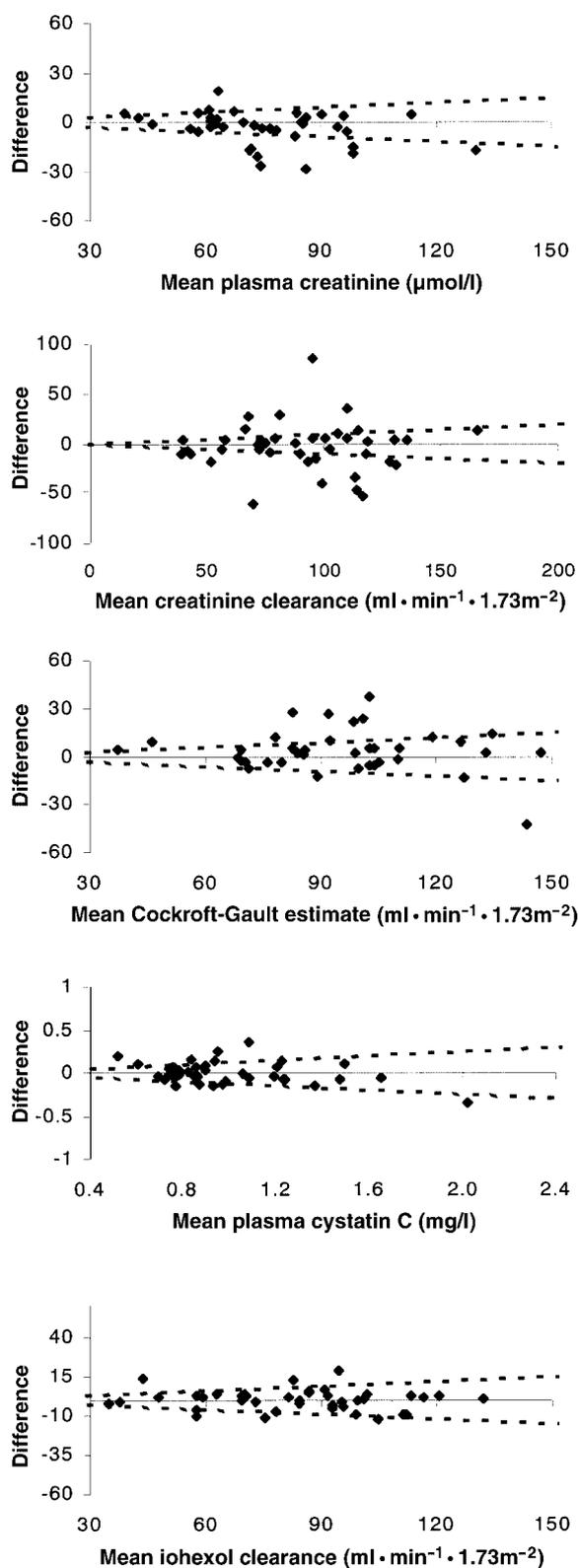
higher correlation with the 'gold standard' test than plasma creatinine and the Cockcroft-Gault estimation.

The study performed duplicates of each test in a group of subjects spanning a clinically appropriate range of renal function, allowing assessment of the reproducibility and reliability of cystatin C and the other measures. Reproducibility is assessed by the SDW; this is specific to a test and to the units in which it is measured. Reliability, on the other hand, relates the imprecision or the 'noise' of a test to the range of values to which it is applied. It can be expressed as the discriminant ratio, the ratio of the between-subject SD to the SDW; this is dimensionless and can be compared between tests (20). Accuracy was assessed by determining correlations with the reference method, performed with single determinations to be applicable in the clinical setting. It was also possible to establish an unbiased equation relating cystatin C concentrations to iohexol clearance, based on the mean of duplicate determinations to provide the best estimation of the underlying relationship. The simultaneous assessment of reliability allowed correction of the regression dilution inherent in the standard least-squares method, which assumes perfect precision in the independent variable (22). This has not been addressed in previous studies of cystatin C (6,12,23–29). Receiver operator curves were not used here because it is an inappropriate method for the comparison of continuous variables, although it has been used by others in the assessment of cystatin C (6,26,28–30).

Because we were interested in the early nephropathy of type 1 diabetes, we

Table 2—Measures of reproducibility

	Mean (range)	Within-subject SD	Between-subject SD	Within-subject CV	Discriminant ratio (95% CI)	Discriminant ratio: P versus iohexol clearance	Discriminant ratio: P versus cystatin C
Plasma creatinine (μmol/l)	75 (39–131)	7.4	19.0	9.9%	2.5 (1.9–3.2)	<0.001	0.065
Creatinine clearance (ml · min · 1.73 m <sup>-2</sup> )	91 (40–166)	17.6	29.6	19.2%	1.5 (1.1–2.1)	<0.001	<0.001
GFR-CG (ml · min · 1.73 m <sup>-2</sup> )	102 (37–203)	10.1	33.7	9.9%	3.3 (2.5–4.2)	<0.001	0.43
Cystatin C (mg/l)	0.98 (0.52–2.03)	0.1	0.3	8.9%	3.4 (2.6–4.4)	<0.001	—
Iohexol clearance (ml · min · 1.73 m <sup>-2</sup> )	84 (35–132)	4.7	23.2	5.6%	4.9 (3.9–6.3)	—	—



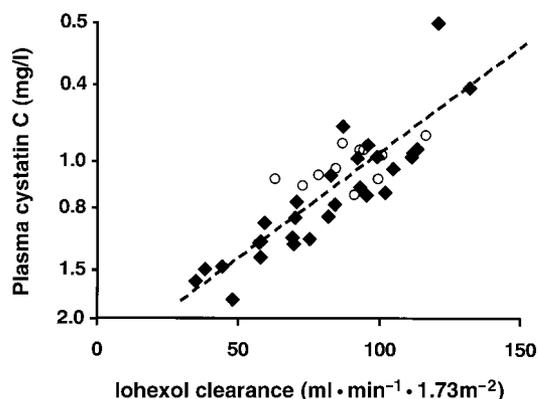
**Figure 1**—Bland-Altman plots illustrating within-subject variability of cystatin C, plasma creatinine concentration, creatinine clearance, Cockcroft-Gault estimation of GFR, and iohexol clearance. The vertical axis represents the difference of two duplicate tests (second minus first). In each case, the ranges of the vertical and horizontal axes are equal. The dotted lines represent 10% deviations above and below the mean value.

selected subjects to provide a range of GFRs in the presence of a 'normal' plasma creatinine. In the absence of a prior determination of GFR, we recruited subjects with type 1 diabetes and a range of urinary albumin excretion rates. Because we could not exclude abnormalities of GFR in diabetic patients with normal albumin excretion, we chose to include a subgroup of healthy nondiabetic subjects to ensure that the spectrum of GFR we studied ranged from normal to moderate impairment. The data presented in Fig. 2 supports that the two groups behaved similarly with respect to cystatin C as a measure of GFR. The two subjects with the highest iohexol clearance were, in fact, diabetic subjects and might be regarded as 'hyperfiltrators' (31).

The principal drawback of plasma creatinine concentration as a measure of creatinine clearance is that, because its concentration is influenced by several covariates, a significantly impaired GFR may be compatible with a creatinine concentration within the normal population range. A plasma marker less subject to such influences, by having a correspondingly narrower normal population range, would allow easier identification of individuals with an abnormal GFR. Although our study includes too few normal population members to define a normal range, an indication that it might be useful in this regard comes from the fact that, of eight diabetic patients with GFR-IO less than the minimum value in the nondiabetic subjects, only two were so identified using plasma creatinine, whereas all eight subjects had cystatin C concentrations above the nondiabetic range. Again, measured against external reference ranges (32), 14 diabetic subjects had an increased cystatin C concentration compared with only 1 subject with an increased creatinine concentration.

The strengths of this study are the detailed and simultaneous evaluation of reproducibility, reliability, and accuracy in comparison with a 'gold standard' test of GFR in an adequately powered, targeted study. The methodology used allows appropriate statistical comparisons of reliability in contrast to most other previous evaluations of cystatin C, which have used single determinations only (6,12,23–29). It also provides an unbiased conversion algorithm between plasma cystatin C and iohexol clearance.

The limitations of this study are its



**Figure 2**—Scattergram of the reciprocal of cystatin C versus iohexol clearance expressed per 1.73 m<sup>2</sup>. Each point represents the mean of two duplicate assessments. Open circles represent nondiabetic subjects and solid diamonds represent diabetic subjects. The dashed line represents the unbiased line of equivalence: the estimate of the underlying linear relationship between the two values, calculated as detailed in the text.

limited size and its cross-sectional nature. The size of the study was determined by its power to detect expected differences in reliability (90% power to detect a 1.5-fold difference in DR at  $P = 0.05$ ), but full assessment of the clinical applicability of cystatin C would require a larger prospective study including a wide variety of patients followed over a period sufficient to detect changes in renal function.

In patients with type 1 diabetes, cystatin C is a promising new marker for early renal dysfunction (7). In a thorough comparison study, we have shown that cystatin C has advantages over routine clinical measures of renal function, being more accurate than plasma creatinine and the Cockcroft-Gault estimation of creatinine clearance and more reliable than 24-h creatinine clearance. This study also provides an equation for converting plasma cystatin C to GFR. Larger-scale prospective clinical studies are now needed to confirm its position in routine clinical practice.

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