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Authors

Beshay, Manal
Rhee, Connie M
Kalantar-Zadeh, Kamyar

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Novel monitoring of renal function and medication levels in saliva and capillary blood of patients with kidney disease

Manal Beshay[a], *Connie M. Rhee*[b,c], *and Kamyar Kalantar-Zadeh*[b,c]

Purpose of review
Serum creatinine, urea, and cystatin C are the main biomarkers used to estimate glomerular filtration rates in persons with and without chronic kidney disease (CKD). Frequent measurements of these assays are needed to identify patients with earlier stages of CKD, detect episodes of acute kidney injury (AKI), and monitor for CKD progression. However, the cumbersome, time-consuming nature of conventional laboratory-based kidney function assays limit more frequent monitoring and greater patient self-management.

Recent findings
Noninvasive salivary assessments of creatinine, cystatin C, and urea make it feasible to conduct frequent monitoring of kidney function in point-of-care settings, as well as in nonclinical-care settings such as at home. Additionally, fingerstick sampling can offer an alternative route of blood testing that is suitable for home-based assessments. In this review, we provide an overview of emerging data on various salivary vs. fingerstick blood assessment methods for kidney function; their accuracy in comparison to 'gold-standard' laboratory-based methods; and their respective strengths and limitations in the clinical setting.

Summary
A practical, cost-effective, minimally invasive, multimarker assessment platform has the potential to circumvent the limitation of conventional laboratory blood-based testing approaches, and thereby address a major unmet need in the management of CKD patients.

Keywords
creatinine, cystatin C, fingerstick, saliva, urea

INTRODUCTION

Serum creatinine and urea, and the emerging serum cystatin C, the so-called indicators of kidney filtration function, are clinically proven measures for calculating estimated glomerular filtration rates (eGFRs) in persons with and without chronic kidney disease (CKD). These filtration markers are also measured and serially monitored in patients with acute kidney injury (AKI), including in cases of superimposed AKI on preexisting CKD. Particularly important is the early indication of kidney function deterioration in CKD patients, detection of which can improve disease management of earlier stages of CKD (i.e., stages 3–5 CKD) or in kidney transplant patients. Important target populations include patients at-risk for CKD, such as persons with diabetes, hypertension, and the elderly, as well as those vulnerable to AKI and kidney transplant recipients. Periodic assessment of kidney function is limited by the cumbersome and time-consuming nature of conventional blood-based kidney function assays. Hence, noninvasive salivary assessments of creatinine, cystatin C, and urea have the potential to make it feasible to conduct frequent monitoring of kidney function in point-of-care (POC) settings, and ultimately in nonclinical-care settings such as at home, similar to glucose monitoring (i.e., capillary fingerstick glucose assessments). Additionally, fingerstick sampling can offer an alternative route of

[a]3S Tech, Inc., Torrance, [b]Harold Simmons Center for Kidney Disease Research and Epidemiology, University of California Irvine School of Medicine, Orange and [c]Tibor Rubin Veterans Affairs Long Beach Healthcare System, Long Beach, California, USA

Correspondence to Kamyar Kalantar-Zadeh, MD, MPH, PhD, University of California Irvine, 333 City Blvd West, Suite, 400, Orange, CA 92868. E-mail: kkz@uci.edu

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KEY POINTS

- Frequent measurements of kidney function tests, namely creatinine, urea, and cystatin C, are needed to identify patients with earlier stages of CKD, detect episodes of acute kidney injury (AKI), and monitor for CKD progression.
- The cumbersome, time-consuming nature of conventional laboratory-based kidney function assays limit more frequent monitoring and greater patient self-management.
- Noninvasive salivary and fingerstick blood assessments of creatinine, cystatin C, and urea make it feasible to conduct frequent monitoring of kidney function in point-of-care (POC) settings, as well as in nonclinical-care settings such as at home-based testing.

blood testing that is suitable for home-based assessments. In this review paper, we will provide an overview of salivary vs. fingerstick blood assessments for kidney function.

SALIVA-BASED METHODS OF KIDNEY FUNCTION AND MEDICATION LEVEL ASSESSMENT

Pro's and con's of saliva-based measurements

Saliva testing has attracted the attention of researchers and clinicians alike because of its noninvasiveness, sampling convenience, and rapid analysis. Some preliminary assessments of saliva compared to serum levels for creatinine, cystatin C, and urea have been reported with promising correlations with blood levels [1▪,2,3▪,4▪]. These findings indicate that saliva is a promising medium for monitoring kidney filtration markers in CKD and AKI patients, including kidney transplant recipients, with testing frequency superior to that of blood. This is critically important in such patients because of their inherent risk of CKD progression, as well as unexpected episodes of kidney function deterioration. Because of the drawbacks and limitations of existing testing methods for POC kidney function monitoring, development of novel techniques for evaluating kidney function are critical targets, including adoption of noninvasive biomarkers for more frequent kidney function assessment of CKD patients.

