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Diagnostic potential of simplified methods for measuring glomerular filtration rate to detect chronic kidney disease in dogs

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Abstract

Background: Glomerular filtration rate (GFR) is the most sensitive indicator of initial renal function decline during chronic kidney disease (CKD), but conventional protocols for measuring GFR are labor-intensive and stressful for the dog.

Objectives: To assess the diagnostic potential for detecting CKD with simplified GFR protocols based on iohexol plasma clearance.

Animals: Seventeen CKD-positive and 23 CKD-negative dogs of different breeds and sex.

Methods: Prospective nonrandomized study. Plasma iohexol was measured 5, 15, 60, 90, and 180 minutes after injection. Glomerular filtration rate was calculated using 5 samples (GFR₅) or simplified protocols based on 1, 2, or 3 samples. The GFR₅ and simplified GFR were compared by Bland-Altman and concordance correlation coefficient (CCC) analysis, and diagnostic accuracy for CKD by receiver operating characteristic curves. A gray zone for each protocol was bounded by the fourth quartile of the CKD-positive population (lower cutoff) and the first quartile of the CKD-negative population (upper cutoff).

Results: All simplified protocols gave reliable GFR measurements, comparable to reference GFR₅ (CCC >0.92). Simplified protocols which included the 180-minutes sampling granted the best GFR measure (CCC: 0.98), with strong diagnostic potential for CKD (area under the receiver operating characteristic curve ± SE: 0.98 ± 0.01). A double cutoff including a zone of CKD uncertainty guaranteed reliable diagnosis outside the gray area and identified borderline dogs inside it.

Conclusions: The simplified GFR protocols offer an accurate, hands-on tool for CKD diagnosis in dogs. The gray zone might help decision-making in the management of early kidney dysfunction.

Abbreviations: AUC, area under the curve; AURC, area under the ROC curve; BSA, body surface area; BW, body weight; CCC, concordance correlation coefficient; CKD, chronic kidney disease; CKD−, CKD negative; CKD+, CKD positive; CV, coefficient of variation; estVd, estimated Vd; GFR, glomerular filtration rate; GFR₁, single-sample GFR; GFR₂, 2-sample GFR; GFR₃, 3-sample GFR; GFR₅, 5-sample GFR; HPLC-UV, high-performance liquid chromatography-ultraviolet; IRIS, International Renal Interest Society; ROC, receiver operating characteristic; RS-GFR, reduced-sampling GFR; Scr, serum creatinine; SS-GFR, single-sampling GFR; Vd, volume of distribution.
1 | INTRODUCTION

Chronic kidney disease (CKD) is an irreversible, progressive deterioration of renal function, with a poor prognosis.1 Dogs with CKD can be classified in stages according to the International Renal Interest Society (IRIS) system, which is based on the concentration of serum creatinine (Scr), with substaging based on the urinary protein/urinary creatinine ratio and blood pressure. However, Scr sensitivity is low and shows alterations only after two-thirds of functional renal mass have been lost, so it cannot detect early kidney dysfunction.2,3 Glomerular filtration rate (GFR) is currently considered the best indicator of renal function and the most sensitive and specific test for early diagnosis of CKD.4,5

In veterinary practice, a routine method for measuring GFR is based on monitoring plasma clearance of iohexol by high-performance liquid chromatography-ultraviolet (HPLC-UV).6-9 One major limitation is that conventional iohexol clearance protocols require repeated blood sampling over several hours, which is labor-intensive, time-consuming, and stressful for the animal. Numerous studies have attempted to determine the smallest number of blood samples needed for accurate GFR measurements in dogs and cats.10-12 and single-point GFR methods have been investigated.13-17 However, most of these methods were not considered sufficiently reliable for CKD diagnosis.

Chronic kidney disease is a progressive disease, with a gradual decline in GFR. Currently, for CKD diagnosis, the GFR cutoff, giving the best compromise between sensitivity and specificity, is usually selected.15,18,19 However, this approach transforms the GFR into an artificially “black or white” statistical index and can easily lead to misclassification of borderline cases. To tackle this problem in humans, a 3-zone partition based on 2 cutoffs including a middle “gray zone” of uncertain diagnosis has been proposed.20,21 The GFR gray zone approach for CKD diagnosis has not yet been investigated in veterinary clinical practice.

The primary aim of the present study was to assess the reliability of a panel of simplified iohexol plasma clearance protocols to measure GFR in dogs and investigate the application of a gray-zone strategy to identify subjects at risk of CKD.

