Supplemental Data

Monitoring the effect of hemodialysis on salivary nitrate and uric acid in end-stage renal disease patients using colorimetric test strips: A proof of principle

Timothy M. Blicharz\textsuperscript{1}, David M. Rissin\textsuperscript{1}, Michaela Bowden\textsuperscript{1}, Ryan B. Hayman\textsuperscript{1}, Christopher DiCesare\textsuperscript{1}, Jasvinder S. Bhatia\textsuperscript{2}, Nerline Grand-Pierre\textsuperscript{3}, Walter L. Siqueira\textsuperscript{3}, Eva J. Helmerhorst\textsuperscript{3}, Joseph Loscalzo\textsuperscript{4}, Frank G. Oppenheim\textsuperscript{3}, and David R. Walt\textsuperscript{1}\textsuperscript{*}

\textsuperscript{1}Department of Chemistry, Tufts University, 62 Talbot Avenue, Medford, MA 02155
\textsuperscript{2}Department of Medicine, Boston University School of Medicine, 715 Albany Street, Boston, MA 02118
\textsuperscript{3}Department of Periodontology and Oral Biology, Boston University Goldman School of Dental Medicine, 700 Albany Street, Boston, MA 02118
\textsuperscript{4}Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA 02115

Preliminary Analyte Screening

All analytes that were initially considered as potential markers for dialysis efficacy in ESRD are listed in Table S1(1). Results from various screening experiments showed inconsistent trends for several of these analytes, thus they were excluded from further consideration. Other biomarkers, including NO\textsubscript{2}\textsuperscript{-}, UA, Na\textsuperscript{+}, Cl\textsuperscript{-}, total protein, pH, amylase, and lactoferrin, appeared to show differences between pre- and post-dialysis saliva (data not shown) and were examined further. This additional screening showed UA, NO\textsubscript{2}\textsuperscript{-}, Na\textsuperscript{+}, and Cl\textsuperscript{-} had the most consistent correlations with dialysis; therefore, these analytes were subsequently analyzed in saliva samples collected at designated hourly intervals throughout the dialysis procedure as discussed in the main text.

Test paper for NO\textsubscript{2}\textsuperscript{-} was made by first preparing a solution of 50 mmol/L sulfanilamide, 330 mmol/L citric acid, and 10 mmol/L N-(1-naphthyl) ethylenediamine in methanol. The solution was transferred to a Petri dish and a \(\sim 7 \times 9\) cm sheet of chromatography paper was immersed in the solution and allowed to fully saturate. The paper was then removed from the solution and placed on a watch glass and dried in a 65\textdegree C oven for \(\sim 10\) min. Once dried, the NO\textsubscript{2}\textsuperscript{-} test paper was stored protected from light in a container with desiccant (2, 3).
Test paper for UA was made by preparing a solution of 2.56% (w/v) 2,2′-biquinoline-4,4′-dicarboxylic acid disodium salt hydrate in deionized water and a solution of 20 mmol/L sodium citrate and 0.08% (w/v) copper (II) sulfate. The two solutions were combined in a 1:1 mixture and transferred to a Petri dish. A sheet of chromatography paper was immersed in the solution, dried in a 65°C oven, and subsequently stored as described above for NO$_2$- test paper preparation (4, 5).

Table S1. Analytes initially screened for correlation with dialysis in saliva.

<table>
<thead>
<tr>
<th>Analytes Included in Further Screening</th>
<th>Analytes Excluded from Further Screening</th>
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<tbody>
<tr>
<td>Amylase</td>
<td>Esterase</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Glucose</td>
</tr>
<tr>
<td>pH</td>
<td>Nucleic Acids</td>
</tr>
<tr>
<td>Total Protein</td>
<td>Thiols</td>
</tr>
<tr>
<td>NO$_2$-</td>
<td>Ca$^{2+}$</td>
</tr>
<tr>
<td>UA</td>
<td>PO$_4^{3-}$</td>
</tr>
<tr>
<td>Na$^+$</td>
<td>Mg$^{2+}$</td>
</tr>
<tr>
<td>Cl$^-$</td>
<td>K$^+$</td>
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</tbody>
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*Analytes shown in red exhibited the best correlations and were analyzed in saliva samples collected throughout the dialysis procedure.

