ORIGINAL ARTICLE

Performance of soluble Klotho assays in clinical samples of kidney disease

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ABSTRACT

Background. Soluble Klotho has multiple systemic salutary effects. In animals, both acute and chronic kidney disease models display systemic Klotho deficiency. As such, there is considerable interest in investigating soluble Klotho as a biomarker in patients with different types and severity of kidney diseases. Unfortunately, there remains uncertainty regarding the best method to measure soluble Klotho in human serum samples.

Methods. Using human serum samples obtained from several clinical cohorts with a wide range of kidney function, we measured soluble Klotho using a commercial enzyme-linked immunosorbent assay (ELISA) as well as with an immunoprecipitation–immunoblot (IP–IB) assay utilizing a synthetic antibody with high affinity and specificity for Klotho. Recovery of spiking with a known amount of exogenous Klotho was tested. A subset of samples was analyzed with and without the addition of a protease inhibitor cocktail at the time of collection or after the first freeze–thaw cycle to determine if these maneuvers influenced performance.

Results. The IP–IB assay was superior to the ELISA at recovery of exogenous Klotho (81–115% versus 60–81%) across the spectrum of kidney function. Klotho measurements by IP–IB were highly correlated with estimated glomerular filtration rate (eGFR) ($R = 0.80$, $P < 0.001$) in comparison with the commercial ELISA, which exhibited minimal correlation with eGFR ($R = 0.18$, $P = 0.12$). Use of a protease inhibitor cocktail neither improved nor impaired performance of the IP–IB assay; however, subsequent freeze–thaw cycle resulted in a significant reduction in Klotho recovery and dissipated the correlation between Klotho levels and eGFR. With the ELISA, the use of protease inhibitor cocktail resulted in an increase in intrasubject variability.

Conclusions. The IP–IB assay is preferable to the commercial ELISA to measure soluble Klotho concentrations in never-thawed serum samples of humans with varying severity of kidney disease. However, due to the labor-intensive nature of the IP–IB assay, further research is needed to secure an assay suitable for high-throughput work.

Keywords: assays, chronic kidney disease, Klotho, measurements

INTRODUCTION

Alpha Klotho (referred to here as Klotho) is frequently described as an antiaging protein [1–3] and is highly conserved across multiple species including mice and humans [4]. Klotho is most highly expressed in the kidney as a transmembrane protein. In membrane-bound form, Klotho serves as a cofactor for fibroblast growth factor 23 (FGF-23) and FGF receptors [5]. Klotho is also cleaved and released from the kidney into circulation, both as a full-length protein or as Kl1 and Kl2 fragments [6, 7]. These circulating isoforms of Klotho are collectively called 'soluble' Klotho.

Chronic kidney disease (CKD) and acute kidney injury (AKI) in rodents are well documented to be conditions of Klotho deficiency in the kidney, blood and urine [8–13]. In soluble form, Klotho influences multiple metabolic pathways including insulin release and sensitivity, and the renin–angiotensin system [14–16], with higher expression associated with less pathologic evidence of aging such as kidney fibrosis and systemic markers of oxidative stress [13, 17–19]. Multiple rodent models of CKD demonstrated significantly lower soluble Klotho [10, 20, 21], suggesting that nephron loss may result in decreased production and release of soluble Klotho [22, 23]. If similar biology is observed in humans, one would anticipate a direct correlation between kidney function determined by estimated glomerular filtration rate (eGFR) and soluble Klotho levels.

Human studies of soluble Klotho have generally shown that patients with lower eGFR also have lower soluble Klotho levels [8, 9], though some studies have reported no change in Klotho depending on the level of kidney function [24–30]. These discrepancies may, in part, be due to problems with current commercially available assays [31, 32] where the same reagents have been licensed to several commercial laboratories [33]. Furthermore, a recent study showed that the most widely used commercial enzyme-linked immunosorbent assay (ELISA) may experience a drop in expected recovery (capture) in patients with advanced kidney failure as well as when samples have undergone multiple freeze–thaw cycles [8]. Taken together, these reports suggest that the current assays may not be optimal for large cohort studies in which samples are collected at different sites, may have been exposed to varying times at room temperature before cryopreservation, may have been subjected to multiple freeze–thaw cycles and may have been in storage for variable durations.

Due to the potential for low soluble Klotho to be a biomarker of kidney disease and its associated complications [34], better methods of detection are needed to generate reliable human databases. In this context, an alternative novel soluble Klotho assay has been described using a synthetic recombinant antibody with high affinity for Klotho and immunoprecipitation–immunoblot (IP–IB). This assay demonstrated progressive decline of serum Klotho with increasing stages of CKD in a small study using fresh samples [8]. However, the prior study used samples from a single center and its performance relative to the ELISA method in serum from patients with different etiology and severity of kidney disease has not been fully elucidated.

We therefore compared a commonly used commercial ELISA with the IP–IB assay using stored serum samples from healthy volunteers, and participants with a broad spectrum of kidney function, AKI and end-stage renal disease (ESRD). Known quantities of exogenous recombinant Klotho were added to samples to determine recovery (capture) characteristics. We then examined the correlation of Klotho levels with eGFR, the impact of freeze–thaw cycles and whether the addition of ethylenediaminetetraacetic acid (EDTA) affected assay performance. Due to the potential for soluble Klotho to be degraded by endogenous proteases [8], we also determined if the addition of a protease inhibitor cocktail improved assay performance. Overall, efforts were directed at establishing the best way to measure serum Klotho in historical human samples.