There is debate in the scientific community about the best way to calculate the iohexol concentration in plasma by HPLC-UV and to normalize clearance to canine body size. High-performance liquid chromatography-UV detects 2 separate iohexol isomers: exo- and endo-ihexol, and discrepancies have been reported by calculating GFR based on the plasma clearance of the isomers separately6,8,19,22,23 or the total iohexol.24 In addition, differences have been reported between estimated levels of renal function standardized to body size (body weight [BW] versus body surface area [BSA]).6,11,15,24 Therefore, the secondary aim of this study was to investigate which method to compute iohexol plasma concentration and standardize GFR to canine body size fitted best in our clinical settings.

2 | MATERIALS AND METHODS

2.1 | Animals

Forty privately owned dogs of different breeds and sex were included. Body weights ranged from 3.9 to 46.0 kg (mean ± SD, 25.1 ± 9.7 kg). The dogs were aged 6 months-16 years (mean ± SD, 5.4 ± 3.5 years).

None of them received medical treatment before the GFR assessment, and they were fed their usual food, with water ad libitum. Each dog had a complete physical examination shortly before GFR was measured. One blood and 1 urine sample were collected before the iohexol injection, for CBC, serum biochemistry profile, and routine urinalysis. Exclusion criteria included abnormal diagnostic screening test results or dogs receiving medications. Healthy was defined as the absence of any clinical signs or relevant abnormalities on physical examination, CBC, serum biochemistry profile, routine urinalysis, and ultrasound examination. Chronic kidney disease was assessed according to the IRIS guidelines.25 Healthy dogs and IRIS stage 0 were considered CKD-negative (CKD−, 23 dogs) and those with CKD IRIS stages 1 or higher CKD−positive (CKD+, 17 dogs).

2.2 | Iohexol injection and blood sampling

The protocol was based on Lippi et al26 with some modifications. Briefly, food was withheld from each dog for at least 12 hours before the procedure. Dogs were allowed free access to water throughout the study. Dogs were weighed, and indwelling catheters were placed in the right and left cephalic veins. A commercially available iohexol formulation (Omnipaque; Nycomed Amersham Sorin, Milan, Italy) was used. The nominal dose of iohexol was 64.7 mg/kg, and the exact dose was determined from the difference between the weights of the syringe before and after the injection. Iohexol was injected as a 60-second IV bolus into the catheter in the left cephalic vein. Two milliliters of blood were directly sampled from the right cephalic vein, transferred to a heparinized tube, and centrifuged. Samples were immediately centrifuged at 2000g for 15 minutes, and plasma was stored at –30°C until use. Samples were taken 5, 15, 60, 90, and 180 minutes after injection of the marker.

2.3 | Iohexol HPLC measurements

Iohexol was determined using a Waters 626 HPLC system with a 996 photodiode array detector (Waters, Milford, Massachusetts) (1 spectrometer/second; wavelength 200-320 nm, extracting the chromatogram at 254 nm). Iohexol was separated in a Simmetry100 C18 column, 3.5 μm,
2.1 x 150 mm (Waters) using a mixture of CH$_3$CN and 0.1% orthophosphoric acid in water (3:97, vol/vol) at a flow rate of 0.3 mL/min. During separation, the column was held at 30°C. Standard iohexol (Omnipaque 350; 755 mg/mL iohexol) was added to untreated dog plasma to obtain the following standard solutions: 5, 20, 50, 200, and 500 μg iohexol/mL. Plasma samples were deproteinized with 5% perchloric acid (1:1, vol/vol), centrifuged at 11000 g for 10 minutes at 5°C, and 10 μL of supernatants were injected into the HPLC column. Data was processed using Millennium software (Waters). The peak areas of both iohexol isomers were used to calculate the iohexol concentrations and plasma clearance. The long-term stability of iohexol in plasma was tested by reanalysis after 36 months in a freezer at −30°C.

2.4 | Calculation of GFR: multisample methods

Multisample GFR was determined by calculating the rate of iohexol clearance using Phoenix WinNonlin software (version 8.0; Certara L.P., St. Louis, Missouri). Plasma clearance was determined with the following formula

\[
\text{Clearance} = \frac{\text{dose of iohexol injected}}{\text{AUC}}.
\]

where AUC is the area under the curve calculated from plasma iohexol disappearance curves after an IV bolus.

Reference GFR values (GFR$_5$) were calculated by plotting the iohexol concentration against the sampling time for 5 samples (5, 15, 60, 90, and 180 minutes after iohexol), and AUC was calculated by the trapezoidal method with a non-compartmental pharmacokinetic model (linear log trapezoidal with extrapolation to infinity).

To calculate GFR with reduced sampling (RS-GFR), AUC was calculated using a 1-compartment model during the mono-exponential time-part of the curve, defined by samples collected at 60, 90, and 180 minutes. The missing area due to the early fast drop of the disappearance curve was corrected by a current dog formula for 1-compartment assumption, according to Heiene et al.27

Reduced-sampling GFR were calculated either with 3 blood samples (GFR$_3$: 60, 90, and 180 minutes after injection) or a combination of 2 sampling times (GFR$_2$:60-90, GFR$_2$:90-180, and GFR$_2$:60-180). Samples taken before 60 minutes were not used for 1-compartment estimates because the terminal mono-exponential slope was often not reached before the 1-hour sample.

