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# Urine soluble CD163 (sCD163) as biomarker in glomerulonephritis: stability, reference interval and diagnostic performance

https://doi.org/10.1515/cclm-2020-0466
Received April 7, 2020; accepted September 27, 2020; published online October 19, 2020

**Abstract**

**Objectives:** Soluble (s) CD163 is a well-established macrophage biomarker, and recent data suggests urine sCD163 to reflect disease activity in crescentic glomerulonephritis (GN). Other types of GN may also be associated with glomerular inflammation but the potential usefulness of urine sCD163 as a general biomarker of GN remains unaddressed.

**Methods:** An in-house sCD163 enzyme-linked immunosorbent assay (ELISA) was validated for urinary use and compared to a frequently used commercial ELISA. The pre-analytical stability of urine sCD163 was assessed and a reference interval was established according to the CLSI guidelines using specimens from 253 healthy individuals. Urine samples from 64 patients with different types of renal disorders were also analysed.

**Results:** Urine sCD163 was highly stable during storage. An upper reference limit of 5.1 µg/L (1.9 µg/mmol, normalised to creatinine) was established using the in-house ELISA. Urine sCD163 was generally increased in GN patients (3.9 µg/mmol, $p<0.0001$, AUROC=0.97) and decreased upon treatment, but did not perform better than urine albumin (AUROC=1.00). Patients with proliferative GN had higher urine sCD163/albumin ($p=0.0001$) ratio. The commercial assay had a higher detection limit, and patient levels were 4–6 times lower than in the in-house assay.

**Conclusions:** Urine sCD163 is a stable biomarker that can be measured with acceptable accuracy using our in-house ELISA. Its pre-analytical characteristics makes it a reliable biomarker and our findings point towards the use of urine sCD163 as a biomarker of specific subtypes of GN.

**Keywords:** biomarker; ELISA; glomerulonephritis; inflammation; macrophages; reference values; soluble CD163; urine albumin.

## Introduction

The haemoglobin scavenger receptor CD163 is a 130 kDa transmembrane protein, expressed by mononuclear phagocytes, and shed from the phagocyte surface as soluble (s)CD163 upon inflammatory activation [1]. The level of sCD163 in blood reflects the macrophage activity in systemic inflammatory diseases [2–5] and several studies have also demonstrated the usefulness of sCD163 as a biomarker in other biological fluids such as cerebrospinal- and synovial- fluid and aqueous humour [3, 4, 6–9].

Glomerular infiltration by macrophages is a part of the inflammatory response in glomerulonephritis (GN) [10–14] and recent studies suggest a potential role for urine sCD163 also as a biomarker of glomerular inflammation in GN, particularly in relation to lupus nephritis and anti-neutrophil cytoplasmic antibody (ANCA)-associated renal vasculitis [15–17]. Specifically, urine sCD163 is able to reflect the histological disease activity in lupus nephritis [15], identify active renal vasculitis and discriminate this from active extrarenal vasculitis [16, 17].

During GN, sCD163 is proposed to be actively shed into the urinary space by glomerular macrophages and hereby constitute a urine biomarker of glomerular inflammation [18]. Urine sCD163 could thus serve as a non-invasive tool to monitor local inflammatory activity in GN and may possibly reduce the need for kidney biopsies. The clinical application of urine sCD163 measurements, however, is hampered by the lack of assay optimisation, standardisation and knowledge regarding its pre-analytical properties and normal values. Furthermore, it remains

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unaddressed if the promising biomarker capability is restricted to certain GN subtypes or if urine sCD163 could serve as a general biomarker across different GN diagnosis.

This study aims to characterise and validate an in-house sCD163 enzyme-linked immunosorbent assay (ELISA) for measurement of urine sCD163, to examine pre-analytical properties of the analyte and to establish its reference interval. Furthermore, it investigates if urine sCD163 is a general biomarker of GN.

# Materials and methods

### Urine and blood specimens: healthy individuals

The urine sCD163 reference interval was established using urine specimens collected among the staff of the Department of Clinical Biochemistry, Aarhus University Hospital (May–June, 2017) and among blood donors of the Department of Clinical Immunology, Aarhus University (June–August, 2017). Only healthy individuals >18 years were eligible to participate, and donors were excluded if taking medication for chronic disease or suffering from diabetes, hypertension or kidney disease. Approximately 1/3 of the female donors were excluded due to haematuria. Specimens were processed anonymously and only information regarding age and gender was collected. All donors were instructed in writing to perform a midstream clean catch procedure following the CLSI guideline GP16-A3 [19]. Collection and analysis of the anonymous urine specimens used for the establishment of the urine sCD163 reference interval and for pre-analytical testing is exempted from approval by national law. The exception from approval was confirmed by the Regional Ethical Committee (VEK34/2017) (March 6th, 2017) and the Data Protection Agency (April 6th, 2017).

Urine and serum specimens for pre-analytical stability testing and assay validation were collected from surplus anonymous samples at the routine laboratory at the Department of Clinical Biochemistry, Aarhus University Hospital.

