CLASSIFYING KIDNEY DISEASE IN A VERVET MODEL USING SPATIALLY ENCODED CONTRAST-ENHANCED ULTRASOUND PERFUSION PARAMETERS

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Abstract—Early stages of diabetic kidney disease (DKD) are difficult to diagnose in patients with type 2 diabetes. This work was aimed at identifying contrast-enhanced ultrasound (CEUS) perfusion parameters, a microcirculatory biomarker indicative of early DKD progression. CEUS kidney flash-replenishment data were acquired in control, insulin resistant and diabetic vervet monkeys (N = 16). By use of a mono-exponential model, time–intensity curve parameters related to blood volume (J), velocity (β) and flow rate (perfusion index [PI]) were extracted from 10 concentric kidney layers to study spatial perfusion patterns that could serve as strong indicators of disease. Mean squared error (MSE) was used to assess model performance. Features calculated from the perfusion parameters were inputs for the linear regression models to determine which features could distinguish between cohorts. The mono-exponential model performed well, with average MSEs (±standard deviation) of 0.0254 (±0.0210), 0.0321 (±0.0242) and 0.0287 (±0.0130) for the control, insulin resistant and diabetic cohorts, respectively. Perfusion index features, with blood pressure, were the best classifiers between cohorts (p < 0.05). CEUS has the potential to detect early microvascular changes, providing insight into disease-related structural changes in the kidney. The sensitivity of this technique should be explored further by assessing various stages of DKD. (E-mail: kennita@email.unc.edu) © 2022 The Author(s). Published by Elsevier Inc. on behalf of World Federation for Ultrasound in Medicine & Biology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Key Words: Contrast ultrasound, Perfusion imaging, Diabetic kidney disease, Segmentation, Model parameters, Regression analysis.

INTRODUCTION

Diabetic kidney disease (DKD) is characterized by persistent albuminuria and a progressive decline in renal function (Selby and Taal 2020). Heterogeneous structural changes in the kidneys of patients with type 2 diabetes (T2D) make early stages of DKD less predictably associated with clinical presentations and, thus, harder to recognize or diagnose (Alicic et al. 2017; Futrakul et al. 2015). Chronic hypoxia or inadequate oxygenation is a main mechanism underlying kidney disease (Okada et al. 2020). Diabetes complicates the oxygen (O2) balance in the kidney, increasing demand for oxygen while decreasing generation (Hesp et al. 2020). As the metabolic balance shifts, adjustments to tubuloglomerular feedback and microvascular perfusion occur to compensate, but the resulting cycle only leads to further degradation of kidney processes. Capillary rarefaction, a microvascular alteration classified as either structural (reduced number of capillaries) or functional (decreased perfusion in capillaries), is a common indicator of developing disease (Okada et al. 2020; Krishnan et al. 2021; Schoina et al. 2021). Capillary rarefaction in DKD is associated with altered microcirculatory blood flow, reduced O2 delivery and hypoxia, which aid DKD progression (Matsumoto et al., 2004; Okada et al., 2020). As a result, changes in microcirculation can serve as a biomarker of DKD.
Ultrasound is a frontline imaging modality commonly used to assess kidney health (Dong et al. 2014; Torres et al. 2018; Granata et al. 2021). The addition of ultrasound contrast agents (microbubbles) to aid in kidney diagnosis is on the rise and holds promise in assessing patients with kidney disease (Dong et al. 2014; Chang et al. 2017; Girometti et al. 2017; Srivastava et al. 2022). Contrast-enhanced ultrasound (CEUS) can characterize blood flow with high temporal and spatial resolution, which is useful for assessing microcirculation (Erlichman et al. 2020). Further, this portable, real-time modality is well tolerated by patients and less expensive than computed tomography and magnetic resonance imaging, and microbubbles (MBs) are safe for compromised kidneys (Torres et al. 2018; Erlichman et al. 2020; Wymer and Wymer 2020; Granata et al. 2021). MBs remain in the vascular space and have increased sensitivity to vascular complications (Erlichman et al. 2020).