Clearance (mL/min) was normalized to BW and BSA (0.101 x [BW in kg]$^{0.71}$) to obtain GFR, which was expressed as mL/min/kg or mL/min/m$^2$, respectively.

2.5 | Calculation of GFR: single-sample methods

The iohexol concentrations in blood samples collected at 60, 90, or 180 minutes were used to derive the equations to predict GFR for the single-sampling protocol (SS-GFR: GFR$_{60}$, GFR$_{90}$, and GFR$_{180}$).

We followed a 3-step procedure, previously described.4,16 The procedure is based on the following Jacobson formula:

\[
\text{GFR} = \frac{1}{0.016 + 0.0015 \times t} \times \ln \left( \frac{Dose}{Vd \times Ct} \right)
\]

where Vd is the volume of distribution (mL) at sample collection time t (min), Ct the iohexol concentration measured at t, and dose is the amount of iohexol injected for each dog (mg/kg).

First, the iohexol Vd at 60, 90 and 180 minutes (Vd$_{60}$, Vd$_{90}$, and Vd$_{180}$) for individual dogs were calculated by substituting the reference GFR$_5$ calculated as described in the previous section and the plasma iohexol concentrations (Ct) at 60, 90, or 180 minutes into the Jacobson formula and solving the formula with the “Goal-Seek” command of Microsoft Office Excel (Microsoft 2007, Microsoft Co.).

Second, the Vd$_{60}$, Vd$_{90}$, and Vd$_{180}$ and the plasma iohexol concentrations at 60, 90, or 180 minutes for each of the 40 dogs were plotted in scatter diagrams, and 3 exponential equations fitting the data were calculated, as follows:

\[
\text{estVd}_t = C_0 e^{-TIC_t},
\]

where C$_0$ is the estimated plasma iohexol concentration at time 0; C$_t$ is the plasma iohexol concentration 60, 90, or 180 minutes after injection; b is the elimination rate constant, and e is the base of the natural logarithm. These equations were used to calculate an estimated Vd (estVd) in each dog from the iohexol concentrations found in single samples collected at 60 minutes (estVd$_{60}$), 90 minutes (estVd$_{90}$), or 180 minutes (estVd$_{180}$).

Third, the estVd$_{60}$, estVd$_{90}$, and estVd$_{180}$ and the iohexol dose injected in individual dogs were put back into the Jacobson formula to obtain GFR$_{60}$, GFR$_{90}$, and GFR$_{180}$.

2.6 | Validation data set—Testing the estVd$_{180}$ formula

The estVd$_{180}$ formula determined using the 40 dogs (training data set) was validated in an independent group of dogs (validation data set). Clinical examinations and GFR$_5$ protocol were the same as for the training data set dogs. The validation data set consisted of 11 client-owned dogs, aged 2-14 years (mean ± SD: 6.6 ± 3.2 years), with BWs from 9.2 to 40.3 kg (mean ± SD, 25.4 ± 10.7 kg). Seven dogs were CKD− and 4 CKD+. Estimated Vd$_{180}$ (estVd$_{180val}$) was calculated by inserting the iohexol concentration at 180 minutes for each dog into the estVd$_{180}$ formula, and GFR (GFR$_{180val}$) was calculated by substituting estVd$_{180val}$ into the Jacobson formula. The GFR$_{180val}$ and the reference GFR$_5$ (GFR$_{5val}$) were then compared.

2.7 | Data analysis

Statistical analyses were done using Graphpad Prism 5.0 (Graphpad Software, San Diego, California) and MedCalc 18 (MedCalc Software, Mariakerke, Belgium). Differences between iohexol isomer peak areas
in fresh and stored plasma samples were analyzed using the paired Student's t test. Glomerular filtration rate absolute values for all 40 dogs, measured with each of the methods, were compared by repeated-measure analysis of variance followed by post hoc Tukey's test. Significance was set at \( P \leq 0.05 \).