### Urine and blood specimens: patients with suspected GN

Paired urine and blood samples were collected from patients with suspected GN at the Department of Renal Medicine, Aarhus University Hospital. The GN diagnosis was subsequently confirmed by histological analysis of a kidney biopsy. Of the 64 patients with suspected GN, 56 received a specific GN diagnoses after renal biopsy, five were diagnosed with other renal diseases than GN and three patients were not biopsied. Among the 56 GN patients, 27 had non-proliferative GN while 29 patients were diagnosed proliferative GN (Supplementary Material, Tables 1 and 2) [20]. Urine and plasma specimens were obtained at three hospital visits: At inclusion and diagnosis (January 2010–April 2012), at a second visit four months after inclusion (May 2010–August 2012) and a third visit 12 months after inclusion (January 2011–November 2012). Patient characteristics, including information on renal diagnosis, steroid treatment, disease status and P-creatinine (P-crea) were obtained from hospital records. The analysis of collected urine specimens from patients enrolled at the Department of Renal Medicine, Aarhus University Hospital, was approved by the Regional Ethics Committee (VEK 1-10-72-378-17) and the Data Protection Agency (2012-41-0561). Informed consent was obtained from all GN patients included in this study.

### Processing of specimens

Urine specimens for the reference interval were collected between 8 AM and 3 PM and processed within 2 h of donation. All samples were screened for blood, leucocytes and nitrite by dip-stick analysis (Siemens Multistix®7, Ref: 2305) and positive samples were discarded. After screening, each specimen was centrifuged (3,000 × *g*, 5 min), aliquoted and stored at −80 °C until further analysis.

Randomly collected urine specimens for assay validation and evaluation of pre-analytical properties were centrifuged (1,800 × *g*, 5–10 min) and processed according to the protocols described in "*Assay validation and evaluation of urine sCD163 pre-analytical properties*".

Urine specimens from GN patients were collected as morning urine and processed within 30 min of donation (stored at room temperature (RT)). Samples were centrifuged at 4,000 × *g* at 4 °C for 20 min and stored at −80 °C until final analysis. Blood specimens from GN patients were collected in pre-chilled ethylene-diamine-tetraacetic acid (EDTA) tubes and plasma was separated by centrifugation at 3,500 × *g* at 4 °C for 10 min. Plasma samples were stored at −80 °C until final analysis.

### ELISA

The in-house ELISA for urine sCD163 was a modification of a previously described serum-assay [21]. Polystyrene microplates (F96 Maxisorp, Thermo Scientific, Waltham, Massachusetts, USA) were coated overnight at 4 °C with capture antibody (Polyclonal rabbit anti-CD163 IgG, Søren Moestrup, Aarhus University, Denmark) diluted to a working concentration of 1.75 mg/L in a 20 mM carbonate-bicarbonate buffer (pH 9.6). After wash with phosphate buffered saline (PBS)-Tween (T) buffer (2.5/7.5 mM phosphate buffer, 0.5 M NaCl, 0.25% Tween, pH 7.2), diluted calibrators, controls and samples in PBS/albumin buffer (PBS-T/0.2% BSA (Sigma-Aldrich, St. Louis, Missouri, USA)) were added and incubated for 1 h under agitating conditions at RT. After a second wash, a biotinylated detection antibody (Monoclonal mouse anti-human CD163, clone MAC2-158, IQ-products, Houstoun, USA) was diluted 1:500 in PBS/albumin, added and incubated for 1 h. After a third wash, avidin-POD (Avidin-peroxidase conjugate, Sigma-Aldrich) and lysozym (Sigma-Aldrich) diluted 1:2000 and 1:100, respectively, in PBS/albumin, were added and incubated for 1 h. After a final triple wash, TMB (3,3′,5,5′-tetramethylbenzidine) ONE (KEM-EN-TEC, Taastrup, Denmark) was added as substrate solution and after 20 min incubation, 1 M phosphoric acid (85% $H_3PO_4$ concentrate, Cat.No.: 20621.290, VWR Chemicals, Radnor, Pennsylvania, USA) was added to stop the reaction. The microtiter plate was read at 450 and 620 nm (Multiskan™ FC Microplate Photometer, Thermo Scientific). All samples were analysed as a batch in duplicate and the concentration of each sample was calculated from the standard curve using the Cubic Spline approach.

The standard curve was generated from serial dilutions of a serum pool (In-house serum pool made from multiple blood donors from the Department of Immunology, Aarhus University, 2015) whose concentration of sCD163 is traceable to purified CD163 [21]. A low

control sample, K1 (HK12 Human Longterm EGQ serum, Nordic Clinical Chemistry Quality Control, Lot.No.: 120028AA), and a high control sample, K2 (In-house citrate plasma pool made from two blood donors from the Department of Immunology, Aarhus University Hospital, 2015), were included in each run. K1 was diluted 1:1000 while K2 was diluted 1:200.

The in-house ELISA was performed manually as well as automated on a BEP 2000 Advance System ELISA robot (Siemens Healthcare Diagnostics, Erlangen, Germany). Analyses on urine sCD163 pre-analytical properties and assay validation were performed manually whereas the analysis of urine sCD163 in the reference population and in GN patients was performed on the BEP 2000 Advance System. The BEP 2000 Advance System used a protocol similar to the manual procedure, except for the following minor modifications: Samples were adsorbed for 1.5 h on the microtiter plate and all incubations were performed in the dark at 37 °C.