For instance, patients with DKD had decreased perfusion with delayed time to peak enhancement in a previous CEUS study (Erlichman et al. 2020; Ma et al. 2012). Here, we use flash-replenishment imaging to assess kidney perfusion in control, insulin resistant (IR) and diabetic non-human primates (NHPs). In flash-replenishment imaging (Fig. 1a), continuous intravenous MB administration to achieve steady state is combined with low-intensity ultrasound interrupted by a short high-intensity pulse (flash) to observe MB replenishment (reperfusion) until steady state is re-established. The reperfusion portion of the time-intensity curve (TIC) (Fig. 1b) is fit with a model to extract parameters related to blood flow.

Commercial software packages such as VueBox® and SonoPerf® (Bracco Suisse SA, Geneva, Switzerland) and the feature CHI-Q (Canon Medical Systems, Tustin, CA, USA) are commonly used to extract blood flow parameters from reperfusion data (Greis 2011; Schneider et al. 2012; Stock et al. 2018; Mannucci et al. 2019; Liu et al. 2020). These software apply motion correction and curve fitting to let the user quantify perfusion in selected regions of interest (ROIs). However, this analysis fails to comprehensively assess kidney perfusion while maintaining the spatial integrity of the distinct kidney compartments. One ROI encircling the entire kidney merges unique blood flow kinetics for the specialized subregions into a single measure. Separate ROIs isolating subsections of the cortex and medulla relate perfusion to anatomic location, but still overlook disease-related alterations outside the analyzed regions. To fully use microcirculation as a biomarker for DKD, blood flow parameters should correlate to anatomic features in the kidney.

Our objective was to generate perfusion parameters linked to kidney location. We accomplished this by segmenting the kidney into concentric layers. Flash-replenishment TICs from each layer were fit to a monoexponential model, extracting features for use in a regression model with blood pressure (BP) to distinguish kidney status in an NHP diabetic model. Ultimately, this tool could be employed as an indicator of which patients will develop progressive DKD, an important distinction as approximately 40% of T2D patients will develop DKD (Okada et al. 2020).

**METHODS**

**Chlorocebus aethiops sabaeus diabetic model**

Non-human primates, specifically Chlorocebus aethiops sabaeus or vervets, are a naturally occurring
model in which to study diabetic disease (Wagner et al. 2006; Rhoads et al. 2017). Sixteen female vervets housed at Wake Forest University were stratified into three cohorts—control (n = 5), IR (n = 5) and diabetic (n = 6)—on the basis of health status. Vervets were classified as control (a), IR (b) or diabetic (c) according to the following criteria: (a) fasting blood glucose (FBG) < 80 mg/dL and glycosylated hemoglobin (HbA1c) < 5%; (b) FBG 80–125 mg/dL and HbA1c 5%–6%; or (c) FBG > 125 mg/dL and HbA1c > 6%. These criteria were taken from the definition of diabetes in humans (American Diabetes Association (ADA) 2016) and modified to account for vervet physiology (Wagner et al. 2006; Chichester et al. 2015). FBG and HbA1c were determined to confirm the clinical presentation of disease. Additionally, NHP age, body weight, waist circumference, systolic BP and diastolic BP were recorded during each session. The significance of these parameters was evaluated using a two-way analysis of variance testing the effects of both imaging session and NHP cohort. Follow-up analysis consisted of Tukey’s multiple comparisons between cohort means. p values < 0.05 were considered to indicate significant differences.

Ultrasound data acquisition

Prior to imaging, vervets were sedated using ketamine and midazolam and underwent catheterization by research personnel to provide access for MB injections. All vervet care was provided by skilled technicians according to Wake Forest University Institutional Animal Care and Use Committee guidelines for research involving NHPs. This study was approved under the Wake Forest University School of Medicine Animal Use Protocol No. A18-185. CEUS data were collected in triplicate for each vervet over a 6-mo period (Fig. 2). From stratification to imaging, one vervet progressed to the diabetic state. As a result, this diabetic vervet was imaged only during session 1, and a new IR vervet was introduced for sessions 2 and 3 (Fig. 2). A Wake Forest University LOGIQ S8 ultrasound scanner (General Electric, Boston, MA, USA) was used with a curvilinear array (C1-5) for all data collections (Fig. 3). Ultrasound parameters defined prior to imaging were kept constant throughout data collection: image depth (7 cm), focal depth (5.6 cm), frequency (3 MHz), mechanical index (0.18), gain (30 dB), dynamic range (57 dB), frame rate (18 Hz) and flash-replenishment scheme (30 s). The transducer was positioned over the left kidney and secured in a custom holder to reduce motion from probe handling during data acquisition (Fig. 3). A continuous MB infusion containing 0.77 mL of Perflutren Lipid Microspheres (DEFINITY®, Lantheus Medical Imaging, North Billerica, MA, USA) in 30 mL of saline was delivered into the bloodstream at 2 mL/min using an 11 Plus syringe pump (Harvard Apparatus, Holliston, MA, USA). During the MB infusion, one flash-replenishment video of the kidney midplane was acquired per vervet for each imaging session (Fig. 3).