Agreement between simplified GFR protocols and reference GFR was calculated using Lin's concordance correlation coefficient (CCC) as an indicator of the degree to which paired observations fell on the line of identity. According to McBride, CCC >0.99, 0.95, 0.90, and <0.90 were defined as almost perfect, substantial, moderate, and poor degrees of agreement between methods, respectively. The agreement was further checked graphically by plotting the difference between

**FIGURE 1** HPLC-UV chromatogram of a deproteinized plasma sample from a representative dog after intravenous iohexol

**FIGURE 2** Representative plasma profiles of iohexol concentrations (mean ± SEM) in 3 dogs with high (white circles) and low (black circles) glomerular filtration rate after an IV bolus. Time 0 was designated as time of injection. A, Arithmetic plot of iohexol plasma concentration versus time. B, Semilogarithmic plot of the same plasma profiles limited to the iohexol elimination phase (60-180)

**FIGURE 3** Effect of standardizing iohexol clearance to body weight (BW) or body surface area (BSA). Clearances were calculated using the 5-sample protocol in 23 dogs negative to chronic kidney disease. A, Correlation between clearance and BW. B, Correlation between glomerular filtration rate (GFR) expressed as mL/min/kg and BW. C, Correlation between clearance and BSA. D, Correlation between GFR expressed as mL/min/m² and BSA
GFR\textsubscript{5} and the GFR values derived by each simplified protocol against the average of the 2 values for each dog (Bland-Altman plots). Bias was defined as the group mean difference between 2 GFR values, and the absolute limits of agreement were defined as the group mean difference \( \pm 1.96 \) SD. Data were analyzed on unit differences and percentage differences plots.

To determine the diagnostic consistency for CKD diagnosis of the different GFR methods, we analyzed receiver operating characteristic (ROC) curves. The calculated sensitivity was plotted against 100-specificity for different cutoff points, and the area under the ROC curve (AURC) was used to compare the sampling protocols. The overall accuracy of the different methods was compared according to DeLong\textsuperscript{30} with \( P \) values considered significant at \( P < .05 \).

To establish the GFR gray zone of diagnostic uncertainty for CKD, 2 cutoff points were identified for each GFR protocol. The gray-zone borders were identified as the fourth quartile of the CKD+ population (lower cutoff) and the first quartile of the CKD− population (upper cutoff).

3 | RESULTS

3.1 | Iohexol measured by HPLC

The HPLC conditions provided good peak shapes (Figure 1), with the stereoisomers eluting at 5.44 and 6.18 minutes for endo-iohexol and exo-iohexol, respectively. The total run was accomplished in 21 minutes, including equilibration of the column. The specificity of the method was tested by analyzing plasma samples before the iohexol injection. No interfering peaks were observed at the elution times as iohexol isomers. The limit of quantification was 1.80 \( \mu \)g/mL, and the assay was linear over the concentration range of 5-500 \( \mu \)g/mL, with an average regression coefficient of 0.99 (\( n = 22 \)). For all calibration curves, the \( y \)-intercepts were virtually zero, indicating the absence of endogenous interferences. Precision, expressed as inter-day coefficient of variation (CV\textsubscript{%}), ranged from 4.4% to 7.8% and the intra-day CV\textsubscript{%} from 3.2% to 5.9%. Accuracy ranged from 92% to 116%.

In the Omnipaque solution, the mean ratio of the isomer peak areas (calculated at different iohexol concentrations) was 18.99 \( \pm \) 0.98 for endo-iohexol and 81.04 \( \pm \) 0.96 for exo-iohexol. The ratio was the same in all plasma samples after iohexol injection analyzed within 2 months from collection (\( P \leq .001 \)). However, in plasma samples stored for longer (36 months at \( -30^\circ \)C), about 5% of the endo-iohexol peak area was shifted to the exo-iohexol peak area, independently from the iohexol concentration. In the same samples before and after storage, the mean ratios were 20.23 \( \pm \) 0.83 and 15.83 \( \pm \) 0.79 for endo-iohexol, and 79.77 \( \pm \) 0.83 and 84.17 \( \pm \) 0.80 for exo-iohexol (mean \( \pm \) SD; \( P \leq .001 \); 60). The combined isomer area did not change, independently from the iohexol concentration (\( P \leq .001 \)). We therefore used the combined peak area of the 2 isomers for the quantification of iohexol in plasma.

3.2 | GFR\textsubscript{5} and relation to body size

No adverse clinical signs were observed during or after the injection of iohexol in any dog. The GFR was first assessed using the reference GFR\textsubscript{5} method, based on the iohexol concentrations measured during the distribution and elimination phases, using the plasma samples collected 5, 15, 60, 90, and 180 minutes after injection. Figure 2A shows a representative example of the curves for plasma iohexol concentrations plotted against time in dogs with high and low GFR. The 60-minute sample was considered the starting point of the elimination phase, on the basis of the linearity of the semilogarithmic plot of plasma iohexol concentrations against time for the last 3 samples (Figure 2B).