MATERIALS AND METHODS

Study design and participants

We conducted a two-phase, experimental study using human sera to compare the performance of two soluble Klotho assays, a commercially available ELISA (Immuno-Biological Laboratories, IBL) and the IP–IB method offered by the O'Brien Kidney Research Center at UT Southwestern [8]. We tested these two assays under different prespecified conditions.

Phase 1. Phase 1 of the study included stored serum samples from (i) 77 participants recruited from the University of California San Diego Medical Center (UCSD), who were also enrolled in the Systolic Blood Pressure Intervention Trial [35] (SPRINT); (ii) 20 patients with ESRD on maintenance hemodialysis from Tufts Medical Center who were enrolled into the Cognition and Dialysis Cohort [36]; and (iii) 15 patients with AKI Kidney Disease: Improving Global Outcomes (KDIGO) Stage ≥2 from UT Southwestern Medical Center who were enrolled in the Klotho in Acute Kidney Injury (KLAKI) study [37].

Phase 2. Phase 2 of the study included prospectively collected serum samples from (i) five patients with CKD Stage 3 (ambulatory); (ii) five patients with ESRD (hospitalized); (iii) five patients with AKI Stage ≥2 (hospitalized); and (iv) five healthy volunteers. All the samples in Phase 2 were obtained at UT Southwestern Medical Center.

Study procedures

All human serum samples (Phases 1 and 2) had not been previously thawed prior to this study. Serum samples were collected according to corresponding biospecimen handling protocols from SPRINT [38], Cognition and Dialysis Cohort [36] and KLAKI [37]. SPRINT is a multicenter randomized controlled trial comparing two systolic blood pressure targets (<140 mmHg versus <120 mmHg); participants from the UCSD site who agreed to additional biospecimen collection at baseline are included in this study. The Cognition and Dialysis Cohort comprised 314 maintenance hemodialysis patients from Boston-area dialysis clinics

in whom baseline and longitudinal cognitive testing were performed. Stored baseline serum samples from both of these cohorts were utilized for this study (vintage period, the age of samples in reference to date of collection, was 2013–15 for SPRINT and 2015–16 for the Cognition and Dialysis Cohort). The KLAKI study is an ongoing prospective study that has included ~200 critically ill patients with and without AKI for the longitudinal examination of Klotho levels and postcritical illness outcomes. Stored serum samples collected at the time of AKI diagnosis were utilized for this study (vintage period, the age of samples in reference to date of collection, was 2015–16).

Biospecimens (Phase 2) were centrifuged at 1000g, 4°C for 10 min. Serum was aliquoted in codified cryovials and stored at −80°C until biomarker measurements were done by laboratory personnel blinded to the study design and data (vintage period from sample collection to utilization for Phase 2 of the study was <30 days). Informed consent was obtained for all study participants or their legally authorized representatives through regulatory procedures from the corresponding studies.

Serum Klotho levels were measured by (i) IP–IB technique using synthetic anti-Klotho sb-106 (Fab-48) antibody (high affinity for Kl2 domain of Klotho) for pull-down and KM2076 antibody, originally developed by Kuro-o [6], now also commercially available (TransGenic Inc., Kobe, Japan) was used for detection by immunoblot as reported [8] (Supplementary data, Figures S1 and S2) and (ii) the commercial IBL ELISA kit per manufacturer's instructions (Immuno-Biological Laboratories Co., Minneapolis, MN, USA).

The following data were collected for study participants when available: demographics, baseline kidney function (eGFR and proteinuria), kidney function at the time of sampling (eGFR and serum creatinine), cause of kidney disease and comorbidity (diabetes, hypertension, cardiovascular disease, congestive heart failure and cancer). AKI was characterized as per maximum KDIGO stage [39] and the need for acute renal replacement therapy (RRT).

Laboratory quality control procedures for the synthetic anti-Klotho sb-106 (Fab-48) antibody

An anti-Klotho sb-106 (synonymous with Fab-48) antibody batch-to-batch titration assay was performed. This consisted of titration testing of serial dilutions of antibody concentrations ranging from 5 to 50 nM to determine the optimal concentration of Klotho detected from 50 μL of normal human sera. The optimal and expected antibody concentration resulting in the strongest 130 kDa band signal is normally between 20 and 30 nM. The inter-assay coefficient of variation (CV) was estimated from the same patient samples assayed in distinct plates with independent calibrators for the standard curve. The laboratory technician was blinded to the study design and clinical data. A detailed description of the generation and validation of the anti-Klotho sb-106 antibody was previously published [8].

Assay experiments and prespecified conditions for the study

Phase 1. In Phase 1 (77 participants also enrolled in SPRINT; 20 ESRD patients; and 15 AKI Stage ≥2 patients), we tested the following conditions (Table 1): (i) control (no additives); (ii) addition of protease inhibitor cocktail [4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), aprotinin, bestatin, E-64, leupeptin and pepstatin]; (iii) addition of protease inhibitor cocktail plus 20–50 pM of exogenous recombinant Klotho protein

Table 1. Phase 1 conditions applied by type of Klotho assay

IP–IB assay
    Native Klotho concentration (control)
    Addition of 50 pM of exogenous Klotho to thawed sample
    Addition of protease inhibitor cocktail to thawed sample[a]
    Additional freeze–thaw cycle (2 total)
ELISA
    Native Klotho concentration (control)
    Addition of 50 pM of exogenous Klotho to thawed sample
    Addition of protease inhibitor cocktail to thawed sample[a]

[a]AEBSF, aprotinin, bestatin, E-64, leupeptin and pepstatin.