Data processing and analysis

Multiframe CEUS data (K frames) were imported into MATLAB® (The MathWorks, Natick, MA, USA) for analysis using the following workflow: kidney contouring, kidney segmentation, data extraction and data fitting (Fig. 4). All analysis was performed on the exported DICOM files (i.e., log compressed data).

Kidney contouring. First, a frame was extracted, and MATLAB® image enhancement tools, including histogram equalization, image filtering and image binarization, were used to brighten the image. Next a contour of the kidney boundary was manually delineated (Fig. 5a).

Kidney segmentation. A binary mask was created from the contour. A segmented line, with the number of segments defined by the user, between the mask center and a point on the contour boundary was generated (Fig. 5b, 5c). Concentric layers (l), corresponding to the number of segments, were created by repeating segmentation between the mask center and all points along the kidney boundary. This method generated 10 concentric layers (Fig. 5d).

Data extraction. A binary mask $M_l$ was formed for each layer $l$ using the inner and outer contours for

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Fig. 2. Study timeline from initial cohort selection (month 0) to final imaging session (month 10). *From stratification (month 0) to the first imaging session (month 6), one vervet progressed to the diabetic state. As a result, an extra diabetic vervet was imaged during session 1 and one less insulin resistant (IR) vervet was imaged. Between sessions 1 and 2, cohorts were re-balanced and a new IR vervet was introduced for sessions 2 and 3, while the extra diabetic vervet was removed.
that layer (Fig. 5e, 5f). Next, $M_l$ was multiplied by frame $K$ of the CEUS data to isolate pixel intensities in layer $l$ (Fig. 5g). The sum of isolated intensities was divided by the sum of pixels in $M_l$ to calculate a data point, $I_l(K)$. This step was repeated for all layers before moving to the next frame, $K + 1$, tracking intensity over time.

**Data fit.** Reperfusion TIC data for each layer was fit with a mono-exponential model (Fig. 6a) first suggested by Wei et al. (1998):

$$I_l = A(1 - e^{-k})$$
\[
\Delta I(t) = I(t) - I(t_5) = A \left( 1 - e^{-\beta(t-t_5)} \right) \tag{1}
\]

Here, \(I(t)\) is the signal intensity in the ROI; \(A\) is the steady-state intensity, related to blood volume; and \(\beta\) is the slope of the curve, interpreted as blood velocity. The product \(A\beta\) denotes the perfusion index (PI) and is proportional to flow rate (Dietrich et al. 2012). From \(I_i(K)\) data, the index with minimum intensity after the flash sequence was allocated (Fig. 6a). Reperfusion data were shifted by subtracting the allocated index from its start point and all subsequent values (Fig. 6b). Finally, by use of the least-squares method, which minimizes the error between the data and the target model, mono-exponential parameters \(A\) and \(\beta\) were deduced and fitted TIC data were computed. The process was repeated for each kidney concentric layer (KCL) from the outer boundary (KCL 1) to the innermost region (KCL 10) (Fig. 7). The fit for each KCL TIC was evaluated using the mean squared error (MSE), where zero values indicate a strong fit and large values a poor fit. The coefficient of determination (\(R^2\)) was also provided.

