A label-free approach by infrared spectroscopic imaging for interrogating the biochemistry of diabetic nephropathy progression

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Routine histology, the current gold standard, involves staining for specific biomolecules. However, untapped biochemical information in tissue can be gathered using biochemical imaging. Infrared spectroscopy is an emerging modality that allows label-free chemical imaging to derive biochemical information (such as protein, lipids, DNA, collagen) from tissues. Here we employed this technology in order to better predict the development of diabetic nephropathy. Using human primary kidney biopsies or nephrectomies, we obtained tissue from 4 histologically normal kidneys, 4 histologically normal kidneys from diabetic subjects, and 5 kidneys with evidence of diabetic nephropathy. A biochemical signature of diabetic nephropathy was derived that enabled prediction of nephropathy based on the ratio of only 2 spectral frequencies. Nonetheless, using the entire spectrum of biochemical information, we were able to detect renal disease with near-perfect accuracy. Additionally, study of sequential protocol biopsies from 3 transplanted kidneys showed biochemical changes even prior to clinical manifestation of diabetic nephropathy. Thus, infrared imaging can identify critical biochemical alterations that precede morphologic changes, potentially allowing for earlier intervention.

Kidney transplantation is often an effective treatment for end-stage renal disease; however, transplant failure is common. In 2012, 28% of those receiving a kidney transplant had diabetic nephropathy as the primary cause of end-stage renal disease. Unfortunately, this group also has the lowest 5-year graft survival compared to end-stage renal disease from hypertension, cystic disease, glomerulonephritis, or other causes. Allograft function is monitored using serum creatinine to monitor for complications, but unfortunately, they are usually detected only after an irreversible decline in renal function. Some institutions use surveillance biopsies performed over the first year for early identification of subclinical complications. Histologic evaluation of renal biopsies by a pathologist remains the gold standard for obtaining critical diagnostic and prognostic information after transplantation, and has been shown to impact subsequent treatment. However, early histologic evidence of diabetic nephropathy including glomerular basement membrane and mesangial thickening is not seen until 2 or more years after transplant, thus highlighting the lack of advanced tools that can be used for the prediction of graft outcome.

Recent dramatic advances have transformed infrared (IR) imaging from a research tool into a potentially powerful clinical tool, providing insight in a label-free, non-perturbing manner into the biochemistry of tissue. IR imaging represents a potentially powerful adjunct to current pathology techniques due to its ability to provide complementary biochemical information that would otherwise not be accessible using conventional staining approaches. IR spectroscopy is based on the principle that different regions of mid-IR light are quantitatively absorbed by different biomolecules present within tissues such as proteins, lipids, DNA, RNA, collagen, glycogen, and carbohydrates. Different tissue biomolecules have different characteristic IR absorption spectra. The principle for much of the work using IR imaging for tissue pathology has focused on examining these IR spectra, also termed spectral fingerprints, and using these fingerprints to predict cell type or disease status or replicate staining patterns with a high level of accuracy. Image resolutions approaching 1 μm are now available permitting visualization of key structures in renal biopsies and subsequent extraction of biochemical information (Figure 1). In visible microscopy, an image typically has 3 channels for every pixel, typically a red, a blue, and a green channel. In IR imaging, every single pixel within the image comprises an entire IR
**Figure 1** | **Schematic of a typical infrared (IR) imaging system.** An IR system has both visible and IR light source and detection systems. (a) The visible light is used to visualize the sample and find the area of interest on the tissue section. The charge-coupled device (CCD) in the system collects the visible light and creates a data cube with red, green, and blue (RGB) channels (3 bands, Z) coupled with spatial dimension (X,Y). After the region is found, the mirrors in the system flip to the IR configuration, and an IR source is used to obtain an image. (b) As mid-IR passes through the sample, different regions are absorbed by different biomolecules (glycogen, DNA, proteins, etc.) to give a biochemical fingerprint of the tissue, which is collected using a detector sensitive to IR. This creates an image with possibly more than 200 channels of data (Z). This also is coupled with spatial data (X,Y) for each spectral band recorded in the Z-axis.