(synthesized in our laboratory, as described) [8] at time of first sample thawing; and (iv) additional freeze–thaw (no additives but with one additional freeze–thaw cycle). Only the IP–IB assay in the SPRINT group was tested for prespecified condition (iv) due to a limited amount of residual serum.

Phase 2. In Phase 2 (five CKD Stage 3 patients; five ESRD patients; five AKI Stage ≥2 patients and five healthy volunteers), we tested the following conditions (Table 2): (i) control [addition of phosphate-buffered saline (PBS) only]; (ii) addition of the same protease inhibitor cocktail described above; (iii) addition of the protease inhibitor cocktail plus EDTA; and (iv) both prior conditions (ii and iii) with the addition of 20 pM of recombinant exogenous Klotho protein (only IP–IB was tested for this prespecified condition); applied at two different time points: (a) immediately after specimen collection (fresh sample), while processing the sample before storage and (b) after thawing the stored sample for the first time for Klotho measurement. The study was designed as such because if protease inhibitors improve assay performance, it is critical to assess if their addition is required to be immediate, or could be applied to stored samples, as the latter are the only sources available from biorepositories from large-scale studies.

Statistical analysis

Categorical data were reported as percentages and continuous data as means ± SD or median (25th–75th percentile) according to data distribution. Inter-assay coefficients of variation were calculated for the IP–IB assay in both phases. Percent recovery of exogenous Klotho was calculated for both assay methods in both Phase 1 and Phase 2 by dividing the measured Klotho concentration by the expected Klotho concentration (the native sample Klotho concentration plus the known quantity of added exogenous Klotho) and multiplying it by 100. Bland–Altman plots were constructed to graphically represent the agreement among these two concentrations (expected versus measured). In Phase 1, a paired Student's t-test was used to compare Klotho concentrations with and without addition of protease inhibitor cocktail for both assay methods. Spearman correlation analysis was utilized to determine the correlation of each assay method to the other as well as each method with eGFR (in samples that had eGFR available). In Phase 2, measured Klotho concentrations obtained under each prespecified condition permutation within each assay method were compared through the use of analysis of variance (ANOVA). In addition, measured Klotho concentrations across levels of kidney function were compared using ANOVA. R version 3.5.1 was used for database construction, statistical analyses and generation of figures.

Table 2. Phase 2 conditions by type of Klotho assay at two different time points: applied to fresh samples before storage and applied to stored samples after the first thaw

IP–IB assay
  Applied to fresh samples before storage
    Addition of PBS (control)
    Addition of protease inhibitor cocktail[a]
    Addition of protease inhibitor cocktail plus EDTA[b]
    Addition of protease inhibitor cocktail plus 20 pM of exogenous Klotho
    Addition of protease inhibitor cocktail plus EDTA plus 20 pM of exogenous Klotho
  Applied to stored samples after the first thaw
    Addition of PBS (control)
    Addition of protease inhibitor cocktail[a]
    Addition of protease inhibitor cocktail plus EDTA[b]
    Addition of protease inhibitor cocktail plus 20 pM of exogenous Klotho
    Addition of protease inhibitor cocktail plus EDTA plus 20 pM of exogenous Klotho
ELISA
  Applied to fresh samples before storage
    Addition of PBS (control)
    Addition of protease inhibitor cocktail[a]
    Addition of protease inhibitor cocktail plus EDTA[b]
  Applied to stored samples after the first thaw
    Addition of PBS (control)
    Addition of protease inhibitor cocktail[a]
    Addition of protease inhibitor cocktail plus EDTA[b]

[a]AEBSF, aprotinin, bestatin, E-64, leupeptin and pepstatin.
[b]EDTA.

RESULTS

Clinical characteristics

Demographics and clinical characteristics of the participants in the two phases of the experiment are shown in Table 3 (Phase 1) and Table 4 (Phase 2). When converted to the same units (pM or pg/mL), Klotho concentrations were overall higher for all groups with the IP–IB assay compared with the ELISA. For the IP–IB, the highest mean Klotho concentrations were seen within the SPRINT group, followed by the AKI group, with the lowest concentrations in the ESRD group. This was in contrast to the ELISA, which yielded the highest concentrations in the AKI group, followed by the SPRINT group and then the ESRD group (Table 3).

Assay performance in Phase 1

The IP–IB Klotho assay displayed an inter-assay CV of 4.8% ($n = 112$). The commercial ELISA inter-assay CV is reported by the manufacturer to be between 2.9% and 11.4% [33]. Recovery of exogenous Klotho (20 pM for SPRINT samples and 50 pM for ESRD and AKI samples) was greater for the IP–IB assay compared with the commercial ELISA across all kidney disease groups. However, we observed lower recovery with both assays in the ESRD and AKI groups relative to participants in the SPRINT trial, which could represent some degree of susceptibility of exogenous Klotho to degradation in uremic sera or impaired retrieval by the capturing antibodies (Figure 1). The agreement between the expected Klotho concentrations (the native sample Klotho concentration plus the known quantity of

added exogenous Klotho) and the actual measured Klotho concentration is represented by Bland–Altman plots in Figure 2.

Within the SPRINT group, there was no difference between Klotho concentrations obtained by the IP–IB assay with and without addition of the protease inhibitor cocktail to samples after the first thaw (mean Klotho concentration 13.7 ± 4.0 pM versus 14.1 ± 3.7 pM, respectively, $P = 0.18$), whereas the commercial ELISA returned significantly higher concentrations when a protease inhibitor cocktail was added (mean Klotho concentration 5.1 ± 4.1 pM versus 3.5 ± 2.3 pM, $P < 0.001$).

