Assessment of Measurement of Salivary Urea by ATR-FTIR Spectroscopy to Screen for CKD

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Key Points
• Salivary urea can be quantitated rapidly and accurately by infrared spectroscopy in the range relevant to that found in patients with CKD.
• The measured urea concentrations allowed discrimination of patients with stages 3, 4, or 5 CKD from each other and from healthy controls.
• This provides a proof of concept that the technique could be developed as a novel, sensitive, and cost-effective screening method for CKD.

Abstract
Stages of CKD are currently defined by eGFR and require measurement of serum creatinine concentrations. Previous studies have shown a good correlation between salivary and serum urea levels and the stage of CKD. However, quantitative salivary urea assays in current clinical use require costly and labor-intensive commercial kits, which restricts the advantage of using saliva and limits wider applicability as a quick and easy means of assessing renal function. Attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy has been shown to provide a potentially straightforward, reagent-free method for the identification of a range of disease-related biomarkers and is in current clinical use for analyses of the chemical composition of kidney stones. We assessed the feasibility of ATR-FTIR spectroscopy as an alternative method to measure salivary urea in patients with different stages of CKD. The ATR-FTIR spectra of dried saliva samples from six healthy controls and 20 patients with CKD (stages 1–5) were analyzed to provide their urea concentrations. The lower limit of detection of salivary urea by the ATR-FTIR spectroscopy method was 1–2 mM, at the lower end of the clinically relevant range. Statistically significant differences in salivary urea concentrations were demonstrated between healthy subjects (4.1±0.5 mM) and patients with CKD stages 3–5 (CKD stage 3, 6.8±0.7 mM; CKD stage 4, 9.1±1 mM; CKD stage 5, 14.8±1.6 mM). These salivary urea concentrations correlated well with serum urea levels in the same patients measured by an automated analyzer (Spearman rank correlation coefficient of 0.71; *P<0.001). The ability of the method to detect and stage CKD was assessed from the sensitivity and specificity parameters of a receiver operating characteristics (ROC) curve analysis. This proof-of-concept study demonstrates that quantitation of salivary urea by ATR-FTIR spectroscopy could provide a viable tool for rapid and cost-effective diagnosis of stages 3–5 CKD.

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Introduction
CKD has an estimated prevalence of 8%–16% worldwide and has been reported to contribute increasingly to mortality globally (1) and to be a significant economic burden. Occurrence of CKD is predicted to rise sharply, and it is estimated that 5.4 million people worldwide will be receiving RRT by 2030 (2). CKD causes significant morbidity and mortality, in part due to a lack of unique markers for diagnosis and delay in initiation of treatment. Many deaths caused by CKD may be preventable through more effective screening of high-risk populations, such as patients with diabetes, hypertension, cardiovascular disease, and HIV, or those with limited access to healthcare.

Determination of renal function in current clinical practice requires measurement of serum creatinine, or other biomarkers of kidney function in the blood, to estimate GFR using validated serum creatinine–based formulae, such as the Modification of Diet in Renal Disease (MDRD) equation (3). eGFR and albuminuria are the principal parameters used to define and stage CKD, according to Kidney Disease Improving Global Outcomes criteria (4). Calculation of eGFR is based on serum creatinine measurements and requires venepuncture expertise and facilities for blood sample collection and transfer to a laboratory for later analysis. Venepuncture can be difficult in some patients, especially children, and is associated with a small risk of infection. The process becomes more challenging outside a hospital or clinical setting, particularly in resource-poor countries where there may be a lack of trained personnel and equipment. Hence, recent

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efforts have focused on the potential use of venepuncture-free biofluids, such as saliva, for more rapid “near-patient” testing and detection of kidney disease.