**Estimated parameters**

Estimated parameters, \(A\), \(\beta\) and PI for each KCL TIC were individually averaged from the three sessions for each vervet, then normalized by the fifth KCL (see Tables S1 and S2, online only). Normalized and non-normalized \(A\), \(\beta\) and PI were plotted against the 10 KCLs and each estimated parameter was individually fit with first- and third-order polynomial functions (Fig. 8). First- and third-degree polynomial fitting used, respectively, the equations:

\[
P_1 = p_{10}l + p_{11} \tag{2}
\]

\[
P_3 = p_{30}l^3 + p_{31}l^2 + p_{32}l + p_{33} \tag{3}
\]

where \(p_{10}\) and \(p_{11}\) are first-order coefficients, and \(p_{30}\), \(p_{31}\), \(p_{32}\) and \(p_{33}\) are third-order polynomial coefficients.

**Statistical analysis and linear regression model**

The MATLAB® linear model function was used to create a regression model from the independent variables (features) along with the response variable (category ID). Control, IR and diabetic groups were given category IDs of 1, 2 and 3, respectively. Features were extracted independently from both normalized and non-normalized averaged \(A\), \(\beta\) and PI estimates for all KCLs in each cohort; available systolic and diastolic BP measurements were added to improve the model’s predictive power (Table 1). Input features included the mean, standard deviation (SD), area under the KCL curve (AUC), the fitted first- and third-order polynomial coefficients and systolic and diastolic BPs (see Table S3, online only). The regression cases tested were control versus IR, control versus diabetic, IR versus diabetic and control versus IR versus diabetic. The number of observations per case ranged from 10 to 16 depending on the case considered. Case comparisons with \(p < 0.05\) had the potential to distinguish between groups based on the given input features.

**RESULTS**

**Demographic and clinical measures**

Average age, body weight, waist circumference and relevant clinical measures per cohort are outlined in Table 2. Of the collected demographic and clinical measures, age, FBG and HbA1c were significant factors in the study. Age was not a significant factor between imaging
Fig. 7. Time–intensity curve reperfusion fitting (red dashes) of $I_i(K)$ data (blue) for each kidney concentric layer.

Fig. 8. Polynomial curve fitting of the normalized (left) and non-normalized (right) kidney concentric layer (KCL) least-squares estimated parameters ($A$, $\beta$ and PI [perfusion index]) in a control group vervet. Estimated parameter data points (blue) with first-order fitting (orange) and third-order fitting (yellow dashes).
sessions ($p = 0.968$) but was significant between NHP cohorts ($p < 0.0001$). Multiple comparison testing revealed no significant differences in NHP age for control versus IR ($p = 0.831$) but did find significant differences for control versus diabetic ($p = 0.0002$) and IR versus diabetic ($p = 0.0014$). No significant differences were found by imaging session or cohort for body weight, waist circumference, systolic BP and diastolic BP ($p > 0.05$; see Table S4, online only). Both FBG and HbA1c differed significantly between NHP cohorts ($p < 0.0001$), but not by imaging session (FBG: $p = 0.830$, HbA1c: $p = 0.241$). Multiple comparison testing revealed no significant differences in NHP FBG for control versus IR ($p = 0.710$), but did reveal significant differences for control versus diabetic ($p < 0.0001$) and IR versus diabetic ($p < 0.0001$). All pairwise comparisons were significantly different based on HbA1c ($p < 0.05$; see Table S4).

**Mono-exponential model fit performance**

The mono-exponential model fit the TIC data well at each KCL (see Tables S3–S7, online only). The average MSE ± SD across all KCLs for the control, IR and diabetic cohorts was $0.0254 \pm 0.0210$, $0.0321 \pm 0.0242$ and $0.0287 \pm 0.0130$, respectively (Table 3). By layer, the largest MSE (0.0721) was found in the IR cohort at KCL 7. Except for KCLs 1, 6 and 7, the control group had the smallest MSE per layer (Table 3). All cohorts had an $R^2 > 0.6$, indicating that the mono-exponential model was a moderately strong fit for the TIC data (Table 3). By layer, the strongest fit occurred in the control cohorts at KCL 2 ($R^2 = 0.829$) and KCL 3 ($R^2 = 0.823$).