When examining Klotho concentrations across all kidney disease groups, the IP–IB and commercial ELISA Klotho concentrations only weakly correlated with each other ($r = 0.28$, $P = 0.01$) (Figure 3A). IP–IB was strongly correlated with eGFR in the SPRINT group ($r = 0.8$, $P < 0.001$), while the commercial ELISA did not correlate with eGFR in the same SPRINT group ($r = 0.18$, $P = 0.12$) (Figure 3B and C). The difference between the two assays in percent recovery and correlation with eGFR persisted when examining subgroups with eGFR <60 mL/min/1.73 m^2 or ≥ 60 mL/min/1.73 m^2 (data not shown). For the commercial ELISA, despite the higher Klotho concentrations seen with addition of the protease inhibitor cocktail, there was no corresponding change in correlation with the IP–IB ($r = 0.25$) or with eGFR ($r = 0.19$). An additional freeze–thaw cycle (due to sample volume constraints, only done for the IP–IB assay) resulted in significantly less detection of initial endogenous Klotho [Δ two versus one freeze–thaw cycle: -45.9%, 95% confidence interval (CI) (-40.0% to -51.9%)] and significantly dissipated the correlation between Klotho levels and eGFR (Supplementary data, Figure S3).

Quality control procedures

During the initial handling of the Phase 1 samples, it was noted that the IP–IB assay returned inadequate recovery of exogenous Klotho, including from known standards. The assay also showed poor differentiation across strata of kidney disease, leading to the production of a new batch of synthetic anti-Klotho sb-106 (Fab-48) antibody that was used in all above reported experiments. This highlights the importance of quality control of the reagents.

Assay performance in Phase 2

The IP–IB had an inter-assay CV of 16.6% ($n = 20$). Recovery of exogenous Klotho (20 pM) was consistent (90.8–116.2%) across all preparation methods (Figure 2). However, we observed lower recovery of exogenous Klotho in the CKD, AKI and ESRD groups relative to healthy volunteers, which was only slightly enhanced by the use of the protease inhibitor cocktail (Supplementary data, Figure S4). We also saw no significant difference in absolute Klotho concentrations across sample preparation using different prespecified conditions, including spiking with EDTA, the addition of protease inhibitor cocktail at the time of collection (fresh sample) or after the first thaw ($P = 0.9$) (Figure 4A). Finally, Klotho levels were consistently higher in the healthy volunteer group, followed by the Stage 3 CKD group and the AKI group, and lowest in the ESRD group ($P < 0.001$) (Figure 4A).

In contrast, the commercial ELISA had an inter-assay CV of 44.9% ($n = 18$). Use of various sample preparation methods resulted in significantly different Klotho concentrations ($P = 0.006$ across different prespecified conditions) (Figure 4B). Finally, the commercial ELISA yielded Klotho levels that were significantly higher in the healthy volunteer group ($P < 0.001$),

Table 3. Characteristics of patients (Phase 1)

	SPRINT	ESRD[a]	AKI[b]
Number of patients	104[c]	20	15
Klotho levels, mean ± SD			
IP–IB (pM)	13.6 ± 3.7	5.8 ±1.4	11.6 ± 6.0
ELISA (pM)	3.5 ± 2.2	2.6 ± 2.1	5.7 ± 4.4
IP–IB (pg/mL)	1771 ± 483	750 ± 182	1505 ± 776
ELISA (pg/mL)	384 ± 245	283 ± 224	618 ± 480
Demographics			
Age, years, mean ± SD	74 ± 8	63 ± 18	61 ± 16
Women, n (%)	27 (26)	9 (45)	5 (33)
Race			
White race, n (%)	88 (84.5)	14 (70)	10 (66)
Black race, n (%)	10 (9.5)	5 (25)	1 (7)
Hispanic race, n (%)	4 (4)	–	3 (20)
Other race, n (%)	2 (2)	1 (5)	1 (7)
BMI, kg/m^2, years, mean ± SD	29 ± 6	–	35 ± 13
Baseline kidney function			
eGFR[d], mL/min/1.73 m^2, mean ± SD	66 ± 18	–	86 ± 13
Dipstick proteinuria >30 mg/dL, n (%)	–	–	3 (20)
Urine albumin-to-creatinine ratio, median (25th−75th)	15 (7, 41)	–	–
Kidney function at the time of sampling			
eGFR[d], mL/min/1.73 m^2, mean ± SD	66 ± 18	–	–
SCr at the time of sampling, median (25th, 75th)	–	–	1.5 (1.4, 2.2)
Comorbidity			
Diabetes, n (%)	0 (0)	7 (35)	4 (27)
Hypertension, n (%)	104 (100)	18 (90)	9 (60)
Cardiovascular disease, n (%)	26 (25)	5 (25)	2 (13)
Congestive heart failure, n (%)	–	3 (15)	5 (33)
Cancer, n (%)	–	3 (4)	5 (33)

[a]ESRD etiology: six patients had diabetic nephropathy; eight patients had hypertensive nephrosclerosis; and six patients had other causes of ESRD.
[b]AKI characteristics: seven patients had KDIGO Stage 2 AKI; five patients had Stage 3 AKI; and three patients had Stage 3 AKI that required RRT.
[c]Summary of characteristics based on $n = 104$ patients, of whom only $n = 77$ had available samples for the study.
[d]eGFR estimated using the Chronic Kidney Disease Epidemiology Collaboration equation.
BMI, body mass index; SCr, serum creatinine.

but did not demonstrate differences in Klotho concentrations across the remaining three kidney disease groups (Figure 4B).