A number of studies have shown that various salivary components, including urea, cortisol, creatinine, uric acid, and phosphate, correlate positively with the severity of CKD and are, therefore, potential indicators of renal function (5–8). Of these, salivary urea has been particularly addressed because it correlates positively with elevated serum urea levels found in patients with CKD (9,10). Hence, salivary urea is a potential noninvasive alternative biomarker for detecting CKD. Nevertheless, current methods for detection and quantitation of salivary urea have some limitations. One recent, simple method uses dipsticks to measure salivary urea nitrogen colorimetrically. However, this method is semiquantitative and was shown to be less sensitive in patients with milder kidney disease when compared with serum analysis (11). In contrast, the quantitative kits for detection of salivary urea are much more expensive due to the reagents, which need to be refrigerated, and also require substantially more time to perform the assay. The development of a more rapid and cost-effective method for analyzing salivary urea can improve CKD diagnosis, either by providing an additional parameter or as a potential alternative where blood testing is less convenient or unavailable.

Attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy has been assessed as a diagnostic method for a wide range of diseases, including cancer and kidney disease (12–16). It is a nondestructive analytical technique that can be used with complex biologic materials, including cells, tissues, or biofluids, often without requiring chemical or physical pretreatment. Analyses of the resulting infrared spectra can reveal signatures of disease biomarkers (17). For example, ATR-FTIR spectroscopy can be used to quantitate creatinine and urea in both blood and urine, with an accuracy equivalent to that of assays in routine clinical use (18–21).

The aims of this study were (1) to determine whether salivary urea can be measured in the clinically relevant range by ATR-FTIR spectroscopy, and (2) to test the performance of this method to diagnose and stage CKD.

Materials and Methods
Saliva Sample Collection

Unstimulated saliva samples were collected from six healthy adults and 52 adult patients with CKD attending the Nephrology Outpatient Department of the Royal Free Hospital (London, United Kingdom) between March and August 2018. Subjects were asked to rinse their mouth with water and wait for 15 minutes before depositing 1–3 ml of saliva into plastic collection tubes (in practice, the smallest volume of saliva practicable for centrifugation and collection of a 5-μl test aliquot was 100 μl). For convenience, saliva samples were stored at −20°C after collection until salivary urea was measured, although preliminary tests showed no difference in using fresh samples stored at room temperature for at least 4 hours before analysis. All participants provided written informed consent at enrollment (ethics number 7727 of the Royal Free London National Health Service Foundation Trust Research and Development Committee).

ATR-FTIR Spectroscopy

ATR-FTIR spectra were recorded with a Bruker IFS 66/S FTIR spectrometer equipped with a potassium bromide beam splitter, a liquid nitrogen–cooled mercury-cadmium telluride detector, and an ATR microprism (SensIR, 3 reflection silicon microprism with zinc selenide optics). Spectra were recorded at room temperature versus a clean prism background in the 4000–600 cm⁻¹ range. Power spectra were computed by Fourier transformation of 1000 (background; clean crystal surface) or 500 (saliva sample) averaged interferograms at 4 cm⁻¹ resolution and were converted to absorbance spectra by calculation as log₁₀(Ibackground/I_sample) at each wave number.

Generation of Salivary Urea Calibration Curve and Estimation of Detection Limit

Saliva was provided by a healthy volunteer with normal urinalysis and no personal or family history of kidney disease. A 100-mM urea (purity ≥99.5%, Sigma-Aldrich) stock solution was prepared in ultra-pure water and diluted into the saliva to obtain 500-μl saliva samples with 0–20 mM added urea. Samples were centrifuged at 10,000× g, for 10 minutes at 4°C to remove any particulate material. Aliquots (5 μl) of the resulting supernatants were dried on the ATR prism with a gentle stream of nitrogen gas (200 ml/min) and sample spectra were recorded (Figure 1A). The areas of the 1449 cm⁻¹ urea peaks and were integrated between limits of 1481 cm⁻¹ and 1435 cm⁻¹ and plotted versus added urea concentration to generate an initial calibration curve. The y axis intercept (peak integral of healthy saliva with no added urea) was then used to determine the amount of endogenous urea present, which, in this case, was 3.5 mM. Hence, this endogenous urea was factored into the calculated total final concentrations of samples with added urea (0–20 mM) and were 3.5–22.8 mM. These concentrations provided the plot of peak area versus total urea concentration (Figure 1B), which was used for all subsequent measurements. The accuracy and reproducibility of the plot indicated that concentrations as low as 1–2 mM urea should be detectable. It should be noted that a new calibration curve would be required for other spectrometer configurations, although this has to be done only once for each new device.