**Estimation of perfusion model parameters**

Layer-based parameter estimation resulted in noticeable changes in $A$, $\beta$ and PI between KCLs (see Fig. S1, online only). Visualizing the normalized estimated parameters for a single vervet per cohort (Fig. 9) reveals how blood volume and perfusion change across the kidney layers and with disease state. In these example vervets, $\beta$ decreases by KCL from the cortex to the renal pelvis, while $A$ increases, peaking near the seventh/eighth layer and then decreasing. In form, PI data resemble an asymmetric parabola across KCLs. KCLs with $A$, $\beta$ or PI $> 1$ exhibit larger blood volume, faster

<table>
<thead>
<tr>
<th>Feature</th>
<th>Estimated parameter (normalized, non-normalized)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$A$</td>
</tr>
<tr>
<td>Average</td>
<td>0.980, 0.362</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.0750, 0.0273</td>
</tr>
<tr>
<td>Area under the curve</td>
<td>8.89, 3.28</td>
</tr>
<tr>
<td>$p_{10}$</td>
<td>0.0150, 0.0053</td>
</tr>
<tr>
<td>$p_{11}$</td>
<td>0.898, 0.333</td>
</tr>
<tr>
<td>$p_{30}$</td>
<td>$-0.0015$, $-0.0005$</td>
</tr>
<tr>
<td>$p_{31}$</td>
<td>0.0182, 0.0063</td>
</tr>
<tr>
<td>$p_{32}$</td>
<td>$-0.0300$, $-0.0088$</td>
</tr>
<tr>
<td>$p_{33}$</td>
<td>0.890, 0.326</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>88.7</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>57.4</td>
</tr>
</tbody>
</table>

PI = perfusion index.
Systolic and diastolic blood pressures are input features independent from the ultrasound measurements and estimated perfusion model parameters.

**Table 1. Normalized and non-normalized features calculated from the estimated parameters for one vervet in the control group**

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Control (n = 5)</th>
<th>Insulin resistant (n = 5)</th>
<th>Diabetic (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>16.0 ± 3.13</td>
<td>16.6 ± 2.50</td>
<td>20.8 ± 2.92*</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>5.79 ± 0.610</td>
<td>6.53 ± 1.58</td>
<td>5.73 ± 1.25</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>35.1 ± 1.80</td>
<td>39.1 ± 7.87</td>
<td>37.8 ± 4.42</td>
</tr>
<tr>
<td>Fasting blood glucose (mg/dL)</td>
<td>68.5 ± 10.6</td>
<td>91.9 ± 13.9</td>
<td>342 ± 119*†</td>
</tr>
<tr>
<td>Glycosylated hemoglobin, HbA1c (%)</td>
<td>4.16 ± 0.230</td>
<td>5.27 ± 0.730*</td>
<td>8.60 ± 0.940*†</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>115 ± 20.7</td>
<td>129 ± 34.2</td>
<td>107 ± 10.4</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>64.0 ± 4.88</td>
<td>69.4 ± 18.8</td>
<td>62.2 ± 8.73</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± standard deviation.
* $p < 0.05$ compared with the control group.
† $p < 0.05$ compared with the insulin resistant group.
blood velocity or faster flow relative to the fifth KCL. The first KCL includes the kidney boundary, which may influence values for that layer relative to deeper KCLs.

**Linear regression model fitting**

Initial estimates for input features \( p_{10} \), \( p_{30} \) and \( p_{31} \) were zero. These terms were removed, and the model was re-assessed, but no effect on the performance of the resultant model was observed. Model performance and the significance of estimated parameters \( A \), \( \beta \) and PI varied per case (Table 4). Features extracted from PI data had the largest influence on model performance. The non-normalized PI control-versus-IR regression model \( (p = 0.0768) \) and the normalized PI IR-versus-diabetic model \( (p = 0.0149) \) performed best at distinguishing between vervet cohorts using PI features and BP measurements combined.