**Figure 2** | **Spectral data were extracted from the glomerular basement membrane (GBM), tubular basement membrane (TBM), and mesangium (M) from IR image scans.** The average spectra for diabetic nephropathy (DN), normal diabetic (NLD), and normal nondiabetic (NL) groups are shown in the top row (a–c), while individual spectra are shown below (d–f). The DN groups showed increases in the glycosylation-associated (1030 cm⁻¹) and DNA- and glycosylation-associated (1080 cm⁻¹) peaks in all 3 structures compared to the control groups (NLD and NL).
spectrum typically composed of hundreds of biochemical channels (Figure 1). IR imaging can potentially provide additional diagnostic and prognostic information in renal biopsies of value for treatment and prognosis. Fourier transform IR spectroscopy is routinely applied to the analysis of renal stones, and it has recently been used for the detection of 2,8-dihydroxyadenine crystals located in renal tubular lumen.15 Previous work has demonstrated that spectroscopic differentiation of pathologic conditions can be achieved in the absence of visual clues.16,17

RESULTS

We first identified the signature of isolated diabetic nephropathy in native kidney tissue. Periodic acid–Schiff (PAS)–stained kidney biopsies and nephrectomy tissue were grouped into 3 categories: histologically normal nondiabetic (NL, n = 4), histologically normal diabetic (NLD, n = 4), and diabetic with evidence of diabetic nephropathy (DN, n = 5) (patient details in Supplementary Table S1 online). PAS staining is primarily used to stain carbohydrate macromolecules in renal biopsies. Formalin-fixed paraffin-embedded tissues were serially sectioned onto a glass slide (for PAS staining) and a barium fluoride slide (for IR imaging). High-resolution IR images were acquired and IR spectra extracted from the glomerular basement membrane, tubular basement membrane, and mesangium. An average IR spectrum was derived from up to 6 glomerular or tubular cross sections of each specimen (Figure 2). In all 3 glomerular components, a pronounced difference was particularly found in the 1120 to 1000 cm⁻¹ region from the entire mid-IR spectral range (3850–900 cm⁻¹, Figure 2). Increases are seen in the glycosylation-associated (1030 cm⁻¹) and the DNA- and glycosylation-associated (1080 cm⁻¹) peaks in the glomerular basement membrane, tubular basement membrane, and mesangium of the DN biopsies. Importantly, the NL and NLD cohorts showed very similar spectra. Thus the diabetic signature we were detecting was not due to the patient’s diabetic status but due to diabetic nephropathy.

One feature of diabetic nephropathy is the increase in glomerular mesangial fraction of surface area (MFSA).18 MFSA was computed from the PAS-stained sections for up to 6 glomeruli per patient (Figure 3a). The IR spectrum of the mesangium from adjacent tissue sections of the same glomerulus (each glomerulus is a single point). Each patient was assigned a unique symbol within each class (thus 5 or 6 symbols per patient). Quadrants were subjectively drawn to visualize separation. Patient 11 (inverted red triangles), who showed low mesangial expansion but had a high 1030:1080 spectral ratio, was classified as having diabetic nephropathy based on electron microscopy, which showed a mild thickening of the GBM and TBM.
glomerulus) was extracted and a simple spectral ratio determined (Figure 3b). Biopsies with diabetic nephropathy had markedly increased MFSA and spectral ratio of 1030 cm to 1080 cm (Figure 3a and b). A small earlier pilot study (data not shown) found that Kimmelstiel–Wilson nodules of the mesangium had an increase in the 1030:1080 ratio. A plot of the 1030:1080 ratio against the MFSA showed that the DN biopsies (high MFSA and high spectral ratio) and the histologically normal biopsies (NL and NLD, low MFSA and low spectral ratio) formed distinct clusters (Figure 3c). Interestingly, there were some DN glomeruli with a low MFSA but high spectral ratio, showing that IR spectral analysis can give information not available from the MFSA analysis alone. The 1030 cm$^{-1}$ peak has been very well correlated with glycosylation. The increase of this peak would be expected in the case of diabetic nephropathy, where the principal cause of tissue damage in diabetic patients is an uncontrolled level of glucose circulating in the blood. This leads to production of advanced glycation end products by non-enzymatic glycosylation typically by covalent bonding to proteins and lipids in tissues causing damage. The 1080 cm$^{-1}$ peak is traditionally associated with DNA; however, we notice increases in the 1080 cm$^{-1}$ peak in renal tissue structures that would not be expected to contain DNA. The 1080 cm$^{-1}$ is also associated with glycogen, and thus it is expected that we are again detecting tissue glycosylation (possibly of a different type). Future work will focus on developing a model in vitro system to better characterize what this biomarker is specifically related to in the tissue.