To overcome the limitations of current blood-based testing approaches, and building upon saliva's usefulness as an alternative for measuring creatinine, with cystatin C and urea integrated on the same panel, salivary platforms have been proposed to (1) accurately measure salivary levels of target markers (with accuracy of at least 85%), and (2) validate salivary levels with serum-based measurements to establish their correlation and compensate for possible physiologic and measurement biases.

Most current predictive assays and laboratory analyses rely on the detection of biochemical species (biomarkers) excreted into the bloodstream or urine. Large-scale blood and urine sampling are logistically impractical in a nonclinical setting, requiring large and expensive equipment, or multiple sample preparation steps by trained personnel. This makes saliva an attractive alternative diagnostic fluid. Whole saliva is a mixture of the secretions of the major and minor salivary glands as a complex fluid containing an entire library of hormones, proteins, enzymes, antibodies, antimicrobial constituents, and cytokines. These constituents pass from the blood into the saliva by transcellular and passive intracellular diffusion, by active transport, paracellular routes by extracellular ultrafiltration within the salivary glands, or through the gingival crevice [5,6]. Research has unveiled large numbers of medically valuable saliva biomarkers for disease conditions, including cancer and autoimmune, viral, bacterial, cardiovascular, and metabolic diseases [7]. CKD markers have been studied by many researchers [2,10–13], with a strong correlation of creatinine and urea levels in saliva compared to blood levels (Fig. 1).

Salivary urea

Urea measurement in saliva has been studied for decades. It has been shown that urea can be detected in saliva, although the sensitivity was rather low compared to serum levels [8]. Secretion of urea in saliva is significantly affected by age and body mass index, but mostly correlates with serum urea [9]. Higher correlations between serum and saliva concentrations of urea have been observed in CKD patients ($r = 0.99$) than in healthy individuals ($r = 0.74$). This may be due to the substantially lower levels of urea in the healthy population's saliva. However, a three- to five-fold increase in urea levels was noticed in CKD patients' saliva, at levels >90 mg/dL, compared to healthy controls with levels <20 mg/dL. Salivary urea levels were also reported to be more sensitive markers for CKD patients, particularly in earlier stages of kidney disease [10].

Salivary creatinine

Similarly, creatinine secretion is significantly lower in healthy subjects than in CKD patients, with about