The commercial ELISA kit, Human CD163 Duoset (Cat.No.: DY1607, R&D Systems, Minneapolis, Minnesota, USA), was performed according to the manufacturer's instructions. A blank calibrator (0 µg/L, reagent diluent) and controls (K1 and K2 diluted 1:1,000 and 1:200 in reagent diluent, respectively) were included in every run.

For urine sCD163 analysis, urine samples were diluted 1:2 and analysed by in-house and R&D ELISA whereas for the analysis of plasma sCD163, plasma samples were diluted 1:101 and analysed by in-house ELISA applicable for plasma [21].

### Assay validation and evaluation of urine sCD163 pre-analytical properties

For comparison of the in-house and R&D ELISA, five serum samples and five urine samples were serially diluted and analysed by both methods. Possible urinary matrix effects at dilutions 1:2, 1:3 and 1:5 were examined in 17 randomly collected urine specimens and analysed by in-house ELISA.

Short term stability of urine sCD163 during storage at either 4 °C or RT (22–24 °C) was tested in six randomly collected urine specimens. Specimens were aliquoted into two tubes and subsequently stored at either 4 °C or RT after which a sample was taken from each aliquot after 24, 48, 72, 96, and 120 h and frozen at −80 °C until further analysis by in-house ELISA. Long-term stability of sCD163 in urine samples stored at −20 °C and −80 °C was tested in five urine pools made from a mix of several random urine samples. Specimens were randomly collected and aliquoted into five pools. A sample from each pool was analysed freshly while the remainder was stored at −20 °C and −80 °C in aliquots until further analysis after 3, 6 and 12 months of storage by in-house ELISA.

The effects of repeated freeze-thaw cycles on urine sCD163 were tested in six randomly collected urine specimens. Specimens were aliquoted and subjected to one or up to eight freeze-thaw cycles at −80 °C and analysed by in-house ELISA.

### Biochemical analyses

Urine creatinine, albumin and protein were measured by standardised, automated assays at the Department of Clinical Biochemistry, Aarhus University Hospital (Cobas 6000 Analyzer, Roche, Basel, Switzerland and ADVIA Chemistry XPT, Siemens Healthineers, Erlangen, Germany). Limit of Detection (LoD) for urine albumin was <3 mg/L and levels below were set to 2.99 mg/L. LoD for urine total protein was <0.02 g/L and levels below were assigned a value of 0.019 g/L.

### Statistical analyses

The data distribution was examined by inspection of inverse QQ-plots. Normal distributed data are presented as mean with standard deviation (SD) whereas non-normally distributed data are shown as median with interquartile range (IQR). Pair-wise comparison was done by Student's t-test or the Mann-Whitney U-test whereas the comparison of more than two groups was done by one-way analysis of variance (ANOVA) with Dunnett post-test or by Kruskal-Wallis (matched data) or Friedman (un-matched data) test followed by Dunn's post-test as appropriate. Correlation analyses were performed using the Spearman Rank Correlation test.

Assay Limit of Blank (LoB) and LoD were calculated following the CLSI guideline EP17 [22]. The urine sCD163 reference interval was established using the non-parametric approach from the CLSI guideline C28-A3 [23]. Statistical analyses were performed using GraphPad Prism 8 (GraphPad software, San Diego, CA, USA).

# Results

## Performance of sandwich sCD163 ELISAs

The standard curve of the in-house ELISA consisted of nine calibrators ranging from 0 to 50 µg/L. The linearity of the calibrators 0–8 was $R^2$=0.9986 and increased to $R^2$=0.9991 after exclusion of calibrators 0 and 1. The LoB was 0.40 µg/L and the LoD estimated to 0.45 µg/L. The CV for calibrators 0–8 was 367.6, 8.3, 3.2, 2.0, 1.8, 2.6, 2.3, 1.9 and 0.8%, respectively. Based on this, calibrator 2 (0.78 µg/L) was judged to be a reliable LoQ. Reproducibility (intra-assay variability) and repeatability (inter-assay variability) were calculated on the basis of the controls of 20 assays. Mean intra-assay variability for K1 was 8.5 and 4.7% for K2. The inter-assay variability for K1 was 11.0 and 6.2% for K2.

The standard curve of the commercial ELISA consisted of eight calibrators and ranged from 0 to 10 µg/L. The linearity of the calibrators was $R^2$ = 0.9944. The LoB and LoD were calculated on the basis of 10 duplicates of calibrators 0 (0 µg/L), 1 (0.156 µg/L) and 2 (0.313 µg/L) and was found to be 0 µg/L and 0.066 µg/L, respectively. The corresponding CV for the calibrators 0–2 was 0.0, 27.0 and 12.1%, respectively. Based on this, calibrator 1 (0.156 µg/L) was found to be the most reliable LoQ. Assay reproducibility and repeatability were calculated by examining the controls, K1 and K2, from every of the eight assays performed in this study. The mean intra-assay variability for K1 was 9.3 and 3.6% for K2. The inter-assay variability for K1 was 17.0 and 10.4% for K2.