### Table 3. Mono-exponential model performance by cohort for each KCL with overall average

<table>
<thead>
<tr>
<th>KCL</th>
<th>Control Model performance (MSE, ( R^2 ))</th>
<th>Insulin resistant Model performance (MSE, ( R^2 ))</th>
<th>Diabetic Model performance (MSE, ( R^2 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0062, 0.786</td>
<td>0.0060, 0.735</td>
<td>0.0096, 0.774</td>
</tr>
<tr>
<td>2</td>
<td>0.0052, 0.829</td>
<td>0.0059, 0.733</td>
<td>0.0122, 0.764</td>
</tr>
<tr>
<td>3</td>
<td>0.0086, 0.823</td>
<td>0.0099, 0.708</td>
<td>0.0189, 0.751</td>
</tr>
<tr>
<td>4</td>
<td>0.0182, 0.789</td>
<td>0.0193, 0.698</td>
<td>0.0278, 0.729</td>
</tr>
<tr>
<td>5</td>
<td>0.0345, 0.739</td>
<td>0.0372, 0.642</td>
<td>0.0356, 0.754</td>
</tr>
<tr>
<td>6</td>
<td>0.0555, 0.689</td>
<td>0.0569, 0.607</td>
<td>0.0459, 0.758</td>
</tr>
<tr>
<td>7</td>
<td>0.0618, 0.654</td>
<td>0.0721, 0.611</td>
<td>0.0464, 0.713</td>
</tr>
<tr>
<td>8</td>
<td>0.0382, 0.768</td>
<td>0.0599, 0.649</td>
<td>0.0391, 0.751</td>
</tr>
<tr>
<td>9</td>
<td>0.0180, 0.765</td>
<td>0.0360, 0.674</td>
<td>0.0277, 0.776</td>
</tr>
<tr>
<td>10</td>
<td>0.0074, 0.690</td>
<td>0.0175, 0.656</td>
<td>0.0238, 0.671</td>
</tr>
<tr>
<td>Average ± Standard deviation</td>
<td>0.0254 ± 0.0210, 0.753 ± 0.0591</td>
<td>0.0321 ± 0.0242, 0.672 ± 0.0463</td>
<td>0.0287 ± 0.0130, 0.744 ± 0.0321</td>
</tr>
</tbody>
</table>

KCL = kidney concentric layer; MSE = mean squared error.
The superior performance of the normalized PI IR-versus-diabetic model in classifying vervet cohorts was clear from the $t$-statistic $p$ values for each model term estimated coefficient. Seven of the nine model coefficients had a $t$-statistic with $p < 0.07$, with six $< 0.05$ (see Table S8, online only). Normalized PI KCL average, KCL AUC, $p_{32}$, $p_{33}$, systolic BP and diastolic BP were all significant features ($p < 0.05$) in distinguishing IR and diabetic vervets, while $p_{11}$ failed to achieve significance ($p = 0.067$) (see Table S8). The non-normalized PI control-versus-IR model performance was similarly explained. Seven of the nine estimated coefficients had a $t$-statistic with $p < 0.074$, but only one, the model intercept, was significant ($p = 0.048$), which explains why the model overall did not achieve statistical significance (see Table S8). Non-normalized PI KCL SD, $p_{11}$, $p_{32}$, $p_{33}$, systolic BP and diastolic BP all exhibited the potential to distinguish between cohorts but failed to achieve significance ($p > 0.05$).

**DISCUSSION**

To monitor functional changes in the kidney, we developed several tools to ensure accurate data extraction representing kidney perfusion. Previous studies observed the influence of depth, lateral position and transducer orientation on TIC parameters, suggesting standardized criteria to compensate (Ignee et al. 2010; Kogan et al. 2011; Kasoji et al. 2017; Xie et al. 2018). Here, the kidney was segmented into standardized KCLs to address heterogeneous blood flow within kidney compartments. KCL parameters were normalized to guarantee more precise depth-independent measurements. We propose that our segmentation method offers an easy-to-implement solution to the current variability in TIC analysis. From a single user-defined ROI and grid, our technique automatically segments the kidney into concentric layers. Here, as a proof-of-concept we implemented 10 layers with equal thickness between layers. Each layer contained a portion of the whole kidney, with deeper layers containing smaller percentages (see Table S9, online only). Reducing layer width could provide finer sampling of kidney regions, or layers could be defined with variable thickness based on the spatial extent of each kidney region. However, consideration should be given to identifying the ideal number of layers. Implementing too few layers could oversimplify and underestimate the perfusion parameter curves, but too many layers could reduce the ROI size per layer to a point that negatively affects the analyzed signal (see Fig. S2, online only). Segment orientation could be modified to further address signal depth dependence and to better represent kidney compartments (i.e., the cortex, medullary pyramids and columns). The NHP model we used has a relatively simple unipapillate kidney, which allowed us to use concentric layers as a rough representative of internal kidney components. The perfusion parameters calculated from layers defined here were expected to correspond to functional aspects of the kidney, but standardizing segmentation, that is, ROI definition, and spatial patterns of functional and structural variations should be further studied. In more complex systems (e.g., humans), these concentric layers would likely misrepresent the anatomical structure and the segmentation method would need to be revisited.