Dual-analyte (NO$_2$-/UA) test strips were prepared by cutting an 8 × 11.5 cm length of 20 mil vinyl backing material and placing two strips of acid-free double sided tape of 8 mm width down the length of the backing material to act as an adhesive for the NO$_2$- and UA test paper. Strips of NO$_2$- and UA test paper were cut to size and adhered to the exposed adhesive strips that had been placed on the vinyl backing material (Figure S1). Test strips of 6 mm width were then cut by hand and stored protected from light with calcium carbonate desiccant.
Figure S1. Diagram for NO$_2$/UA test strip preparation. Two lengths of acid-free double sided tape were adhered to a sheet of vinyl backing material, to act as an adhesive for the NO$_2$ and UA test paper. Strips of NO$_2$ and UA test paper were then cut to size and adhered to the exposed side of the adhesive that had been placed on the backing material. Test strips of ~6 mm width were measured and cut as indicated by the dotted lines in the diagram.

Test Paper Characterization

NO$_2^-$ and UA test papers produced in our laboratory were immersed in serially diluted NO$_2^-$ and UA standard solutions, respectively, and imaged using a digital camera or digital flatbed scanner (Figure S2). The resulting test paper color intensities were quantified using the histogram function of Adobe Photoshop and transferred to Microsoft Excel to obtain calibration curve data (Figure S3). The results indicated that the test paper color intensities exhibited a linear dependence with their respective target analyte concentrations, demonstrating the feasibility of using test strips to make semi-quantitative NO$_2^-$ and UA determinations in unknown samples by visual inspection or simple digital image processing. Test papers showed linear color intensity dependence up to 1000 and 2000 µmol/L for UA and NO$_2^-$, respectively.

Figure S2. Digital photographs of (a) NO$_2^-$ and (b) UA test papers following immersion in serially diluted standard solutions of NO$_2^-$ and UA, respectively.
Figure S3. Representative calibration curves for (a) NO$_2^-$ and (b) UA test papers. Test paper color intensities were calculated using the histogram function of Adobe Photoshop and normalized to the color intensity of blank measurements made by immersing test papers in deionized water. Insets within the graphs show images of test paper squares following immersion in deionized water and the highest concentrations of NO$_2^-$ or UA standard. Coefficient of variation calculations (n = 3) for the 62.5, 125, and 250 µmol/L measurements were 7.46, 8.99, and 1.70% for NO$_2^-$; and 1.77, 12.71, and 17.13% for UA, respectively.

Test Strip Characterization

Cross-analyte interference of the NO$_2$/UA test strips was examined by preparing serially diluted standard solutions of either NO$_2^-$ or UA with the other analyte held constant at 250 µmol/L. Test strips were immersed in the solutions and imaged (Figure S4). We observed negligible change in the test strip color due to the presence of the other analyte (Figure S5).
Figure S4. (a) Test strips following immersion in solutions containing 250 μmol/L NO$_2^-$ and varying concentrations of UA. (b) Test strips following immersion in solutions containing 250 μmol/L UA and varying concentrations of NO$_2^-$.

Figure S5. Normalized color intensities of test strips shown in Figure S4a (a) and Figure S4b (b) after immersion in solutions containing varying concentrations of NO$_2^-$ with 250 μmol/L UA and varying concentrations of UA with 250 μmol/L NO$_2^-$, respectively.

Standard solutions containing combinations of both NO$_2^-$ and UA were tested using NO$_2^-$/UA test strips to further demonstrate the capability of the test strips to measure both analytes simultaneously (Figure S6). First, NO$_2^-$/UA test strips were immersed in a sample set where a
stock solution containing 2000 µmol/L NO$_2^-$ and 4000 µmol/L UA was serially diluted to a solution containing 1.95 µmol/L NO$_2^-$ and 3.91 µmol/L UA (Figure S6a). Another set of solutions containing complex combinations of NO$_2^-$ and UA was prepared, ranging from a solution containing a high NO$_2^-$ concentration of 2000 µmol/L and a low UA concentration of 3.91 µmol/L, to a solution containing a high concentration UA of 4000 µmol/L and a low NO$_2^-$ concentration of 1.95 µmol/L. These solutions were prepared such that the concentration of NO$_2^-$ would sequentially increase while the concentration of UA would sequentially decrease. NO$_2^-$/UA test strips were immersed in these solutions and imaged (Figure S6b).