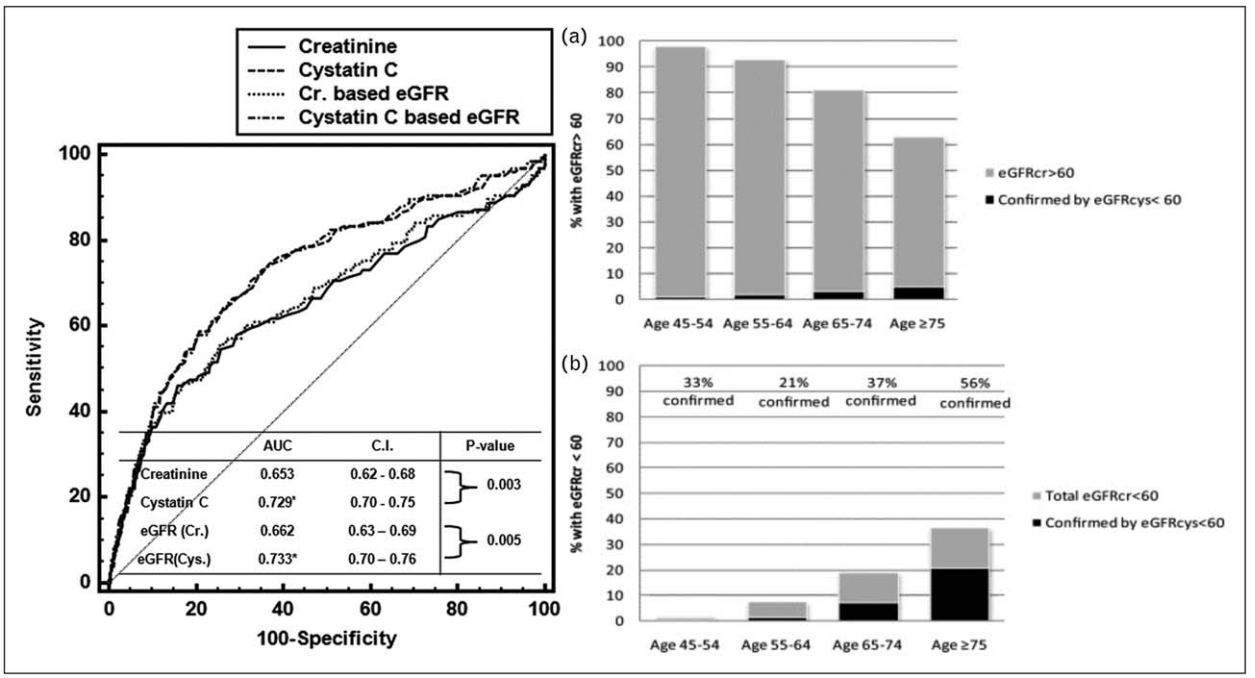

FIGURE 1. Previously reported ROC for creatinine vs. cystatin C with higher AUC (0.729) with cystatin C compared to creatinine: (a) Serum cystatin C showed better diagnostic accuracy than serum creatinine at the cutoff value of <60 mL/min./1.73 m^2 of creatinine clearance [16]. (left panel); (b) Prevalence reported by Shlipak *et al.* of eGFR ≥60 mL/min/1.73m^2, and the proportion missed by creatinine but detected by cystatin C varies by age. The overall prevalence of eGFR <60 mL/min/1.73 m^2 by creatinine, and proportion confirmed by cystatin C varies by age (right panel) [16]. AUC, area under curve; ROC, Receiver Operating Characteristic; eGFR, estimate glomerular filtration rates.

a ten-fold increase in both serum and saliva levels. However, more variability was reported in creatinine correlation in serum than in saliva [11]. This was attributed to creatinine, as a large molecule, having low lipid solubility, which limits its circulation in intercellular junctions and into saliva.

Salivary cystatin C

Cystatin C is a small protein produced at a constant rate by all nucleating cells. It is filtered by the glomerulus, which mainly determines its levels in the bloodstream, hence its usefulness as an indicator for eGFR. Cystatin C activity and levels in whole saliva have been assessed in prior studies [12]. Nonnegligible levels were found in whole saliva from nonsmoking subjects (averaged at 0.7 μg/mL). Additionally, there was no correlation reported with gingival inflammation up to 6 weeks, which suggests no bias from periodontal disease in the proposed enhanced immunoassay-based lateral flow (ELF) studies.

Cystatin C as a reliable marker of chronic kidney disease (with and without creatinine)

Unlike creatinine, cystatin C levels are unaffected by age, muscle mass, sex, or race. As reported by a number of researchers [13,14], cystatin C yields more robust eGFR levels than creatinine-based eGFR (at left in Fig. 1). It is therefore considered a better test for the presence of significant CKD, particularly in stages 3 or 4 CKD (according to the KDIGO 2012 guidelines) [15], in which fluctuation of GFR can cause patients' eGFR to decline faster than before, and specifically in elderly CKD patients who suffer from constant muscle mass loss that affects creatinine measurements. In a study conducted by Shlipak *et al.* [16] examining 11,909 participants in the Multi-Ethnic Study of Atherosclerosis and the Cardiovascular Health Study, mortality, cardiovascular events, heart failure, and ESRD risk among categorized groups based on the biomarkers estimated (eGFR <60 mL/min. per 1.73 m^2) creatinine only, cystatin C only, both, or neither (Fig. 1).

Different testing approaches

Rapid assays are known for their limited sensitivity in comparison with ELISA, chemiluminescence, and qPCR kits, because of their lack of incubation time. Laboratory immunoassay techniques are well established and widely used for monitoring a number of markers and biochemical species in various body fluids. It is now practical to develop portable