Both the in-house and the commercial ELISA exhibited parallelism when blood and urine samples were diluted throughout the respective detection ranges (Figure 1A, B). Generally, samples analysed by the commercial ELISA were 4–6-fold lower in levels of serum and urine sCD163 compared to samples analysed by the in-house ELISA (Figure 1A, B). No urinary matrix effects interfered with the in-house assay at dilutions 1:2, 1:3 and 1:5 (p=0.27, Figure 1C).

## Pre-analytical properties of urine sCD163

Urine sCD163 exhibited stabile pre-analytical properties during short term storage (up to five days) at RT and 4 °C (Figure 2A, B) and during long term storage (up to 12 months) at –20 °C and –80 °C (Figure 2C). Urine sCD163 was also unaffected by multiple freeze-thaw cycles (up to eight cycles) (Figure 2D).

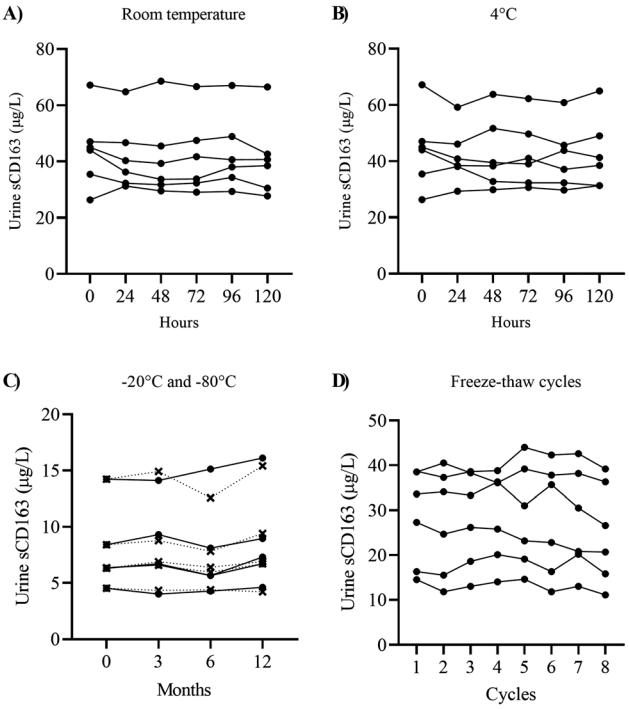


**Figure 2:** Stability of urine soluble (s)CD163 in urine samples, stored at (A) room temperature (RT), (B) 4 °C, (C) –80 °C (filled circles) or –20 °C (crosses and dotted line), and exposed to (D) repeated freeze-thaw cycles.
Each dot and connecting lines represent a sample.

## Urine sCD163 reference interval

The reference population consisted of 253 donors (121 males and 132 females), aged 19–65 years (Median (IQR), 35 (26–47) years). All biochemical renal parameters measured were within the reference intervals and the characteristics of the reference population are summarised in Table 1.

Age and urine sCD163/creatinine did not correlate ($r_s$=–0.05, p=0.4) and the urine sCD163/creatinine ratio did not differ between the 10 youngest the 10 eldest subjects in the reference population (0.4 µg/mmol (0.3–0.8) vs. 0.7 µg/mmol (0.5–1.1)) (p=0.08). The levels of urine sCD163/creatinine were slightly higher in women compared to men (0.6 µg/mmol (0.3–0.8) vs. 0.4 µg/mmol (0.3–0.6)) (p<0.0001).

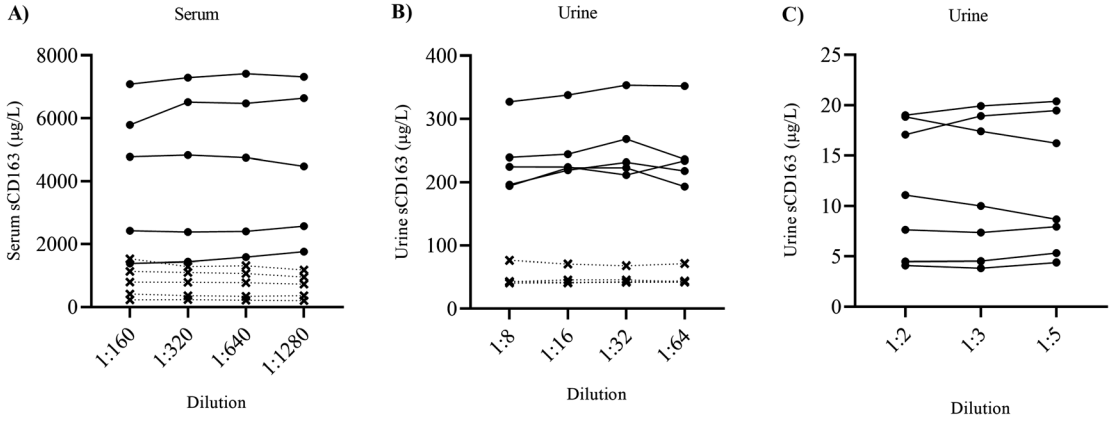


**Figure 1:** Analysis of soluble (s)CD163 in urine and serum.
(A) Serial dilutions of five serum samples, analysed by in-house (filled circles) and R&D enzyme-linked immunosorbent assay (ELISA) (crosses and dotted line). (B) Serial dilutions of five urine samples, analysed by in-house (filled circles) and R&D ELISA (crosses and dotted line). (C) Seven urine samples diluted 1:2, 1:3 and 1:5 to investigate possible urinary matrix effects, analysed by in-house ELISA. Each dot and connecting lines represent one sample.