Finally, we applied a supervised multivariate data analysis technique called linear discriminant analysis (LDA) to the IR data. In LDA, the entire spectral range is used for each glomerulus (3850–900 cm$^{-1}$) and a known class assigned (Figure 4). LDA identifies sources of intergroup variance and maximizes discrimination between groups, allowing for analysis of clustering based on biochemical similarity. Care was taken in selection of principal components to avoid potential overfitting in LDA. A very high level of separation
between the 3 classes was achieved, demonstrating that IR spectroscopy could identify spectral differences between the 3 groups (Figure 4). Interestingly, the NLDs (blue) were distinct from ND glomeruli (black), indicating that IR imaging is powerful enough to identify renal biochemical changes due to diabetes even in patients without histologic changes.

To determine whether the IR spectral signature could also be used in renal transplantation, we studied 5 transplant patients with end-stage renal disease caused by diabetes. These patients with stable allograft function had undergone protocol posttransplant biopsies, both early (3–6 months posttransplant) and late (around 24 months posttransplant), to check for subclinical complications. Three of five patients had diabetic nephropathy–related histologic changes in the late biopsy, with an example of 1 of the patients shown in Figure 5. All 3 had changes in the 1080:1030 spectral ratio (Figure 6a). The 2 biopsies without histologic changes of diabetic nephropathy showed no change in the spectral ratio.

The early (3– to 6-month) biopsies had no clinical or histologic evidence of diabetic nephropathy. The average IR spectrum of the mesangium (measured in up to 6 glomeruli) from these biopsies did not show an altered spectral ratio. The entire spectrum (3850–900 cm\(^{-1}\)) was analyzed using the unsupervised multivariate analysis technique, principal component analysis. Principal component analysis converts every IR spectrum of a given sample into a single point and allows for analysis of clustering based on spectral variance where the closer the 2 points are, the more the spectral similarity. Recurrent and non-recurrent diabetic nephropathy in late biopsies was associated with distinct clusters in the early biopsies (Figure 6b). This phenomenon suggests that underlying biochemical changes occur even in the absence of histologic evidence of diabetic nephropathy. Thus, IR imaging may allow for the detection of diabetic nephropathy in transplanted kidneys earlier than is morphologically evident.

**DISCUSSION**

IR imaging allows for the identification of a biochemical signature of diabetic nephropathy in renal biopsies from a single unstained tissue section. In addition, we can identify early biochemical changes associated with the recurrence of diabetic nephropathy in transplant patients prior to histologic changes. Treatment modalities for diabetic nephropathy are not very effective, possibly because renal involvement is discovered too late. A new diagnostic tool that could detect very early changes of diabetic nephropathy could be useful for preventing further progression of disease, specifically in the posttransplant protocol serial biopsies setting. It could also be used as an intermediate end point in diabetic nephropathy recurrence prevention studies. Future studies are focused on...
using IR imaging for the assessment of interstitial fibrosis and exploiting the derived biochemical information to understand the mechanism of disease. In addition, the emergence of new laser-based IR imaging systems has the potential for real-time imaging. We will also focus on the integration of spatial and morphologic information from stained tissue sections with the spectral information from IR images to maximize the amount of diagnostic and prognostic information from tissue biopsies. It will also be important to expand the number of patients in these studies to allow for the training and validation of a predictive model with the requisite sensitivity and specificity.20