Quantitation of Salivary Urea in Controls and Patients with CKD

After thawing, 500-μl aliquots of normal and patient saliva samples were transferred to 1.5 ml Eppendorf tubes and centrifuged at 4°C for 10 minutes at 10,000× g (Eppendorf 5415R bench microcentrifuge). An aliquot (5 μl) of the supernatant was placed on the ATR prism, dried with a gentle stream of nitrogen gas (200 ml/min), and spectra were recorded after stabilization of the dried state. Each sample was analyzed in triplicate and salivary urea was quantitated using the calibration plot of peak area versus total urea in Figure 1B.
Serum Urea and Creatinine Measurement and CKD Staging

Serum urea and creatinine were measured by an automated analyzer (Cobas 8000 modular analyzer series; Roche), calibrated according to the manufacturer’s instructions. Serum creatinine, alongside other variables including age, sex, and race, were used to estimate GFR using the MDRD equation and, hence, define stages of CKD (3,4).

Statistical Analyses

Salivary urea concentrations were plotted according to CKD stage and differences between CKD stages were assessed with a nonparametric one-way ANOVA Kruskal–Wallis with Dunn multiple comparison test. Correlation between serum and salivary urea was assessed by calculation of the Spearman rank correlation coefficient. Diagnostic performance of salivary urea to detect stages of CKD was assessed using receiver operating characteristic (ROC) analysis. The optimal threshold concentration of salivary urea to diagnose CKD was determined according to the Youden index (22), and sensitivity and specificity at this threshold are reported. Analyses were performed with GraphPad Prism version 7 (www.graphpad.com). A P value of <0.05 was considered statistically significant.

Results

ATR-FTIR Spectra of Dried Saliva and Quantitation of Salivary Urea

Figure 1A shows the ATR-FTIR spectra of a dried saliva sample from a healthy individual with different concentrations of added urea (0, 1, 2, and 3 mM), together with that of a dried sample of pure urea. Pure urea displays a prominent band at 1464 cm$^{-1}$ (attributable to a v$_{as}$[C--N] mode) when dried in water, but this can be shifted to a lower wave number when urea is dried in complex mixtures, probably due primarily to retention of water molecules (23). This is the case for urea in dried saliva, where this band is centered around 1449 cm$^{-1}$ (Figure 1A). An increase in intensity of this 1449 cm$^{-1}$ urea band is seen in the dried saliva spectra with increasing additions of urea above 1 mM. This urea peak was chosen for quantitation because it is intense and has little interference from other materials in the saliva. A calibration curve was constructed using a healthy control saliva sample with added urea in the physiologically relevant 1–20 mM range, with urea quantitated from the area of the 1449 cm$^{-1}$ peaks integrated between 1481 cm$^{-1}$ and 1435 cm$^{-1}$ (Figure 1B). The peak area of the control saliva with no added urea is 0.8 (y axis intercept in [B]), corresponding to an endogenous concentration of 3.5 mM urea, a concentration within the expected range for healthy saliva samples. Hence, total urea is added urea + 3.5 mM and the plot with these total urea concentrations (lower x axis) was used to determine the total urea concentrations in the patient samples. ΔA, change in absorbance.

Patient Cohort

Of the 52 saliva samples collected from patients with CKD, 32 had either an insufficient saliva sample (n=6) for
analysis or were contaminated with blood (n=7), sputum (n=7), or foreign material (n=12), and were excluded before ATR-FTIR analysis. The remaining 20 patient samples were included in this pilot study, alongside saliva samples from six healthy controls. Demographic and clinical data of the patients with CKD are outlined in Table 1. Causes of CKD were IgA nephropathy (n=5), diabetic nephropathy (n=5), lithium toxicity (n=2), hypertension (n=2), SLE (n=1), minimal change disease (n=1), membranous nephropathy (n=1), membranoproliferative GN (n=1), previous nephrectomy (n=1), reflux nephropathy (n=1), obstructive uropathy (n=1), and unknown (n=1).

Quantitation of Salivary Urea from Normal Subjects and Patients in Different CKD Stages by ATR-FTIR Spectroscopy

Salivary urea concentrations (Supplemental Table 1) were estimated by ATR-FTIR spectroscopy from areas of the 1449 cm⁻¹ urea peak referenced to the calibration plot of Figure 1B. The salivary urea concentrations in controls and in patients with different stages of CKD are shown in Figure 2. There were consistent differences in urea concentration ranges between the control and CKD stage 3–5 groups. The mean±SD values of salivary urea for each group were as follows: control, 4.1±0.5 mM; CKD stage 3, 6.6±0.3 mM; CKD stage 4, 8.7±0.4 mM; and CKD 5, 14.3±0.6 mM, with significance of differences between groups (shown in Figure 2). For CKD stages 1/2 (where eGFR was either normal or very slightly lower than normal), there was only a small increase in average salivary urea levels compared with the healthy control group; a greater sample size would be required to determine whether this difference is statistically significant.

Correlation between Salivary and Serum Urea Levels in Patients with CKD

Serum urea of patients with CKD was measured by an automated analyzer at the same time as the saliva samples were taken. Serum and salivary urea concentrations were positively correlated (Spearman rank correlation coefficient of 0.71; P<0.001; Figure 3). The automated analyzer also provided serum creatinine concentrations, and these also showed the expected positive increase with advancing stages of CKD (Table 1).

Diagnostic Performance of Salivary Urea

The performance of salivary urea measurement by ATR-FTIR spectroscopy for distinguishing different stages of CKD was assessed by ROC curve analyses, which provide estimates of the ability to resolve groups as the threshold value of the distinguishing parameter is varied (22). The areas under such curves range from one for perfect resolution to 0.5 for zero resolution. Firstly, an ROC plot was constructed to test the resolution of a combined group of patients with stages 3–5 CKD (i.e., eGFR <60 ml/min) from a control group of healthy individuals and patients with stage 1 and 2 CKD (i.e., eGFR >60 ml/min) (Table 2).

Table 1. Patient demographic and clinical data at the time of saliva collection

| CKD Stage | Patients, n (Male/Female) | Age, yr, Mean±SD | Serum Urea, mM, Mean±SD | Serum Creatinine, μM, Mean±SD |
|-----------|---------------------------|------------------|------------------------|-------------------------------|
| CKD 1 and 2 | 2/3                       | 42±17.1          | 7.1±1.2                 | 78.2±14.9                     |
| CKD 3     | 5/0                       | 65.2±8.4         | 12.9±2.9                | 159±12.5                      |
| CKD 4     | 5/0                       | 57.6±19.7        | 15.8±1.6                | 298±76.3                      |
| CKD 5     | 1/4                       | 71±5.3           | 23.1±2.1                | 434.2±330                     |

Discussion

Although measurement of BUN is still often used together with serum or plasma creatinine to assess kidney excretory function, it is generally regarded as a less-reliable measure than creatinine, which is used to estimate GFR. An increase in blood urea concentration is not as directly related to changes in GFR because it can be affected by additional factors, including dietary protein intake, gastrointestinal bleeding, dehydration, use of glucocorticoids, or liver dysfunction. That said, measurement of urea levels in saliva is a potentially useful, rapid, convenient, and cost-effective initial screening measure of kidney function in a busy clinical setting, and especially in more remote environments, with the additional advantage of being with or near the patient. Our data illustrate its potential for this purpose and could guide the selection of patients for follow-up serum or plasma creatinine estimates and further investigation of kidney function.

We show here that urea, at physiologically relevant concentrations, can be detected in the spectra of saliva samples. Urea has a prominent and characteristic ν(C–N) urea band at 1464 cm⁻¹ when dried in its pure state. However, this band is usually downshifted when urea is dried in complex mixtures, as is the case here, where the band appears at 1449 cm⁻¹ in the dried saliva samples in Figure 1A. This is probably due to the retention of water in the complex mixtures and the resulting hydrogen-bonding interaction differences in urea-urea and/or urea-water interactions in comparison with those of pure dry urea, previously discussed in relation to urea spectra in dried urine specimens (23,27).
A urea calibration plot was established in the clinically relevant concentration range by using the peak at 1449 cm⁻¹, integrated between 1481 and 1435 cm⁻¹, for a series of saliva samples to which urea was added, and taking into account the 3.5-mM urea present in the original healthy saliva sample. Quantitation of urea from this band allows account the 3.5-mM urea present in the original healthy saliva samples to which urea was added, and taking into account the 3.5-mM urea present in the original healthy saliva sample. Quantitation of urea from this band allows hence enabling quantitation in the clinically relevant range of 3–40 mM (9,24–26).

The concentrations of salivary urea measured by this method were slightly lower than the corresponding serum urea concentrations (Table 1). This is consistent with several published reports in the literature (9,25,26), and, because saliva is an ultrafiltrate of blood, the same components measured in saliva might be expected to be lower than in blood (28). However, a few studies have reported levels of salivary urea that are similar to, or even higher than, serum urea in some patients with CKD (26,29).

Importantly, the urea concentrations measured by the ATR-FTIR protocol are sufficiently accurate to provide a definitive diagnostic method of distinction between healthy subjects and those with stages 3–5 CKD (Figure 2), as defined by their eGFR values. In addition, the urea levels clearly increase with advancing CKD severity and allow each of stages 3–5 to be distinguished from each other.

In this very small sample size, there was no statistically significant difference in concentrations of salivary urea between the healthy control group and patients with stage 1–2 CKD, although the mean salivary urea concentration in the stage 1–2 CKD group was slightly higher. This may warrant further investigation with much larger sample sizes and a more standardized saliva collection protocol to determine statistical significance. However, patients classified with CKD stages 1–2 purely on the basis of eGFR still need other markers of kidney disease (e.g., abnormal urine or abnormal imaging) to be classified as having CKD. As such, a patient with CKD stage 2 may actually have a lower urea (and higher eGFR) than one that has “normal kidney function.”

Despite this proof-of-concept study of its performance quality, to provide a fast, cost-effective method that could replace the more conventional clinical screening methods that require blood samples or other time- and cost-intensive methods, several issues of sample preparation and, in particular, technology development need to be resolved. Firstly, a much larger number of patients at well-defined CKD stages should be included, with both salivary and serum urea levels quantitated also by standard current methods. This would be aided by a more rigorous and consistent method of saliva collection—with the present protocol more than half of the samples were insufficient in terms of volume or were unsuitable because of contaminants that

### Table 2. Diagnostic performance of salivary urea measurements

| Groupings versus Control Group eGFR (CKD stages) | Sensitivity, % | Specificity, % | Area Under Receiver Operating Characteristic Curve | Threshold of Salivary Urea Concentration, mM* |
|-----------------------------------------------|----------------|---------------|---------------------------------|---------------------------------|
| eGFR <60 ml/min (stages 3–5 CKD)               | 87             | 100           | 0.97                            | 6.5                             |
| eGFR <30 ml/min (stages 4–5 CKD)               | 100            | 100           | 1                               | 8.1                             |
| eGFR <15 ml/min (stage 5 CKD)                  | 100            | 94            | 0.95                            | 11.2                            |

Diagnostic performance was assessed from ROC plots of true positives (sensitivity) versus false positives (100−specificity) as urea concentration is varied. Areas under these curves (maximum 1.0) are a measure of diagnostic performance. ROC, receiver operating characteristic.

*Threshold salivary urea concentrations to distinguish the CKD stages listed are those with the optimal Youden index, determined as the urea concentration at which the sum of sensitivity and specificity is maximal.
made sample handling difficult. An improved collection protocol might include using agents, such as chewing gum, to stimulate saliva production (30), or providing subjects more time for appropriate oral hygiene before sample collection. Finally, although no chemical additives or reactions are required, and data acquisition is straightforward, our present protocol has involved centrifugation of samples before drying 5-μl aliquots onto the ATR prism surface. An improved protocol of saliva collection could obviate the need for centrifugation.

Most important is the need for simplification of the technology for clinical application. This requires development of simplified, nonspecialist spectrometers, and/or the development of multiwell plate readers for the preparation and analysis of many samples simultaneously. Automated data analysis methods that can provide urea concentrations are easily added, hence allowing operation by staff with minimal training. Further enhancement of sensitivity and specificity of urea estimation is also possible with more global spectral analysis methods. The reader is referred to an excellent review by Finlayson et al. (31) on problems and progress in these areas. To date, a number of other techniques have been used to measure salivary urea and the characteristics of these tests are compared to the method used in this study in Supplemental Table 2 (10,11,32-36).

In summary, our results demonstrate that ATR-FTIR spectroscopy can be used to detect urea in saliva with an accuracy that is sufficient to identify and categorize patients as having stages 3, 4, or 5 CKD. Hence, the measurement method has the potential to be a rapid, preliminary screening tool for stages 3–5 CKD, particularly for populations at high risk (e.g., those with obesity and diabetes) of developing CKD, or in countries with limited resources. It might also be used more widely in conjunction with the current clinical parameters of albuminuria and eGFR, which alone may not always provide sufficient accuracy (37,38).

Disclosures

J. Norman reports serving as associate editor of BMC Nephrology, as associate editor of the European Journal of Clinical Investigation, as committee member of the Kidney Research UK Research Strategy Committee, as committee member of Medical Research Council Genetically Engineered Mice for Medicine, on the editorial board for Scientific Reports, and as cochair of the UK Renal Fibrosis Network; having other interests in/relationships with Kidney Research UK (as a trustee); and receiving research funding from UCB Pharma. R. Unwin reports being employed by AstraZeneca Biopharmaceuticals R&D, Cardiovascular, Renal and Metabolism (Cambridge, United Kingdom, and Gothenburg, Sweden); having ownership interest in AstraZeneca; and having served on the scientific advisory board of the European Renal Association–European Dialysis and Transplant Association. All remaining authors have nothing to disclose.

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Author Contributions

R.D.R. Evans, J.T. Norman, P.R. Rich, and R.J. Unwin reviewed and edited the manuscript; T.-L. Lin wrote the original draft and was responsible for investigation; J.T. Norman was responsible for project administration; J.T. Norman and P.R. Rich were responsible for resources; P.R. Rich was responsible for methodology; and P.R. Rich and R. Unwin conceptualized the study and provided supervision.

Data Sharing Statement

All data are included in the manuscript and/or supporting information.

Supplemental Material

This article contains the following supplemental material online at http://kidney360.asnjournals.org/lookup/suppl/doi:10.34067/KID.0004362021/-/DCSupplemental.

Supplemental Table 1. Concentrations of urea in healthy controls and CKD patient saliva samples.

Supplemental Table 2. Test characteristics of methods for measurement of salivary urea.

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