DISCUSSION

We evaluated two methods of measuring soluble Klotho concentrations in human serum: IP–IB and a frequently utilized commercial ELISA. When assays were compared in serum samples of patients with different types and severity of kidney disease, the overall performance of the assay favored IP–IB. In particular, the IP–IB assay exhibited a stronger direct correlation with eGFR in patients with a wide range of kidney function, better recovery (capture) of exogenous Klotho, less susceptibility to variability from sample additives and larger differentiation across different kidney disease groups (AKI, CKD and ESRD) in reference to healthy volunteers. Importantly, we did not find improvement in the performance of the IP–IB assay when a protease inhibitor cocktail was added, suggesting that this may not be a necessary step for assaying soluble Klotho in larger studies. Nonetheless, the IP–IB assay performance was susceptible to additional freeze–thaw cycle, suggesting that use of never previously thawed specimens is important for future studies examining soluble Klotho measurements [8].

These findings are important and timely because the perceived lack of a reproducible and reliable assay for measurement of soluble Klotho levels in human blood samples has been a major limitation in performing large-scale studies of patients with kidney disease or other conditions. While decreased levels of both renal tissue Klotho and soluble Klotho have been associated with adverse outcomes in some clinical and preclinical studies, the results have been inconsistent [24–30] and these inconsistencies may be partially explained by limitations in current Klotho ELISAs [32]. Heijboer et al. showed poor inter-assay as well as intra-assay agreement between three different commercial Klotho ELISAs, suggesting that problems may exist beyond just the one ELISA that we tested in our study [32]. Moreover, a prior study reported the IBL ELISA yielded different results in fresh versus stored serum samples, and Klotho levels measured by ELISA were less stable after repeated freeze–thaw cycles when compared with the IP–IB assay [8]. However, using a larger sample size than the prior study, we found that the IP–IB assay can also be susceptible to lower Klotho yields with a second freeze–thaw cycle, suggesting that Klotho itself may be unstable with additional freeze–thaw processing. Due to lack of sample availability, we did not examine the performance of the ELISA after repeated freeze–thaw cycles in the present study, thus our study demonstrates changed test characteristics after freeze–thaw with the IP–IB method but whether or not the magnitude of these effects is similar with the ELISA remains uncertain.

We confirmed a direct relationship between soluble Klotho and eGFR, with a stronger correlation with the IP–IB assay when compared with the ELISA. This result is consistent with animal data showing decreased Klotho gene expression and Klotho

Table 4. Characteristics of patients (Phase 2)

	CKD Stage 3	ESRD	AKI[a]	HV
Number of patients	5	5	5	5
Klotho levels,[b] mean ± SD				
IP–IB (pM)	11.0 ± 5.3	5.7 ± 1.3	9.0 ± 3.8	22.4 ± 1.1
ELISA (pM)	0.8 ± 0.6	1.6 ± 1.4	1.1 ± 0.5	2.2 ± 1.7
IP–IB (pg/mL)	1195 ± 573	623 ± 144	985 ± 409	2437 ± 123
ELISA (pg/mL)	91 ± 68	173 ± 153	122 ± 57	243 ± 182
Demographics				
Age, years ± SD	57 ± 7	53 ± 21	54 ± 30	42 ± 14
Women, *n* (%)	4 (80)	2 (40)	4 (80)	3 (60)
Race				
White race, *n* (%)	1 (20)	1 (20)	3 (60)	4 (80)
Black race, *n* (%)	1 (20)	2 (40)	0 (0)	1 (20)
Hispanic race, *n* (%)	3 (60)	2 (40)	1 (20)	0 (0)
Other race, *n* (%)	0 (0)	0 (0)	1 (20)	0 (0)
BMI, kg/m^2, years, mean ± SD	35 ± 7	28 ± 5	26 ± 3	24 ± 4
Baseline kidney function (prehospitalization)				
eGFR,[c] mL/min/1.73 m^2, mean ± SD	50 ± 7	10 ± 6	86 ± 22	–
Dipstick proteinuria >30 mg/dL, *n* (%)	1 (20)	5 (100)	1 (20)	–
Kidney function at the time of sampling				
eGFR,[c] mL/min/1.73 m^2, mean ± SD	51 ± 8	11 ± 6	–	–
SCr, mg/dL, median (25th, 75th)	1.40 (1.15, 1.46)	6.01 (4.03, 7.13)	2.82 (1.88, 3.06)	–
Comorbidity				
Diabetes, *n* (%)	2 (40)	4 (80)	1 (20)	–
Hypertension, *n* (%)	5 (100)	5 (100)	3 (60)	–
Cardiovascular disease, *n* (%)	0 (0)	1 (20)	1 (20)	–
Congestive heart failure, *n* (%)	0 (0)	2 (40)	1 (20)	–
Cancer, *n* (%)	1 (20)	0 (0)	1 (20)	–

[a]AKI characteristics: two patients had KDIGO Stage 2 AKI; two patients had Stage 3 AKI; and one patient had Stage 3 AKI that required RRT.
[b]Klotho levels reported correspond to control samples (no additives).
[c]eGFR estimated using the Chronic Kidney Disease Epidemiology Collaboration equation.
BMI, body mass index; HV, healthy volunteer; SCr, serum creatinine.

production in models of AKI [11, 26] and CKD [8–10]. We believe the strong correlation with eGFR seen with the IP–IB assay, when coupled with the superior exogenous Klotho recovery (capture) characteristics, is indicative of overall superior assay performance relative to the ELISA evaluated here. We also saw little difference in results when using EDTA or a protease inhibitor cocktail with the IP–IB assay, implying that use of these additives does not appear to significantly improve assay performance. These results also suggest that the IP–IB assay may be useful to measure Klotho in stored EDTA plasma rather than serum; however, this question was not directly assessed in our analysis and requires future study.