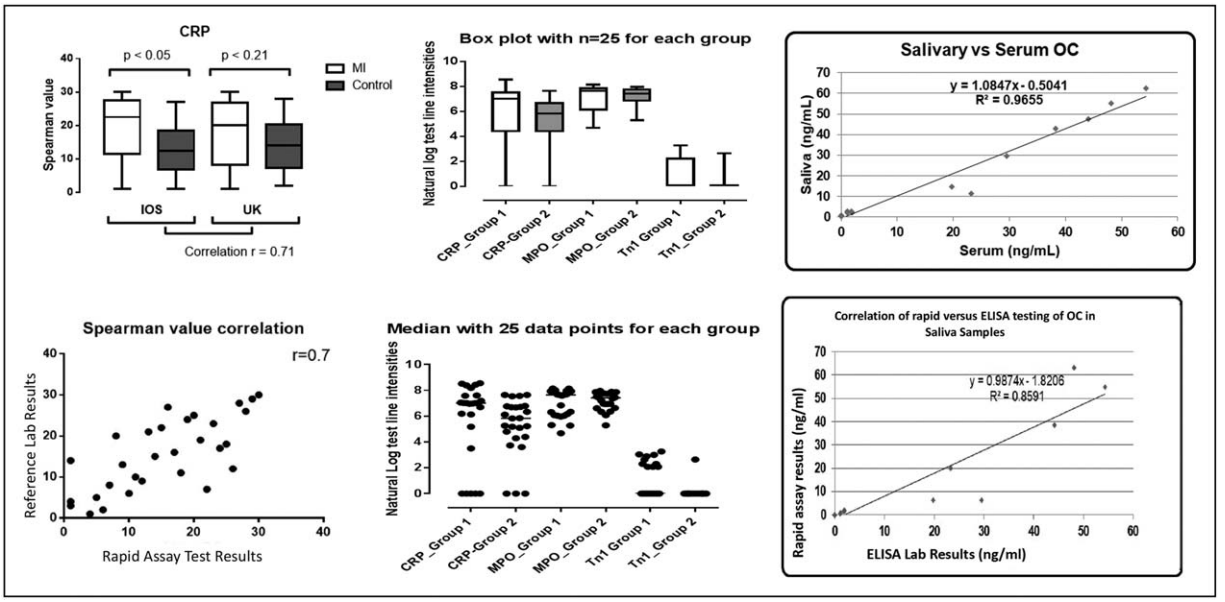

FIGURE 2. Correlation between CRP levels assayed using ELISA vs. LFA rapid assays (left panel); Spearman's comparison between CRP, MPO, and TnI levels in myocardial infarction (group 1) and control patients (group 2) (middle panel); correlation of osteocalcin salivary assay with serum (top) and saliva (bottom) lab measurements (right panel). CRP, C-reactive protein; ELISA, enzyme linked immunoassay; LFA, lateral flow assay; MPO, myeloperoxidase.

instruments for immunoassays and DNA-based assays, and many technologies have been employed for this purpose. One product that is miniaturized and is relatively simple to use, is the Triage System from Biosite (San Diego, CA). This technology transports reagents by microcapillary action. Detection is by fluorescence energy transfer and has been applied by Biosite in products to triage patients suffering from heart dysfunction.

The rapid lateral flow assay (LFA) developed by Response Biomedical Corp. (Burnaby, Canada) is also suitable for multiplexed immunoassays. This technology is similar to conventional lateral flow immunoassays, but with fluorescent labels that achieve semi-quantification measured with a miniature fluorescence reader. Additionally, super paramagnetic particles have been used in some LFA, such as those being developed by Quantum Design (San Diego, CA), though with limited sensitivity.

Other groups have developed miniaturized devices based on surface enhanced Raman spectroscopy, up-conversion phosphor-based immunoassay, and portable flow cytometry, but none of these has reached the market [17,18]. POC tests are available to measure established biomarkers, but minimally invasive, quantitative, rapid testing with sample treatment and sensing assays in a miniaturized format with the least user interface is the target of this proposed development, to fill a technology gap in the monitoring of immunosuppressant drugs.

Beshay *et al.* have developed a number of saliva-based biomarker assay panels based on rapid flow test platforms for qualitative and quantitative detection, including testing for bone metabolism and cardiac biomarkers, and alcohol and drugs of abuse. Salivary diagnosis has been an active field for many researchers and is considered the future fluid for general health assessment of systemic illness. Low levels of biomarkers, food effects, saliva flow, viscosity, and sympathetic and parasympathetic effects have been considered as the main challenge in utilizing saliva. Examples of successful clinical validation studies by the abovementioned investigative team are shown in Fig. 2.

Salivary renal panel results

ELF assays for the quantitative measurement of three kidney filtration markers, creatinine, cystatin C and urea have been developed for use with human saliva using highly selective reagents for optimum specificity. The ELF assays show good shelf-life stability at room conditions whereas yielding a coefficient of variation (CV) of <10% through inter- and intra-assay studies (Fig. 3).

Beshay *et al.* have used healthy donor saliva samples spiked with known levels of creatinine and urea. Standard calibration curves (SCCs) for each marker were established with nonlinear 4-parameter logistic curve fitting in triplicate

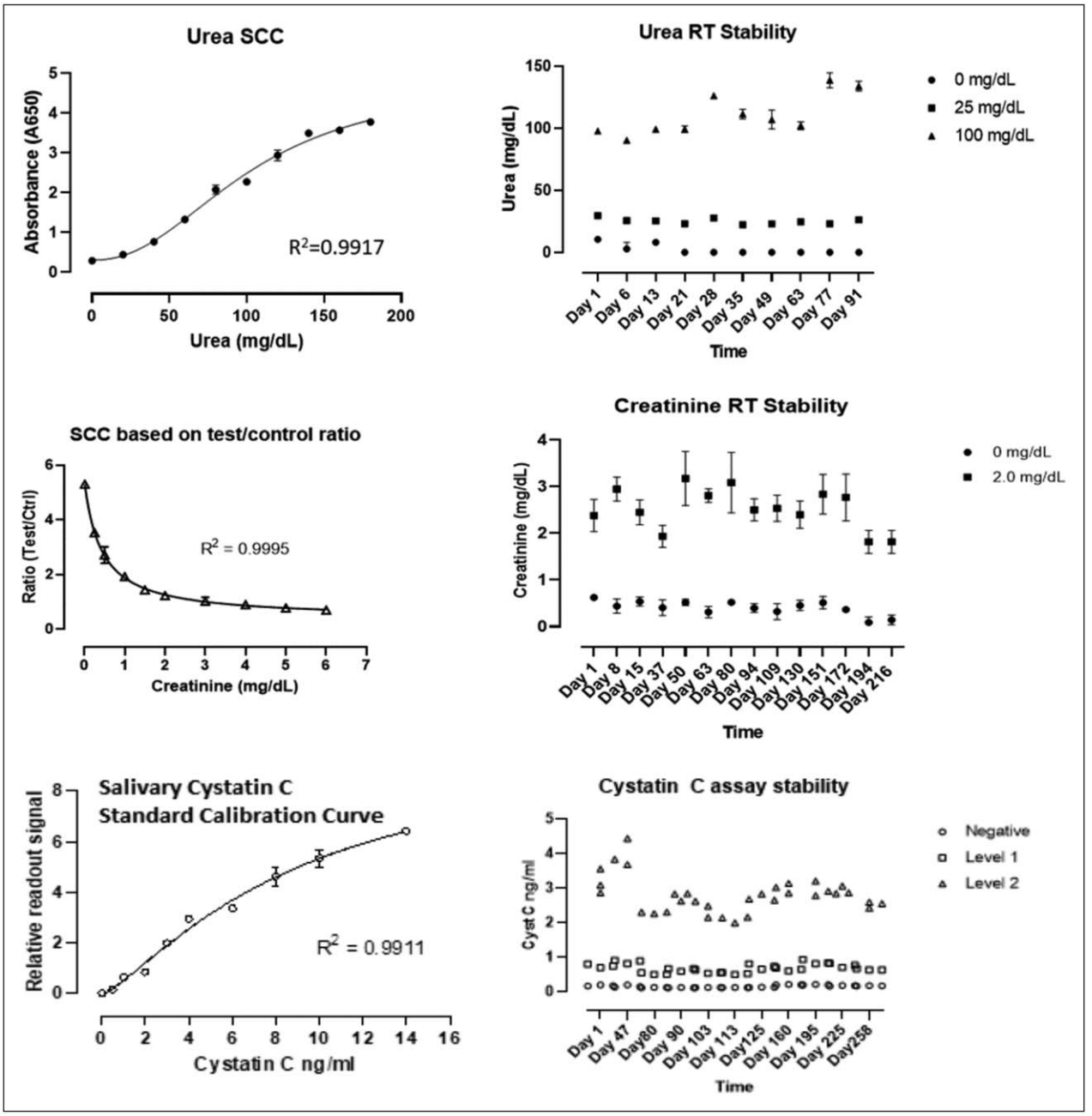

FIGURE 3. Salivary renal markers (urea, creatinine, and cystatin C) calibration (left) and room temperature storage stability (right). All stability studies are ongoing, and have demonstrated promising performance for almost one year.

measurements at each spiked concentration level. Accuracy/fit of the SCC was assessed as the coefficient of determination (R^2). Intra-assay repeatability was assessed as CV, and studies of inter-assay repeatability over time (over ~8 days) examined reproducibility of the whole experimental protocol.

The resulting SCC fitted to relative optical intensities (ratio of test to control lines vs. spiked urea (0–180 mg/dL), creatinine (0–6 mg/dL), and cystatin C (0–14 ng/ml) were excellent ($R^2 = 0.992$, 0.999, and 0.991, respectively). Assessment of intra-assay variation showed that repeatability was very good, with CV <15% for creatinine, and CV < 10% for cystatin C and urea throughout the dynamic range of measurements. Assessment of inter-assay variation (measurements over 8 days) showed that reproducibility was acceptable, with CV < 10% for creatinine, and <13% for cystatin C and urea throughout most of the dynamic range. Preliminary assessment of long-term reproducibility (stability) up to 91 days

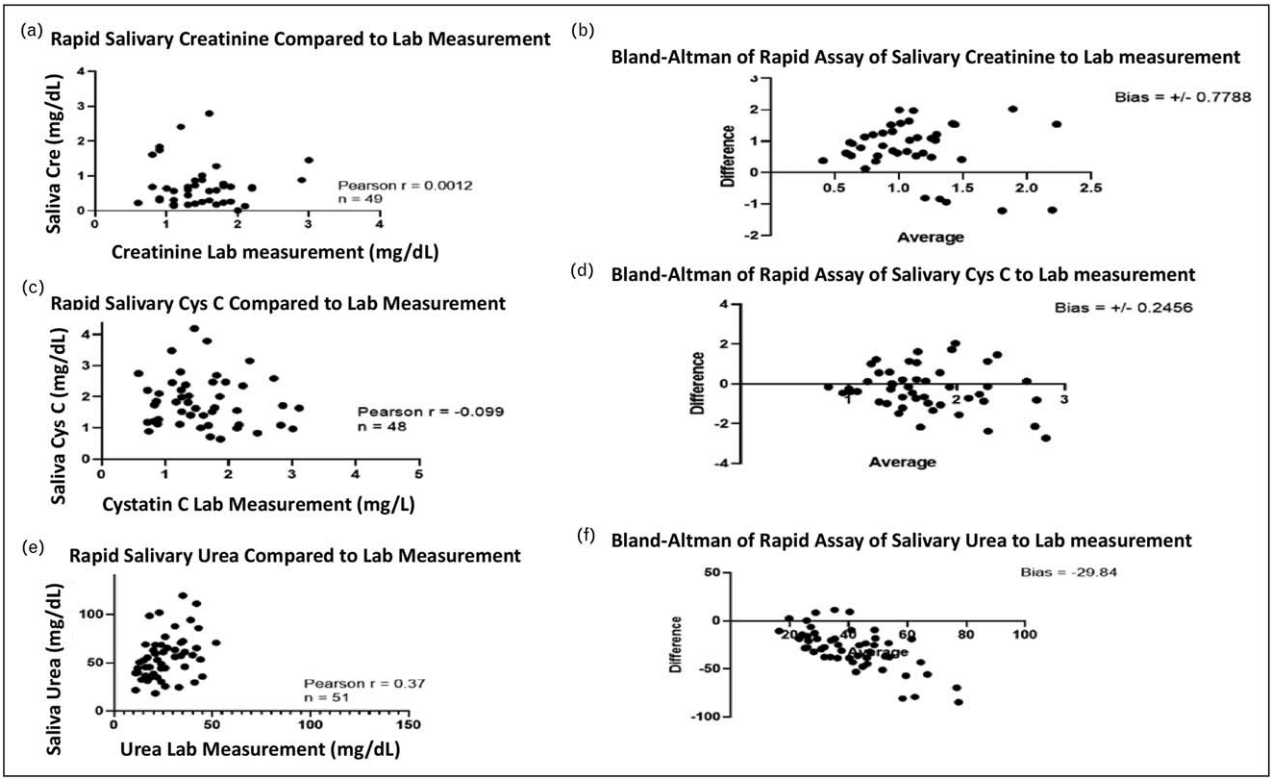

FIGURE 4. Correlation of saliva markers to laboratory analyzed levels for (from top to bottom) creatinine, cystatin C, and urea.

for the urea assay, 216 days for the creatinine assay, and 258 days for the cystatin C assay indicated stable performance.

These results, along with the measurement range testing as outlined in each assay calibration curve (Fig. 3), indicated that creatinine, cystatin C, and urea can be measured in human saliva with acceptable ELF assay characteristics, including accuracy, repeatability, reproducibility, and long-term stability. A clinical pilot study validation to date by Beshay *et al.* included 72 patients with earlier stages of kidney disease (i.e., stages 1–2 CKD).

Saliva measurements: correlation to serum measurement of the same subject

Beshay *et al.* have also compared ELF assay measurement of the three analytes in saliva samples to standard laboratory measurements of serum samples obtained from the same CKD subjects. In a pilot analysis of 40, 48, and 51 saliva samples for creatinine, cystatin C, and urea, respectively, Pearson correlation analysis shows no significant correlations for creatinine and cystatin C, and slightly positive correlations for urea with $R = 0.37$ (Fig. 4). Bland-Altman analysis showed biases of 0.7 mg/dL, 0.2 mg/L, and 20.84 mg/ml for creatinine, cystatin C, and urea, respectively (Fig. 4). However, interpretation of these pilot data may be limited by the number of samples and the limited reference range of analytes in the sample (i.e., analysis of a range of serum creatinine from 1 to 2 mg/dL, instead of a target range of 0.5–6 mg/dL). Considering the highly correlated data when comparing the performance of this assay to that of commercial ELISA in measuring saliva samples, and also the ELF assay to standard laboratory measurements on the same set of serum samples (described in the next section), it can be concluded that this assay performed relatively well despite the low levels of markers in these 'relatively healthy' patient samples. Further study with the measurement of a broader range of serum creatinine levels to better understand the correlation between saliva and serum measurement is needed.

CAPILLARY BLOOD-BASED METHODS OF KIDNEY FUNCTION AND MEDICATION LEVEL ASSESSMENT

Pro's & con's of capillary blood-based measurements

A fingerstick-based assay, including blood sampling, to be applied to the ELF assay platform, which is housed in a customized enclosure for rapid

quantification has been innovated by Beshay *et al.* In a typical LFA test, the sample to be analyzed is applied to the sample membrane, where capillary action pulls it through the membrane to the reagent pad containing detector particles. In their novel ELF assay platform, with completely dried reagents, reconstitution occurs when the sample is applied, which then flows onto a coated membrane, producing highly fluorescent lines when the target markers are present.

Different testing approaches

Kidney function assessment using serum creatinine, urea, and cystatin C is currently focused on infrequent laboratory measurements that require invasive blood draws, followed by laborious and time-consuming blood specimen processing and reporting. Other clearance agent assessment has been used where Iohexol has been shown to be a safe and reliable reagent in the measurement of GFR. However, this approach requires that subjects remain in the clinic for up to five hours. Niculescu-Duvaz *et al.* have described a highly valuable alternative in the outpatient finger-prick method for the determination of iohexol GFR [19]. In this review, we describe a capillary approach for kidney function panel assessment that utilizes the known three markers, creatinine, urea, and cystatin C, in fingerstick capillary sampling compared to laboratory assessment from the same subjects.

Serum measurements: correlation of enhanced immunoassay-based lateral flow assay to laboratory measurements with blood samples

To better understand the performance of our ELF assay, Beshey *et al.* have validated ELF assay performance against standard laboratory measurements of the same set of serum samples. A subsequent ELF assay was custom-designed specifically for serum samples, which differ slightly from saliva. By means of Pearson correlation and Bland-Altman analysis, they assessed the correlation and bias, respectively, of ELF assay measurements to laboratory standard measurements from a set of 70 serum samples obtained from CKD patients at the University of California Irvine obtained by nephrologists (KKZ, CMR). In addition, Receiver Operating Characteristic (ROC) curve analysis was also applied to assess the medical diagnostic value of the ELF assay. For this purpose, ELF assay measurements of 70 CKD samples and 20 healthy reference samples were used to determine the ROC curve and area under curve (AUC) of the assay.

The ELF cystatin C assay measurements showed excellent correlation to standard laboratory measurements, with Pearson $r = 0.94$ (Fig. 5). Bland-Altman analysis showed a bias of -0.5 mg/L for the ELF assay, which could be adjusted if it is consistent when monitoring the assay on a broader concentration set of samples (Fig. 5). The ROC analysis showed excellent diagnostic value, with AUC = 0.83, which has potential to discriminate healthy from CKD subjects (Fig. 5).

Similar to cystatin C measurements, Beshay *et al.* have also observed excellent correlation to standard laboratory measurements of blood urea nitrogen, with Pearson correlations of $r = 0.86$ (Fig. 6). Bland-Altman analysis showed a bias of -1.34 mg/L for the urea ELF assay. The ROC analysis showed excellent diagnostic value, with AUC = 0.85, which has potential to discriminate healthy from CKD subjects.