**Table 1:** Biochemical measures and upper reference limits of the reference population (n=253).

| | All, n=253 | Males, n=121 | Females, n=132 |
|---|---|---|---|
| **Biochemical measures, median (IQR)** | | | |
| Urine pH | 6.0 (6.0–6.0) | 6.0 (6.0–6.5) | 6.0 (6.0–6.0) |
| Urine creatinine, mmol/L | 3.8 (2.2–7.9) | 4.9 (2.5–10.3) | 3.1 (2.0–6.3) |
| Urine albumin, mg/L | 2.99 (2.99–2.99) | 2.99 (2.99–2.99) | 2.99 (2.99–2.99) |
| Urine protein, g/L | 0.019 (0.019–0.020) | 0.019 (0.019–0.030) | 0.019 (0.019–0.020) |
| Urine albumin/creatinine, mg/g | 7.3 (3.8–12.6) | 5.8 (3.0–10.8) | 9.1 (4.6–15.5) |
| Urine protein/ creatinine, mg/g | 48.0 (31.6–76.4) | 39.1 (25.7–67.2) | 56.0 (36.5–84.0) |
| **Upper reference limits, 97.5th percentile (90% CI)** | | | |
| Urine sCD163, µg/L | 5.1 (4.9–7.7) | 3.5 (3.3–7.7) | 4.2 (3.9–6.9) |
| Urine sCD163/creatinine, µg/mmol | 1.9 (1.9–2.2) | 1.1 (1.1–2.2) | 1.9 (1.7–2.2) |
| Urine sCD163/albumin, µg/mg | 1.3 (1.3–2.3) | 1.0 (1.0–1.4) | 1.3 (1.2–2.3) |

Biochemical measures are presented as median with interquartile range, and upper reference limits as 97.5th percentile with 90% confidence interval.

Using our in-house ELISA, 147 (57.7%) of the 253 reference urine samples were below the LoQ. A non-parametric upper limit of the urine sCD163 reference interval was found to be 5.1 µg/L (90% Confidence Interval (CI)=4.9–7.7) before normalisation to creatinine and 1.9 µg/mmol (90% CI=1.9–2.2) after normalisation. All reference intervals established by our in-house ELISA are listed in Table 1. Using the commercial ELISA, only one of the 235 reference urine samples was detectable and thus, no upper limit could be defined.

## Urine sCD163 in glomerulonephritis patients

At inclusion, levels of urine sCD163/creatinine in the collective group of GN patients were significantly above the reference interval (median (IQR); GN (inclusion)=3.9 (1.8–9.0) µg/mmol, reference population=0.4 (0.3–0.7) µg/mmol, p<0.0001, Figure 3A). Mean urine sCD163/creatinine ratios declined at 4 and 12 months (both, p<0.0001); but remained significantly higher than in the healthy population (reference interval vs. GN 4 months; p<0.0001 and reference interval vs. GN 12 months; p<0.001, Figure 3A). Similar tendencies were observed for levels of urine albumin/creatinine (Figure 3B) and protein/creatinine ratios (Data not shown). Compared to the healthy population, urine sCD163/albumin ratios were significantly lower for GN patients at the time of inclusion (p<0.0001) and at 4 (p<0.0001) and at 12 months (p<0.0001) after inclusion (Figure 3C).

The diagnostic ability of urine sCD163/creatinine, albumin/creatinine and sCD163/albumin ratios was examined using the receiver-operating characteristic (ROC) curve and the calculated area under the curves (AUCs) were 0.97 (p<0.0001), 1.0 (p<0.0001) and 0.99 (p<0.0001), respectively (Figure 4A–C).

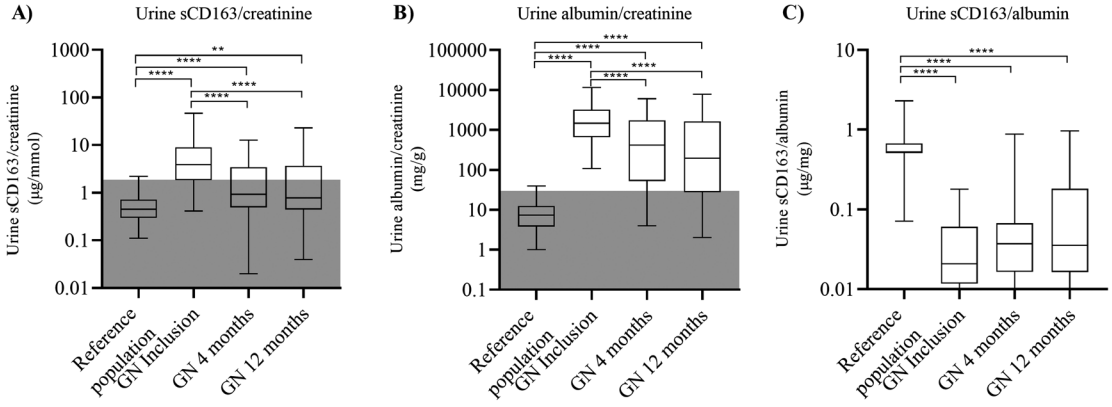


**Figure 3:** Urine ratios in the reference population (n=253) and in glomerulonephritis (GN) patients at different time points (inclusion, n=56; four months, n=45; 12 months, n=40).
(A) Soluble (s)CD163/creatinine ratio, (B) albumin/creatinine ratio and (C) sCD163/albumin ratio. Boxes are median and interquartile range. Whiskers are minimum and maximum. Grey shade marks upper reference limits (97.5th percentile). ****p-value<0.0001. **p-value=0.001.