Although the IP–IB assay has several advantages relative to the commercial ELISA, it also has several shortcomings. IP–IB is labor-intensive and thus requires more time and effort compared with a standard ELISA. The IP–IB assay also requires meticulous quality control procedures due to the large number of steps involved, which includes the production of a synthetic antibody, and thus could be operator-dependent. In comparison, the Klotho measurements performed with the commercial ELISA utilized viable kits and all detailed instructions from the manufacturer were followed by an experienced laboratory technician. Overall, the use of a custom synthetic antibody along with a labor-intensive protocol limits the ability to widely export the IP–IB method to other laboratories. However, we believe the superior performance when coupled with careful and consistent quality control procedures outweighs these shortcomings. Finally, we note that there is a lack of negative controls and published data on the characterization of the specific
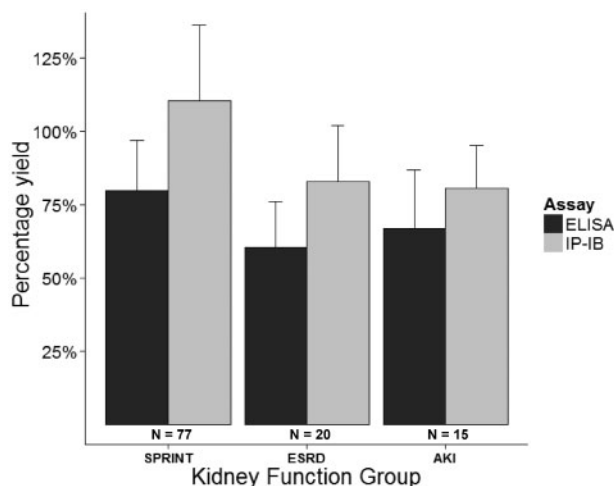

FIGURE 1: Mean percentage recovery after addition of exogenous Klotho (20–50 pM) plus protease inhibitor cocktail in different strata of kidney function by IP–IB and ELISA. Error bars represent SD. SPRINT participants with mean (SD) eGFR of 66 (18) mL/min/1.73 m^2.

antibodies used by commercial Klotho ELISAs, in contrast to the IP–IB assay, for which the protocol, including the specific antibodies utilized, has been previously reported [8].

There are many unanswered questions about Klotho biology and its role in kidney disease. There is a great interest to further define the subtypes of soluble Klotho protein as current assays
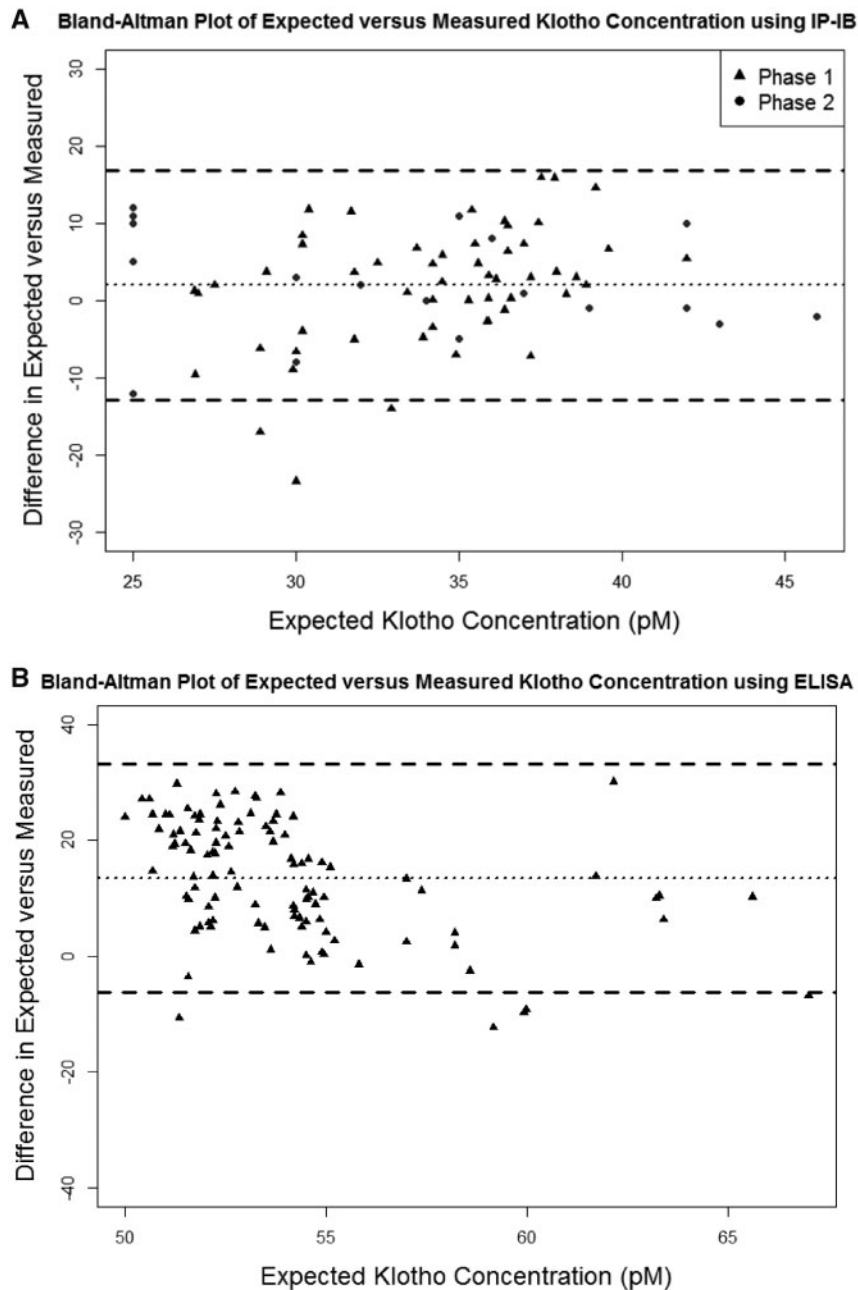
A Bland-Altman Plot of Expected versus Measured Klotho Concentration using IP-IB