Our approach simplifies current methods used to assess renal perfusion. For both flash-replenishment and bolus TIC analysis, common practice involves an experienced user, typically a radiologist or trained researcher, manually defining ROIs. Number and placement vary, but usually multiple ROIs are considered in the cortex and medulla (Kalantarinia et al. 2009; Schlosser et al. 2001; Schneider et al. 2012; Stock et al. 2018; Liu et al. 2020). The spatial distribution of key structural components contributing to underlying mechanisms of disease progression makes evaluation of both cortical and medullary perfusion vital for the early detection of DKD. Mannucci et al. (2019) noted significant differences in medullary peak intensity and AUC in dogs with acute kidney injury compared with healthy dogs, but noted none from cortical measurements. Stock et al. (2018) found that cortical mean transit time and time to peak were significant parameters for distinguishing healthy from CKD cats, while rise time, time to peak and fall time were significant in the medulla. In the future, isolating data from regions with cortical and juxtamedullary nephrons may be informative, as glomerular and tubular mechanisms have been found to alter surrounding

\begin{table}
\centering
\begin{tabular}{|l|c|c|c|c|c|c|}
\hline
Case & Number of observations & Non-normalized & & & Normalized & \\
& & & $A$ & $\beta$ & PI & $A$ & $\beta$ & PI \\
\hline
Control vs. IR & 10 & 0.767 & 0.812 & 0.0768 & & 0.821 & 0.643 & 0.762 \\
Control vs. diabetic & 11 & 0.138 & 0.958 & 0.475 & & 0.186 & 0.844 & 0.853 \\
IR vs. diabetic & 11 & 0.150 & 0.296 & 0.454 & & 0.264 & 0.206 & 0.0149 \\
Control vs. IR vs. diabetic & 16 & 0.213 & 0.733 & 0.597 & & 0.213 & 0.646 & 0.516 \\
\hline
\end{tabular}
\caption{$p$ values for four different regression models based on estimated parameter statistics and blood pressure measurements.}
\end{table}

IR = insulin resistant; PI = perfusion index.
microvasculature. Tubuloglomerular feedback has been linked to both dilation and constriction of glomerular arterioles (Hostetter 2001; Vallon and Thomson 2012; Mora-Fernández et al. 2014; Lin et al. 2018; Barrera-Chimal and Jaisser, 2020). Resulting changes in vascular resistance led to increased intraglomerular capillary pressure causing both intraglomerular and systemic hypertension and contributing to glomerular hyperfiltration (Vallon and Thomson 2012; Mora-Fernández et al. 2014; Amorim et al. 2019; Barrera-Chimal and Jaisser, 2020).

Our method could be further enhanced by fully automating segmentation. Prior work revealed that automatic kidney segmentation can be performed with high efficiency (Zheng et al. 2018; Yin et al. 2020). Yin et al. (2020) used a pre-trained deep neural network and pixel-wise classification scheme to segment clinical kidney images without any user processing steps, while Prevost et al. (2014) developed an automatic co-segmentation method using B-mode and CEUS images to detect the kidney and improved poor results with minimal user input. Kidney segmentation techniques can also be used to identify internal components and disease: Bommanna Raja et al. (2008) implemented a feature-based classification scheme to identify healthy versus diseased kidneys. Differentiating the kidney compartments, primarily the cortex and medulla, remains a challenging problem based on conventional imaging alone; however, some progress has been made toward this goal. Chen et al. (2022) developed an approach to separate the renal parenchyma from the sinus, and Marsousi et al. (2019) trained a neural network to detect the kidney capsule, medulla and pelvis/calyses. Application of these US segmentation techniques to our KCL method, with the ability to automatically generate kidney contours of the outer boundary or multiple regions (e.g., the parenchyma or medulla versus sinus), could further simplify analysis for radiologists and researchers alike, providing more accurate and easily attainable measures of kidney perfusion. Further, super-resolved ultrasound localization microscopy (ULM) images of kidney vasculature may offer a solution by providing clearer delineation between cortical and medullary components. More encouraging is that ULM extends beyond purely structural information and has recently been used to localize hemodynamic changes in the brain, determining the spatial extent of regional vascular involvement during activation (Renaudin et al. 2022). This technique could be incredibly informative for quantifying vascular behavior in tumors or vascular markers of disease in vital organs, such as the kidney.