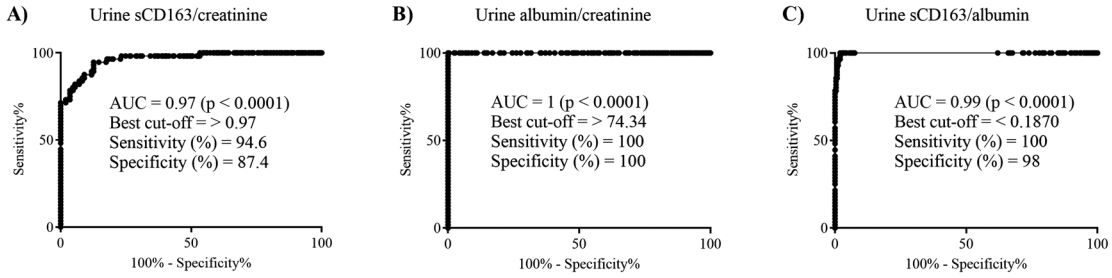


**Figure 4:** Receiver-operating characteristics (ROC) curves of urine ratios in the reference population (n=253) and in glomerulonephritis (GN) patients (n=56) at the time of patient inclusion.
(A) Soluble (s)CD163/creatinine ratio, (B) albumin/creatinine ratio and (C) sCD163/albumin ratio.

When dividing the patients into proliferative and non-proliferative GN at the time of inclusion, a tendency to higher urine sCD163/creatinine ratios were observed among patients with proliferative GN compared with non-proliferative (p=0.3, Figure 5A and Supplementary Material, Table 2). The urine albumin/creatinine ratio was significantly higher in patients with non-proliferative GN compared to proliferative forms (p=0.009, Figure 5B and Supplementary Material, Table 2) and a similar tendency was observed for levels of urine protein/creatinine (Data not shown). Hence, urine sCD163/albumin ratios were significantly higher in patients with proliferative GN compared to the non-proliferative forms (p<0.0001, Figure 5C and Supplementary Material, Table 2).

Levels of urine sCD163/creatinine and albumin/creatinine ratios were significantly reduced 12 months post inclusion in patients with proliferative GN in remission (both, p<0.0001) (Figure 6A, B). Urine sCD163/albumin ratios were higher, although not statistically significant, 12 months post inclusion in patients with proliferative GN in remission (p=0.1) (Figure 6C). For patients with proliferative GN not in remission, levels of urine sCD163/creatinine, albumin/creatinine and sCD163/albumin ratios were all reduced 12 months post inclusion, however, not statistically significant (p=0.3, p=1.0 and p=0.1, respectively) (Figure 6D–F).

The medians of plasma sCD163 at patient inclusion, 4 and 12 months post inclusion were each within the reference interval for plasma sCD163, (i.e. 0.69–3.86 mg/L [5]) and the levels of plasma sCD163 did not differ significantly between the visits (Inclusion=2.4 mg/L (1.8–3.8), four months=2.0 mg/L (1.5–2.8) and 12 months=2.0 mg/L (1.7–3.0)) (p=0.18). A weak positive correlation was found between levels of plasma sCD163 and urine sCD163/creatinine at the time of inclusion ($r_s$=0.3, p=0.049).

## Discussion

Urine sCD163 has been suggested as a useful biomarker in subtypes of GN, in which sCD163 is produced locally due to macrophage infiltration in glomeruli and crescents [15–17].