B Bland-Altman Plot of Expected versus Measured Klotho Concentration using ELISA

FIGURE 2: Bland–Altman plot representing the agreement between expected and measured Klotho concentration after the addition of known quantities of exogenous Klotho using (**A**) IP–IB assay [Phase 1 (triangles) and Phase 2 (circles)] and (**B**) ELISA (Phase 1 only). Dotted lines represent 95% CI. The IP–IB assay exhibited better agreement of expected versus measured Klotho in a wide range of concentrations when compared with the ELISA. Klotho concentrations are expressed in pM.

may not differentiate between full-length soluble Klotho formed from cleavage of the transmembrane form [40–42], further cleaved Kl1 and Kl2 Klotho fragments, and/or Klotho complexes with other proteins. This constitutes an area of ongoing research that can further advance our understanding of the role of Klotho in human kidney disease.

In conclusion, we report a series of experiments in human sera among a group of healthy volunteers and participants with different etiology and severity of kidney disease. Our experiments revealed the better performance of the IP–IB assay compared with the widely used and available commercial ELISA. Until a superior ELISA is developed, we consider the IP–IB assay,

utilizing the antibodies described in the above experiments, the preferred method for serum Klotho measurements in preclinical and clinical studies.

SUPPLEMENTARY DATA

Supplementary data are available at ckj online.

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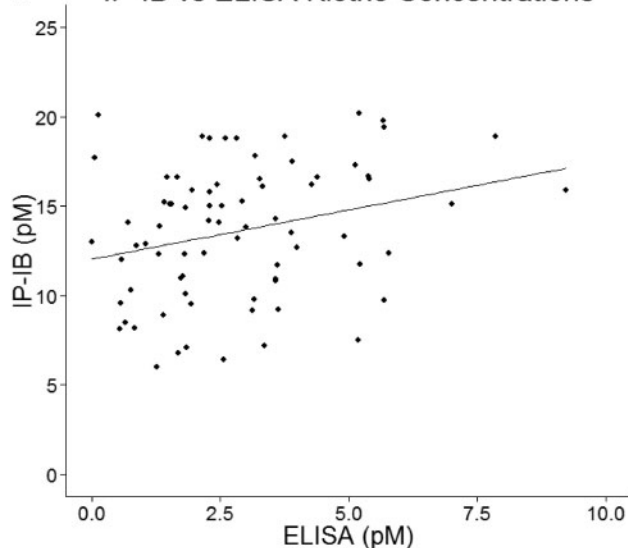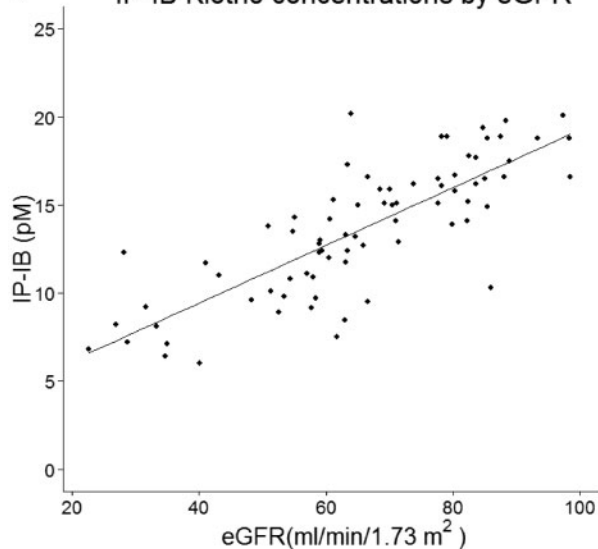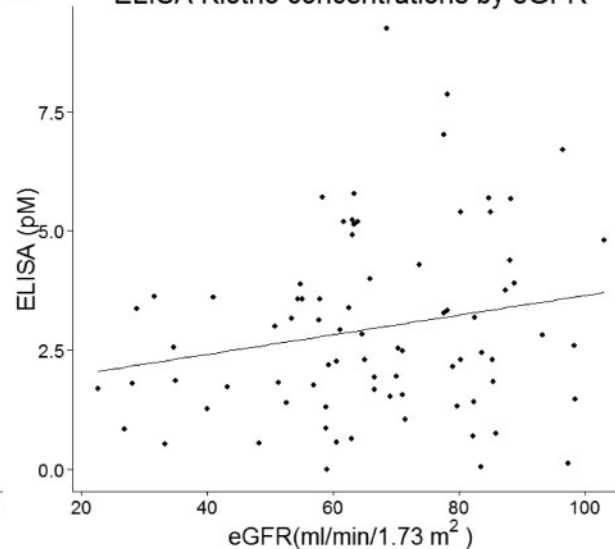Research reported in this publication was supported by the O'Brien Kidney Research Center at the University of Texas

FIGURE 3: (**A**) Correlation between IP–IB and ELISA (R = 0.28, P = 0.01); (**B**) correlation between IP–IB and eGFR (R = 0.8, P < 0.001); (**C**) correlation between ELISA and eGFR (R = 0.18, P = 0.12). All panels represent SPRINT participants (n = 77). Klotho concentrations are expressed in pM.
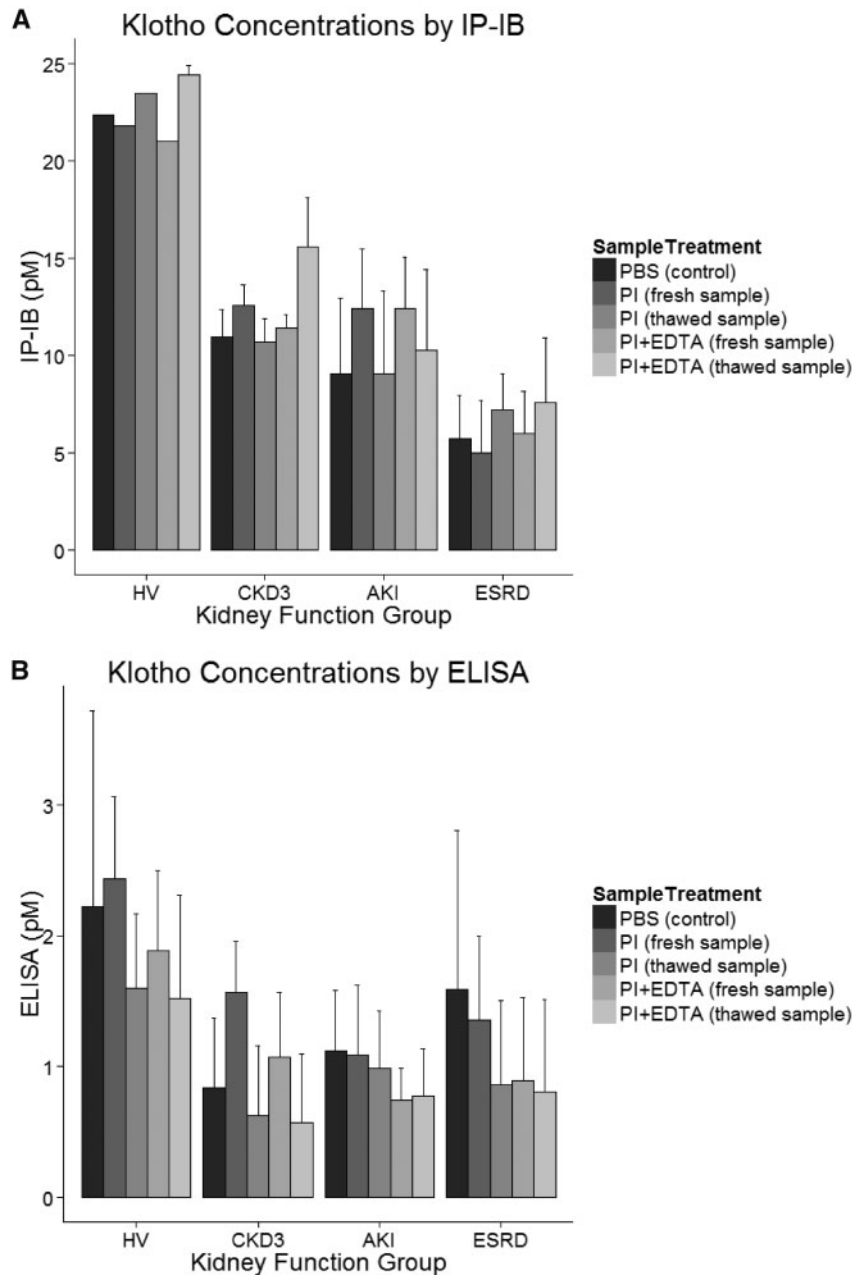
AUTHORS' CONTRIBUTIONS

J.A.N., O.W.M., M.J.S., J.H.I. and D.A.D. designed the study; J.A.N., O.W.M., J.P. and F.G. carried out the experiments; J.A.N. and D.A.D. analyzed the data; J.A.N. and D.A.D. made the figures; J.A.N., O.W.M., S.S.S., M.J.S., J.H.I. and D.A.D. drafted and revised the paper; all authors approved the final version of the manuscript.

FIGURE 4: Mean Klotho concentrations by (**A**) IP–IB and (**B**) ELISA under different prespecified conditions: (i) no additives—PBS (control); (ii) addition of protease inhibitor (PI) cocktail to fresh sample; (iii) addition of PI cocktail to thawed sample; (iv) addition of EDTA + PI cocktail to fresh sample; (v) addition of EDTA + PI cocktail to thawed sample. Error bars represent SD. Klotho concentrations are expressed in picomolar. CKD3 = CKD Stage 3; HV = healthy volunteers.

CONFLICT OF INTEREST STATEMENT

None declared.

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