FUTURE APPLICATIONS AND RESEARCH DIRECTIONS

A prospective clinical pilot trial is being planned to be supplemented with 100 additional subjects

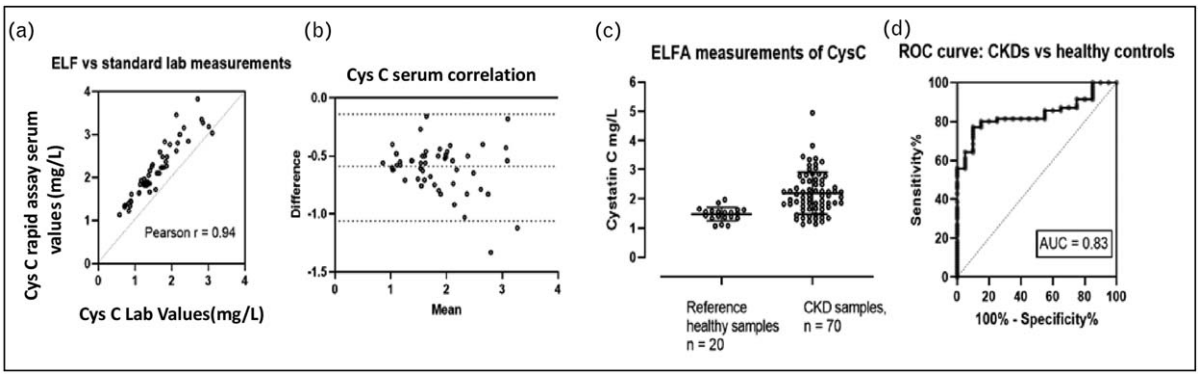

FIGURE 5. Correlation of ELF assay serum-based markers to laboratory analyzed levels for cystatin C. ELF, enhanced immunoassay-based lateral flow.

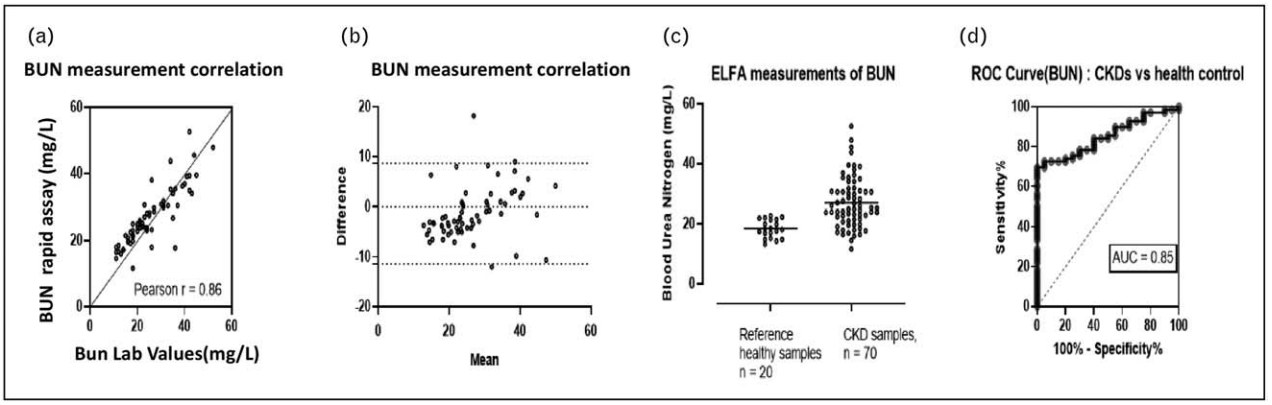

FIGURE 6. Correlation of ELF assay serum-based markers to laboratory analyzed levels for urea. ELF, enhanced immunoassay-based lateral flow.

targeting stages 3–5 CKD. As in the previous analysis process, the assayed fingerstick capillary samples will be compared to laboratory analysis of serum samples for creatinine, cystatin C, and urea to assess agreement of measurements between the two methods using the Bland-Altman method, with a target LoA of 95%. Testing for bias, within-subject, between-subject, and analytical test variation and reliability assessed by intra-class correlation coefficient will also be reported. Other parameters for analysis include age, weight, history of diabetes or hypertension, prior eGFR measurement, time of POC visit, and the time of laboratory testing for target markers.

CONCLUSION

More frequent monitoring and greater self-management enabled by novel POC and home-based kidney function testing methods has the potential to ameliorate the progression of CKD. Hence, a practical, cost-effective, minimally invasive, multimarker assessment platform has the potential to circumvent the limitation of conventional laboratory blood-based testing approaches, and thereby address a major unmet need in the management of CKD patients.

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Conflicts of interest

There are no conflicts of interest.

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- ■ of special interest
- ■■ of outstanding interest

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