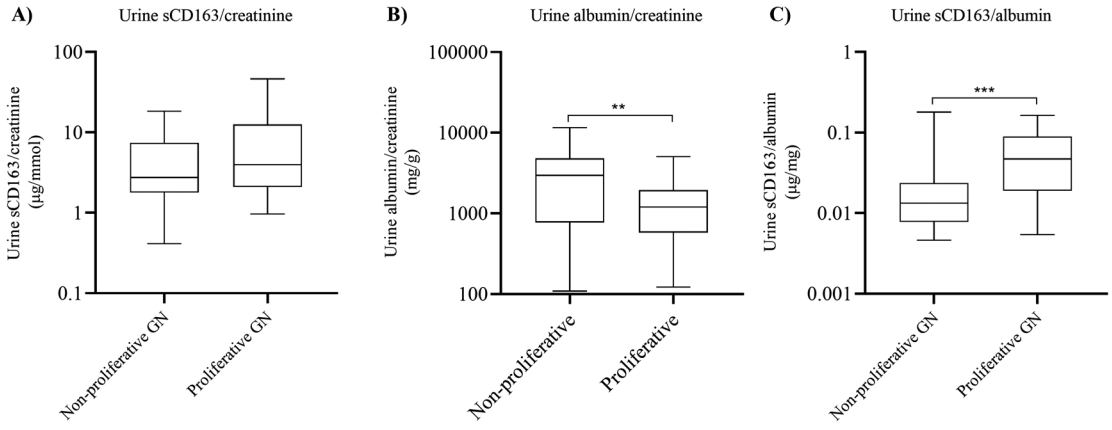


**Figure 5:** Urine ratios in patients with non-proliferative (n=27) and proliferative (n=29) glomerulonephritis at the time of patient inclusion.
(A) Soluble (s)CD163/creatinine ratio, (B) albumin/creatinine ratio and (C) sCD163/albumin ratio. Boxes are median and interquartile range. Whiskers are minimum and maximum. **p-value=0.009. ***p-value=0.0002.

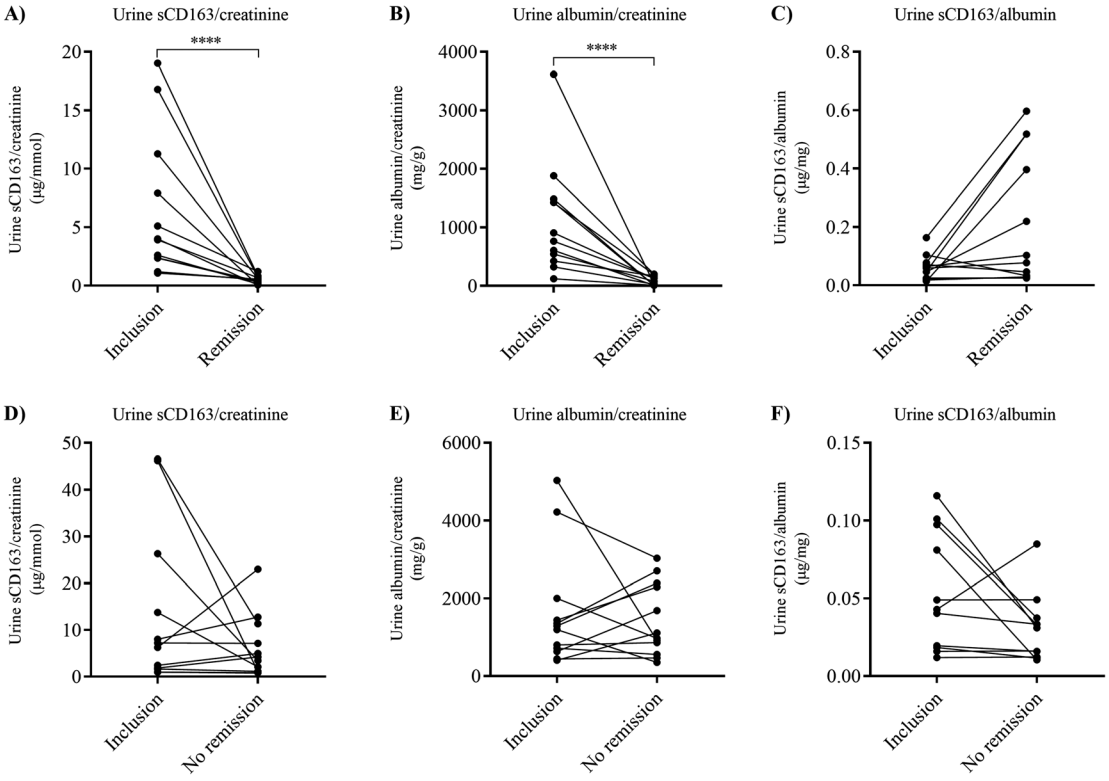


**Figure 6:** Urine ratios in patients with proliferative glomerulonephritis in remission (A–C) (n=12) and not in remission (D–F) (n=12). (A–D) Soluble (s)CD163/creatinine ratio, (B–E) albumin/creatinine ratio and (C–F) sCD163/albumin ratio. Remission defined as urine albumin/creatinine ratio<300 mg/g. ****p-value<0.0001.

In this study, we characterised and validated an in-house ELISA for measurement of urine sCD163 and compared it to a commercially available assay from R&D system. We assessed the preanalytical properties of urine CD163, established its reference interval and evaluated levels of sCD163 in a cohort of patients with mixed GN diagnoses. We also evaluated the diagnostic and prognostic performance of urine sCD163 compared to urine albumin.

The in-house assay had a robust standard curve, excellent intra- and inter-assay coefficients of variation and an acceptable LoQ which was safely below the upper limit of the urine sCD163 reference interval. Compared to the assay from R&D Systems, our in-house ELISA was more sensitive and exhibited less analytical variation. The measured levels of sCD163 were 4–6 times higher than those measured by the commercial assay which is consistent with previous comparison studies [5]. The reason for this difference is probably due to the use of different calibrators, e.g. purified vs. recombinant protein, underscoring the need for standardisation of sCD163 assays. In addition, urine sCD163 exists in very low quantities which may also influence measurements by similar and different assays [15–17, 24].

Urine sCD163 was highly stabile when stored at RT, 4, −20, −80 °C and through multiple freeze-thaw cycles. The observed preanalytical properties of urine CD163 observed in our study is in agreement with the existing literature [5, 16], and our data thus extend the evidence for high protein stability in urine. Our findings also demonstrate that urine sCD163 possess excellent preanalytical properties that make clinical implementation feasible.

More than half of the reference subjects had levels of urine sCD163 below LoD of our in-house assay. This is comprehensible as soluble sCD163 is a bulky molecule (approximately 130 kDa) which should normally be retained by the glomerular filtration barrier and only appear in the urine due to glomerular damage or glomerular macrophage production (crests). Using the R&D ELISA, previous studies on urine sCD163 have reported levels in healthy controls comparable to those observed in our study [15–17]. We detected slightly higher levels of urine sCD163 in women compared to men whereas patient age did not appear to have a significant influence. In serum, levels of sCD163 are conversely related to age but not gender [5, 25]. Since the gender-specific differences in urine sCD163 were of limited magnitude in relation to the reference interval and levels observed in our GN

populations, it is unlikely that the gender difference will be of clinical significance in this context.

Urine sCD163 is expected to originate mainly from serum due to glomerular filtration and therefore to increase in diseases with loss of glomerular barrier integrity. Consistently, levels of urine sCD163 were highly increased in the collective group of GN patients on admission to the hospital compared to the healthy population and in line with a parallel increase in urine albumin. Upon follow-up, levels of both urine sCD163 and albumin decreased significantly but remained above the respective upper reference limits. The observation of declining levels of urine sCD163 after treatment is in line with previous reports [16, 17]. Supporting the serum origin of urine sCD163, a weak correlation between serum and urine sCD163 was evident in the GN patients which has also been reported previously [24]. Due to the larger molecular size of sCD163, albumin is expected to be more sensitive to barrier dysfunction. In addition, this was also apparent in ROC analyses, showing a complete separation between patients and controls.

While urine sCD163 does not perform better than albumin as a marker of barrier dysfunction, sCD163 was reported to be superior to the urine protein/creatinine ratio in the identification of active renal vasculitis in a cohort of patients with crescentic GN [16]. This is expected to be due to local renal production of sCD163, e.g. from macrophage infiltration in glomerular crescent formation [15, 16, 18]. To approach this question, we stratified patients according to histological evidence of cellular proliferation. Of the 29 patients with proliferative GN, 16 were diagnosed IgA Nephritis, seven ANCA + vasculitis and the remaining were of mixed GN diagnoses (e.g. systemic lupus erythematosus) (Supplementary Material, Table 2). Of the 27 patients with non-proliferative GN, 13 were diagnosed with minimal change disease, eight with glomerulosclerosis, four with focal segmental glomerulosclerosis and the rest had mixed GN diagnoses (e.g. type 1 membranous proliferative GN) (Supplementary Material, Table 2).

Patients with proliferative disease had higher urine sCD163/albumin ratios at inclusion compared to those with non-proliferative illness whereas the urine sCD163/creatinine ratios were comparable between the groups. In proliferative GN, this is likely due to an increased production of sCD163 by infiltrating immune cells which adds to the sCD163 filtered over the comprised glomerular filtration barrier.

When patients with proliferative disease went into remission, a strong decrease was observed in both sCD163/creatinine and albumin/creatinine ratios which complies with improvement of the filtration barrier. The sCD163/albumin ratio increased simultaneously which may suggest that a partially restored filtration barrier could retain the larger sCD163 from plasma more efficiently than the smaller albumin and/or that treatment selectively affect the filtration barrier and not the local sCD163 production. Further investigations are required to unravel the exact mechanism behind this observation.

## Conclusions

Urine sCD163 is a biomarker that is present only in minute amounts in healthy adults. Using our in-house ELISA, urine sCD163 can be measured with acceptable accuracy and our data illustrate that the protein holds highly stable pre-analytical properties. The results also support previously published studies that demonstrate the usefulness of urine sCD163 as a biomarker for specific subtypes of GN. However, it highlights the need for development of a standardized reference material before the biomarker is established for clinical use.

**Acknowledgments:** The authors wish to thank Helle Hauser Ryom, Christina Strande Sønderskov, and Lene Dabelstein for exceptional technical assistance.
**Research funding:** The study was supported by Helen & Ejnar Bjørnows Fond, P.A. Messerschmidt og Hustru Fond and Nyreforeningens Forskningsfond.
**Author contributions:** AJN, MCN, HB, PI, HJM, CVBH: Conception and study design. HB, PI: Inclusion of patients. AJN, PI, CVBH: Collection of data. AJN, MCN, HB, PI, HJM, CVBH: Analysis and interpretation of data. AJN, MCN, HB, PI, HJM, CVBH: Revising the manuscript critically. All authors have accepted responsibility for the entire content of this manuscript and approved its submission.
**Competing interests:** Authors state no conflict of interest.
**Informed consent:** Informed consent was obtained from all individuals included in this study.
**Ethical approval:** The analysis of collected urine specimens from patients enrolled at the Department of Renal Medicine, Aarhus University Hospital, was approved by the Regional Ethics Committee (VEK 1-10-72-378-17) and the Data Protection Agency (2012-41-0561).

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**Supplementary Material:** The online version of this article offers supplementary material (https://doi.org/10.1515/cclm-2020-0466).