The GFR\textsubscript{5} in the 23 CKD− dogs were used to establish the best method for standardizing the GFR to body size. The strong correlation between unscaled clearance and BSA and BW (Pearson’s \( r \), 0.91, for clearance against BW and also for clearance against BSA; \( P \leq .001 \); Figure 3A) was lost when clearance was standardized to kg (Pearson’s \( r \), 0.13; \( P = .58 \)), but not when scaled to m\(^2\) (Pearson’s \( r \), 0.66; \( P \leq .001 \); Figure 3B). In our setting, normalization to BW was in fact the better method, so GFR was expressed as min/mL/kg. The descriptive statistic for GFR\textsubscript{5} indexed to BW in the 40 dogs is reported in Table 1.

3.3 | GFR\textsubscript{3} and GFR\textsubscript{2}

Glomerular filtration rate in the 40 dogs was measured using methods based on a reduced number of blood samples. In

| TABLE 1  | Descriptive statistics of the GFR measured by different protocols in 40 dogs |
|----------------|---------------------------------|
|               | Minimum | Maximum | Mean   | Median | IQR       |
| GFR\textsubscript{3} | 0.25    | 3.43    | 2.12   | 2.15   | 1.42-2.80 |
| GFR\textsubscript{3} | 0.18    | 3.76    | 2.12   | 2.13   | 1.36-2.78 |
| GFR\textsubscript{2} 60-90 | 0.15    | 3.72    | 2.14   | 2.20   | 1.50-2.78 |
| GFR\textsubscript{2} 60-180 | 0.16    | 3.83    | 2.13   | 2.14   | 1.38-2.81 |
| GFR\textsubscript{2} 90-180 | 0.20    | 3.65    | 2.12   | 2.17   | 1.39-2.79 |
| GFR\textsubscript{1} 60 | 0.82    | 3.99    | 2.09   | 2.14   | 1.31-2.60 |
| GFR\textsubscript{1} 90 | 0.76    | 4.04    | 2.19   | 2.27   | 1.40-2.77 |
| GFR\textsubscript{1} 180 | 0.52    | 4.03    | 2.16   | 2.14   | 1.29-2.86 |

Values are expressed as mL/min/kg.

Abbreviations: GFR, glomerular filtration rate; GFR\textsubscript{3}, single-sample GFR protocol; GFR\textsubscript{2}, 2-sample GFR protocol; GFR\textsubscript{1}, 3-sample GFR protocol; GFR\textsubscript{5}, 5-sample GFR protocol; IQR, interquartile range.
there were 3 samples (GFR₃, 60-90-180 minutes), and 3 methods used 2 samples, in all the possible time combinations during the elimination phase (GFR₂, 60-90, 90-180, and 60-180 minutes). The descriptive statistics for GFR₃, GFR₂, 60-90, GFR₉₀-180, and GFR₂, 60-180 are shown in Table 1. None of the differences were significant.

Agreement between GFR₃ and RS-GFR was investigated using Bland-Altman plots (Figure 4; Table 2). Biases were close to 0 with the line of equality lying within the confidence interval of the bias, with narrow limits of agreement. Bias values for all 4 methods were constant throughout the range of GFR, both as absolute numbers and percentages. Lin’s CCC between GFR₃ and RS-GFR indicated substantial agreement for all methods except GFR₂, 60-90, for which agreement was moderate (Table 2). Based on the CCC, agreement with GFR₃ followed the order GFR₂, 60-180 > GFR₃ > GFR₉₀-180 > GFR₂, 60-90.

3.4 | The single-sample GFR

Using the GFR₅ measured in the 40 dogs, the following formulae for the estVd at a desired time (e.g., 60, 90, and 180 minutes) were derived from scatter plots (Figure 5).

\[
estVD_{60} = 514.9e^{-0.014C} \quad \text{estVD}_{90} = 499.4e^{-0.012C} \quad \text{estVD}_{180} = 309.1e^{-0.01C}.
\]

From these equations, SS-GFR was back-calculated for each dog at 3 time points (GFR₁₆₀, GFR₁₉₀, and GFR₁₈₀). The descriptive statistic for these 3 GFR₁ is shown in Table 1. None were significant.

The SS-GFRs were compared to the reference GFR₅. Bland-Altman plots showed narrow limits of agreement, biases very close to 0 and consistent across the range of values (Table 3; Figure 6). The line of equality lay within the confidence interval of the bias for GFR₁₆₀ and GFR₁₈₀ but not for GFR₁₉₀. Lin’s CCC between GFR₅ and SS-GFR indicated substantial agreement for GFR₁₈₀ and moderate agreement for GFR₁₆₀, GFR₁₉₀. On the basis of the CCC, agreement within protocols followed the order GFR₁₈₀ > GFR₁₉₀ > GFR₁₆₀.