Figure 6 | Comparison of spectral changes from renal transplant patients who showed either recurrence or no recurrence of diabetic nephropathy. (a) Spectral changes in mesangium from 3 patients associated with recurrent diabetic nephropathy (DN) and 2 patients without recurrent diabetic nephropathy (NLD). Increases in glycosylation-associated (1030 cm\(^{-1}\)) and DNA- and glycosylation-associated (1080 cm\(^{-1}\)) peaks were observed in the patients with recurrent DN, while patients without recurrent DN did not show any increases. This shows promise of tracking progression of a diseased state. (b) Principal component analysis of histologically normal glomeruli from patients who had recurrent diabetic nephropathy within 3 years (3 patients, red) and patients who did not have recurrence (2 patients, blue). While all biopsies were histologically normal, there was clustering of biopsies from patients who later had recurrent DN. While the cohort for this study is small, this shows promise of an underlying biochemical signature that may be able to predict recurrence of DN prior to any histologic DN changes. Please note the differential scaling of the X- and Y-axis of the plot. The age range of the patients was 28 to 59 years.
MATERIALS AND METHODS
Subjects were characterized as diabetic based on a fasting glucose greater than 126 mg/dl or any serum glucose level greater than 200 mg/dl. A clinical diagnosis of diabetic nephropathy was made when the estimated Modification of Diet in Renal Disease glomerular filtration rate was <60 ml/min per 1.73 m² and a spot urine albumin/creatinine ratio was >30 mg/g creatinine. Formalin-fixed paraffin-embedded tissue blocks were retrieved from the University of Illinois at Chicago, Tissue Bank (institutional review board approval protocol number 2014-0267). One section was placed on a standard glass slide and stained using PAS stain for histologic analysis (cut at 3 μm), and an adjacent section was placed on an IR-compatible substrate (cut at 4 μm). Tissues for IR analysis were deparaffinized in hexane for 48 hours prior to imaging. The PAS-stained slide was scanned with Aperio ScanScope CS (Leica Biosystems, Nussloch, Germany) and digitally analyzed using Aperio ImageScope v11.2. The IR images were obtained with an Agilent FT-IR microscope (Santa Clara, CA) collected in transmission mode with a 36X collecting objective and 15X focusing objective with a pixel size of 2.2 μm × 2.2 μm similarly to as previously described. The background was collected with 128 co-adds, and each image was collected with 64 co-adds. Spectral resolution of 8 cm⁻¹ was used over the range of 3850 to 900 cm⁻¹. The data were baseline using standard linear baseline correction, had noise reduction applied using the minimum noise fraction approach, and were normalized to the amide I peak. Multivariate analysis was performed using OriginPro 9.0 (OriginLab, Northampton, MA) for principal component analysis and LDA. The native biopsy study consisted of 13 patients (age range 49–93), classed as NL (n = 4), NLD (n = 4), and DN (n = 5) based on clinical data, light microscopy, electron microscopy, or some combination of these. Each patient had approximately 350 spectra extracted from each glomerulus, with either 5 or 6 glomeruli examined per patient. It took approximately 3 minutes to acquire an IR image of each glomerulus. The transplant biopsy study consisted of 5 patients with an early (~3 months posttransplant) and late (~24 months posttransplant) biopsy. Three of the patients had evidence of diabetic nephropathy recurrence in their late biopsies, while 2 did not. The age range of the patients was 28 to 59 years. Each patient had approximately 350 spectra extracted from 5 glomeruli. The 5 spectra from each of the patients’ glomeruli were then averaged to form a single spectrum per patient of either early- or late-stage biopsy and were then subject to further analysis (Figure 6).

DISCLOSURE
All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL
Table S1. Patient cohorts with clinical and histologic parameters and diagnoses.
Supplemental material is linked to the online version of the paper at www.kidney-international.org.

REFERENCES