We fit a mono-exponential model to flash-replenishment data but could adapt this technique to other established perfusion models, such as the indicator-dilution models used for bolus data (Strouthos et al. 2010; Turco et al. 2020). Krix et al.’s (2003) multivessel model and the Arditi–Hudson model (Dietrich et al. 2012) are alternatives to flash-replenishment data, providing more parameters, which could improve classification. Turco et al. (2020) provides a comprehensive review of available models that could be combined with our kidney layer technique. Although simple in comparison, the mono-exponential model still performed extremely well when fit to our data, confirmed by the low average MSE (<0.033) in each group. One drawback was the lack of linearization of the log compressed data and removal of other non-linearities prior to fitting and analyzing the data. We assessed inaccuracies between the linearized and log compressed data by first implementing a generic linearization scheme (Rognin et al. 2008) and then calculating the percentage error between estimated perfusion parameters and the agreement between the normalized fits for both the log compressed and linearized data (see Tables S10 and S11, online only). We found low error between $\beta$ values and a strong agreement between the fitted curves of both data sets. The ability to analyze log compressed DICOM data with minimal processing is an important step toward the development of a semiquantitative, cost-effective ultrasound toolkit for the detection of DKD and overall assessment of kidney health. If successful, this toolkit could eventually be implemented in low-income areas to help address health disparities.

Normalized and non-normalized PI data were superior to the other perfusion parameters for classifying cohorts. Normalized PI distinguished IR and diabetic vervets ($p = 0.0149$), while non-normalized PI exhibited the potential to distinguish control from IR vervets ($p = 0.0768$). However, these results were obtained only after adding BP measurements to the model. Before including BP data, both cases were non-significant ($p > 0.05$). The small sample size (16 NHPs) likely limited the ability of the regression model to distinguish vervet cohorts based only on CEUS data. Current understanding of the complexity of developing DKD and the interaction between hypertension and altered renal blood flow in DKD support the use of additional features in models classifying DKD. Indeed, Okada et al. (2020) found that changes in microcirculation may indicate DKD, after adjusting for BP. Future work should consider incorporating and weighting by available clinical measures, as this proved potentially useful for detecting changes in renal function (Araújo et al. 2020; Okada et al. 2020). Our small data set also precluded the use of more advanced machine learning techniques to classify the data. However, with a larger data set, neural networks, $K$-nearest neighbor, support vector machine or other machine learning techniques could be implemented.
Artificial intelligence has successfully predicted DKD progression from medical data (Makino et al. 2019), and artificial neural networks have successfully classified kidney disease based on ultrasound images (Wagih et al. 2015). These examples illustrate the power of machine learning for disease classification.

**CONCLUSIONS**

Because 40% of patients with T2D will potentially develop DKD, distinguishing kidneys in the diabetic milieu from those with advancing DKD is important. The CEUS technique evaluated here holds promise, as it was sensitive enough to distinguish control, IR and diabetic vervets. Analysis of blood and urine samples (not shown) revealed that none of the NHPs had overt kidney disease at the time of imaging. However, this provides an opportunity for follow-up imaging to determine the prognostic capability of these methods. Additionally, future studies will implement this method in diabetic models with and without DKD. In summary, our regression model illustrated that a combination of perfusion features and clinical measures could differentiate between the phases of diabetic progression. Improving CEUS sensitivity to microvascular alterations through changes in renal perfusion could be the key to identifying early irregularities predictive of developing disease.

**CONFLICT OF INTEREST DISCLOSURE**

E.H.C. currently receives funding from Lantheus Medical Imaging for projects involving imaging and surveillance of kidney lesions. R.W.W. consulted for SonoVol, Inc., on unrelated imaging projects between November 2021 and April 2022. All other authors declare no competing interests.

**SUPPLEMENTARY MATERIALS**

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ultrasmemedbio.2022.10.015.

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