3.5 | Testing the estVd₁₈₀ formula

In the 11 dogs of the validation data set, the GFR₁₈₀ was ranged from 0.60 to 3.01 mL/min/kg (mean ± SD: 1.90 ± 0.88) and the GFR₁₈₀ was from 0.57-3.01 mL/min/kg (mean ± SD: 1.92 ± 0.86). Agreement between GFR₁₈₀ and reference GFR₁₈₀ was evaluated by CCC and Bland-Altman plots (Figure 7; Table 4). Lin’s CCC indicated substantial agreement (CCC: 0.98). Bland-Altman analysis indicated biases close to 0 with the line of equality lying within the confidence interval of the bias, with narrow limits of agreement. Bias values were constant throughout the range of GFR, both as absolute numbers and percentages.

3.6 | The diagnostic potential of the different GFR methods for CKD

We tested the diagnostic performances for CKD of GFR₅, GFR₃, GFR₂, 60-180, GFR₂, 90-180, and GFR₁₈₀ based on evidence of the
best agreement of these methods with the reference GFR protocol as shown by CCC ≥ 0.95.

Descriptive statistics for GFR in CKD+ and CKD− dogs with the selected protocols are reported in Table 5. The AURC for each of these protocols was ≥ 0.98 (Table 6), indicating strong diagnostic potential for CKD. The AURC did not differ significantly for the different methods.

Table 7 shows the GFR cutoffs employed to define gray zones of diagnostic uncertainty for CKD in different GFR protocols. Figure 8 shows the distribution of the GFR measured by the various protocols in the 17 CKD+ and 23 CKD− dogs in relation to the respective gray zones. Classification of the 40 dogs was consistent between protocols, despite some differences in the gray-zone limits and width. For each protocol, 13 dogs were correctly classified as CKD+ and 18 dogs were correctly classified as CKD−. Nine lay within the gray zone, of which 5 were CKD− and 4 CKD+.

4 | DISCUSSION

We found that CKD in dogs can be diagnosed with satisfactory accuracy using GFR calculated from a limited number of blood samples—from 1 to 3—with flexible sampling schedules.
The GFR measured by the RS- and SS-protocols proved reliable for many clinical situations regardless of the level of renal function. This is in agreement with previous reports that GFR assessed by simplified sampling approaches in companion animals correlates with GFR based on multi-sample investigations.\textsuperscript{13,16,22,27}

There might be several reasons for the overall agreement we found between the GFR\textsubscript{5} and the various RS- and SS-GFR. First, to compute the simplified GFR, we used only samples collected during the terminal mono-exponential phase. This is essential when using simplified protocols for GFR so as to avoid loss of accuracy.\textsuperscript{4,8,24} The timing we considered—as the end of the iohexol distribution phase (ie, 60 minutes after the injection)—agrees with previous reports and with the average half-life of iohexol.\textsuperscript{8,31}

Second, the RS-GFR was calculated employing a dog-specific 1-compartment correction formula according to Heiene et al.\textsuperscript{27} Third, to calculate the SS-GFR, we derived the reference regression curves for Vd estimation using data from dogs with a wide range of GFR. This might be essential to ensure reliable estimates of GFR from single blood samples.\textsuperscript{10}

The GFR measured by the RS- and SS-protocols proved reliable for many clinical situations regardless of the level of renal function. This is in agreement with previous reports that GFR assessed by simplified sampling approaches in companion animals correlates with GFR based on multi-sample investigations.\textsuperscript{13,16,22,27}

The GFR given by the simplified protocols did not significantly differ from GFR\textsubscript{5}, but concordance was best when the calculation included the sample collected 180 minutes after iohexol injection, as indicated by higher CCC and lower biases. This implies that for the best performance, we can reduce the number of blood samples but not the time needed for the clearance test (3 hours). This too is in agreement with previous studies,\textsuperscript{13,19} and the rationale is that the timing of the last sample determines the percentage of AUC extrapolated to infinity by the pharmacokinetic model compared to total AUC. The larger this proportion, the less accurate the clearance estimate.\textsuperscript{12} Furthermore, for SS-GFR methods based on Jacobsson’s formula, if the sampling does not extend to late enough times after injection, the GFR can be overestimated, especially for lower rates.\textsuperscript{13}

The simplified GFR protocols that proved most reliable (GFR\textsubscript{3}, GFR\textsubscript{90-180}, GFR\textsubscript{60-180} and GFR\textsubscript{180}) were then examined for their diagnostic power. The four methods showed strong potential to

| TABLE 4 | Agreement between the GFR\textsubscript{180\textsubscript{val}} and the multisampling GFR reference protocol in the validation data set |
|-----------------|--------------------------------------------------|
|                  | GFR\textsubscript{val} versus GFR\textsubscript{180\textsubscript{val}} |
| Concordance correlation coefficient                  | 0.98 |
| Bias ± SD (mL/min/kg)                                | \(-0.01 ± 0.16\) |
| 95\% lower and upper LoA (mL/min/kg)                | \(-0.33/0.31\) |
| Bias ± SD (%)                                       | 0.80 ± 8.42 |
| 95\% lower and upper LoA (%)                        | \(-15.71/17.30\) |

Abbreviations: GFR, glomerular filtration rate; GFR\textsubscript{1}, single-sample GFR protocol; GFR\textsubscript{5}, 5-sample protocol; LoA, Limits of agreement.
classify CKD+ and CKD− dogs (AURC >0.98). The use of a single cutoff can easily lead to misclassification of borderline cases, especially in the diagnosis of progressive diseases like CKD. Therefore, we tested the concept of a gray zone, identifying an interval where the GFR gave uncertainty about the CKD diagnosis.

Different approaches can be used to establish the cutoffs for a gray-zone. Glomerular filtration rate in dogs is variable, with intra-individual and interindividual CV up to 20%. According to Hazra and Gogtay, when there are wide differences, it is appropriate to use a quartile range to establish reference ranges for a defined population. We therefore defined a fairly wide GFR gray zone, spanning from the fourth quartile of the diseased dogs (ie, CDK+ dogs with the highest GFRs) to the first quartile of the healthy dogs (ie, CDK− dogs with the lowest GFRs). This achieved not only 100% specificity and sensitivity for CKD diagnosis outside the gray zone but also permitted consistent classification of the dogs, independent of which protocol was used for GFR measurement.

Several studies have found that moving away from the dichotomous division of quantitative test scales and identifying intermediate range(s) of test results gave a better understanding of the diagnostic accuracy potential of a test. We therefore suggest that in veterinary clinical practice, this approach—which clearly establishes the lower and upper thresholds—should facilitate clinical decisions. A GFR falling in the gray zone would not be totally uninformative as it could lead

**TABLE 5** Descriptive statistics of GFR measured with different protocols in CKD+ and CKD− dogs

|               | Minimum | Maximum | Mean | Median | IQR       |
|---------------|---------|---------|------|--------|-----------|
| **GFR5**      |         |         |      |        |           |
| CKD−          | 1.98    | 3.43    | 2.65 | 2.69   | 2.18-2.99 |
| CKD+          | 0.25    | 2.02    | 1.34 | 1.38   | 1.12-1.69 |
| **GFR3**      |         |         |      |        |           |
| CKD−          | 2.06    | 3.76    | 2.68 | 2.73   | 2.46-2.94 |
| CKD+          | 0.18    | 2.08    | 1.30 | 1.33   | 0.99-1.77 |
| **GFR60-180** |         |         |      |        |           |
| CKD−          | 2.06    | 3.83    | 2.70 | 2.68   | 2.47-3.00 |
| CKD+          | 0.16    | 2.07    | 1.30 | 1.35   | 0.99-1.80 |
| **GFR90-180** |         |         |      |        |           |
| CKD−          | 2.07    | 3.65    | 2.69 | 2.66   | 2.41-2.93 |
| CKD+          | 0.20    | 2.12    | 1.30 | 1.32   | 0.99-1.76 |
| **GFR180**    |         |         |      |        |           |
| CKD−          | 1.90    | 4.03    | 2.81 | 2.79   | 2.33-3.09 |
| CKD+          | 0.52    | 2.05    | 1.25 | 1.25   | 0.99-1.44 |

Values are expressed as mL/min/kg.

Abbreviations: CKD, chronic kidney disease; CKD−, CKD-negative dogs; CKD+, CKD-positive dogs; GFR, glomerular filtration rate; GFR1, single-sample GFR protocol; GFR3, 2-sample GFR protocol; GFR5, 3-sample GFR protocol; GFR60-180, 5-sample GFR protocol.

**TABLE 6** ROC curve analysis for chronic kidney disease identification with different GFR measurement protocols

|               | GFR5 | GFR3 | GFR60-180 | GFR90-180 | GFR180 |
|---------------|------|------|-----------|-----------|--------|
| AURC ± SE     | 0.99 ± 0.007 | 0.99 ± 0.004 | 0.99 ± 0.003 | 0.99 ± 0.007 | 0.98 ± 0.01 |
| (95% CI)      | (0.90 to 1.00) | (0.91 to 1.00) | (0.91 to 1.00) | (0.90 to 1.00) | (0.87 to 1.00) |

Abbreviations: AURC, area under the ROC curve; CI, confidence intervals; GFR, glomerular filtration rate; GFR1, single-sample GFR protocol; GFR3, 2-sample GFR protocol; GFR5, 3-sample GFR protocol; GFR60-180, 5-sample GFR protocol; ROC, receiver operating characteristic.

**TABLE 7** GFR cutoffs defining the gray zone of diagnostic uncertainty for chronic kidney disease in different GFR measurement protocols

|               | GFR5 | GFR3 | GFR60-180 | GFR90-180 | GFR180 |
|---------------|------|------|-----------|-----------|--------|
| Lower cutoff  | 1.69 | 1.77 | 1.80      | 1.76      | 1.44   |
| 95% CI        | 1.40-1.99 | 1.34-1.97 | 1.36-2.02 | 1.38-1.93 | 1.29-1.94 |
| Upper cutoff  | 2.18 | 2.46 | 2.47      | 2.41      | 2.33   |
| 95% CI        | 2.12-2.50 | 2.12-2.52 | 2.13-2.51 | 2.15-2.54 | 2.17-2.67 |
| Gray zone width | 0.49 | 0.69 | 0.67      | 0.65      | 0.89   |

Values are expressed as mL/min/kg.

Abbreviations: CI, confidence intervals; GFR, glomerular filtration rate; GFR1, single-sample GFR protocol; GFR3, 2-sample GFR protocol; GFR5, 3-sample GFR protocol; GFR60-180, 5-sample GFR protocol.
the veterinarian to seek further evidence of kidney failure and, if necessary, to adopt measures to slow its progression.

We are aware that simplifying sampling method increases the chance of errors in the estimation of GFR, especially single-sample methods because of their empirical character. Indeed, among the GFR protocols based on a limited number of blood samples, GFR\textsubscript{1} were those with the lowest agreement with GFR\textsubscript{5}, with only GFR\textsubscript{1180} giving a clinically acceptable margin of error. Furthermore, when collecting only 1 sample any analytical error will influence the accuracy of the clearance measurement. However, the advantage of reducing physical discomfort and stress for the dog, as well as costs and time, substantially balances the risk of errors in GFR measures. In addition, a gray-zone approach with an interval of uncertainty for overlapping values can reduce the potential for error and thus limit wrong clinical decisions. Indeed, this could lead to a decision to use further diagnostic tools in a smaller group with inconclusive results and, at the same time, offer diagnostic certainty outside the gray zone.

The assessment of GFR from iohexol plasma clearance in dogs still suffers a lack in standardization. Differences in the marker used, the marker concentration assays, sampling times, pharmacokinetic models, and mathematical modeling of the data used for calculating GFR can all lead to wide variability in the GFR reported for healthy and diseased dogs.\textsuperscript{7,24,37} We investigated 2 controversial issues hindering the harmonization of GFR measurement in dogs between laboratories: which of the 2 iohexol isomers detected by HPLC-UV has to be used for plasma clearance calculation and which measure of body size has to be used for GFR normalization.

Discrepancies have been reported by calculating GFR based on the plasma clearance of endo- and exo-iohexol separately\textsuperscript{6,8,19,22,23} and the use of the total iohexol.\textsuperscript{4} Here, we found that in plasma samples frozen for a long time, a significant proportion of the endo-iohexol was shifted to the exo-iohexol. This is in agreement with early reports that the isomers are interconvertible and that rotational conversion is temperature\textsuperscript{38} and storage-dependent.\textsuperscript{39} The isomer shift modified single peak areas but did not influence their sum, in agreement with reports that total iohexol is very stable in plasma\textsuperscript{13,40,41} For GFR measurement in veterinary practice, injection of the tracer and sample analysis are often separate in space and time, so stability is of primary importance. Our results suggest the routine use of the sum of the absorbance peaks of the 2 isomers for calculating iohexol clearance in order to avoid preanalytical errors, especially if the samples need to be frozen or sent by mail to the reference laboratory.

The most commonly used method to normalize GFR in dogs is indexation to BW, but some authors prefer to normalize the measurements to BSA\textsuperscript{9,10,16} and differences have been reported between the levels of renal function standardized to BW or BSA.\textsuperscript{4} In the present study, we observed that the correlation between iohexol plasma clearance and dog body size was lost only when GFR was scaled to BW. This supports the recommendation that indexation to BSA should be abandoned and that the formulae used to estimate BSA in dogs is of questionable accuracy.\textsuperscript{42,43} Our analyses were done on dogs with a wide range and normal BW distribution, but the group was only small, the majority weighing 25-27 kg. This might be a bias when drawing any conclusion that BW is a better method for clearance indexation in dogs. Whether our findings are valid for a general population of dogs with and without CKD needs further study.

In conclusion, we propose a panel of accurate, hands-on, flexible, simplified procedures for estimating GFR in dogs as a practical tool for CKD diagnosis in daily clinical practice. We also recommend the gray zone concept of uncertainty in CKD diagnosis, as it can be especially useful when it is more important to suspect reduced renal function as early as possible than to know the exact GFR.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approval from Animal Care Committee of the University of Milan, opinion n.107_2016.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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