The color stability of test papers following immersion was also examined. A test strip was immersed in a freshly collected, whole saliva sample and imaged at 5 min intervals over a period of 35 min. We observed that the resulting test strip color was stable for at least 5 min after sample immersion and began to noticeably degrade after 10 min (data not shown).

Following characterization of the NO$_2^-$/UA test strips, archived pre- and post- dialysis saliva supernatant samples from ESRD patients were tested using the NO$_2^-$/UA test strips. The strips were immersed in the specimens and imaged using a digital camera. Upon visual inspection, the color for both the NO$_2^-$ and UA test papers appeared more intense for pre-dialysis saliva samples relative to post-dialysis saliva samples (Figure S7).

**Figure S6.** Digital photographs of (a) NO$_2^-$/UA test strips after immersion in serially diluted standard solutions containing NO$_2^-$ and UA, as well as (b) test strips immersed in solutions containing complex combinations of NO$_2^-$ and UA.
Figure S7. Test strips following immersion in saliva supernatant samples collected from two ESRD patients before (“pre”) and immediately after (“post”) undergoing dialysis treatment. Note the relative difference in test strip color intensity between pre- and post-dialysis saliva samples.

Color Chart Development for Semi-Quantitative Concentration Determination

The eyedropper tool in Adobe Photoshop was used to digitally analyze the color from images of test papers following immersion in serially diluted standard solutions, as shown in Figure S2. The color data were utilized to produce a chart for visually determining semi-quantitative NO$_2^-$ and UA concentrations using test strips in a POC application (Figure S8). Concentration determinations could also be completed by determining test strip color intensities from digital images using Adobe Photoshop, as discussed above, and comparing the color intensities to a standard curve. Color chart accuracy was evaluated by comparing the colors of the chart to NO$_2^-$ and UA test papers that were immersed in serially diluted standard solutions (Figure S9).

Figure S8. Color chart for visual semi-quantitative NO$_2^-$ and UA concentration determinations using test strips.
Figure S9. Digital photographs of (a) NO$_2^-$ and (b) UA test papers shown next to a NO$_2^-$ or UA color comparison chart following immersion in deionized water as a blank, and serially diluted NO$_2^-$ (3.91 – 2000 µmol/L), or UA (3.91 – 1000 µmol/L) standard solutions, respectively.

**Point of Care (POC) Test Strip Reading Demonstration**

The feasibility of using the test strips in a POC setting was examined by testing fresh saliva samples collected from ESRD patients and healthy control volunteers in a dialysis clinic. Upon collection of the first 1 mL of stimulated whole saliva, each sample donor was asked to immerse the test strip in the sample for ~1 s, read the strip by determining the square of the color chart that best matched the color of the NO$_2^-$ and UA test pads on the strip, and record their result. The strip was then sequentially handed to two analysts who were more experienced with reading the test strips. Each analyst similarly read the strip and recorded his/her result. Visual readings were completed within 1-2 min of immersing the test strip. The readings from the two analysts were averaged to determine the final value, and sample donor readings were excluded to minimize subjectivity (Figure S10). The POC study demonstrated that salivary NO$_2^-$ and UA levels could be rapidly and easily determined using the test strips and a color chart.

It should be noted, however, that the test strip results completed in the clinic differed irregularly from the test strip results obtained by analyzing digital images of test strips after immersion in archived samples (as discussed in main text). This difference could be due to several factors, such as subjectivity in user-based measurements as well as changes in sample composition between collection and storage at -80°C. The effect of user subjectivity can be determined and accounted for in subsequent studies across a larger sample set. In addition, preliminary experiments conducted on saliva samples stored for several days at RT showed that analyte degradation occurred with increasing time spent at RT, resulting in less intense test strip
color, whereas storage at -20 or -80°C maintained analyte stability for over a month (data not shown). We hypothesize that sample degradation can be avoided simply by testing freshly collected whole saliva or immediately storing samples at -20 or -80°C after collection if testing on a later date is required.

**Figure S10.** Test strip results compiled from examining fresh, stimulated whole saliva samples from ESRD patients and healthy controls at the collection location for (a) NO$_2^-$ and (b) UA. Data are shown as the average of two visual concentration readings from two analysts. Note: Patient #13 completed dialysis and left the clinic prior to collecting a post-dialysis sample and was therefore excluded from the study.
References:


