Multi-photon microscopy for the evaluation of interstitial fibrosis in extended criteria donor kidneys: A proof-of-concept study

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Abstract

Introduction: To evaluate the initial use of label-free second harmonic generation (SHG) imaging with two-photon excitation (2PE) auto-fluorescence in multiphoton microscopy (MPM) for the quantification of collagen/fibrosis on preimplantation biopsies of extended criteria donors (ECD).

Materials and methods: Twenty preimplantation core biopsies were extracted from 10 donor kidney samples, of which originated from seven donors. Kidney Donor Profile Index (KDPI) and Remuzzi scores of biopsies were calculated. Collagen parameters measured included quantification by the Collagen Area Ratio in Total Tissue (CART) and qualitative measurements by Collagen Reticulation Index (CRI).

Results: Biopsies classified with > 85% KDPI scores had significantly higher CART (p = 0.011) and lower CRI values (p = 0.025) than biopsies with ≤ 85% KDPI scores. Increase in CRI values correlated significantly with rise in recipient creatinine levels 1-year post-transplant (p = 0.027; 95% CI: 4.635-66.797).

Conclusion: MPM is an evolving technology that enables the quantification of the amount (CART) and quality (CRI) of collagen deposition in unstained preimplantation biopsies of donor kidneys stratified by KDPI scores. This initial evaluation found significant differences in both parameters between donor kidneys with more or less than 85% KDPI.

KEYWORDS
donor, extended criteria donors, interstitial fibrosis, kidney, kidney transplantation, multi-photon microscopy, transplant

1 INTRODUCTION

Renal anatomy and intrinsic residual function have always been of paramount importance in stratifying deceased donor kidneys for transplantation. This is particularly so for extended criteria donors (ECD) who do not fulfill the criteria of a standard criteria donor (SCD). Given that ECD kidneys have a reportedly 70% higher risk of graft failure than non-ECD kidneys,1 it is imperative to risk-stratify across the...
spectrum of available ECDs. The dichotomous classification of single versus dual implantation has also reaffirmed the need to objectively determine the quality of a donor kidney.\(^2\)

Several frameworks exist to characterize the quality of donor kidneys. One approach is the procurement of a preimplantation renal biopsy for histopathological analysis. Remuzzi et al. pioneered a histological-based selection criteria to evaluate the quality of donor kidneys for single or dual implantation. Four variables are analyzed – glomerular global sclerosis, tubular atrophy, interstitial fibrosis, arterial and arteriolar narrowing.\(^3\) In the same vein, The United Network for Organ Sharing Kidney Transplantation Committee\(^4\) developed the Kidney Donor Profile Index (KDPI) score, which aims to incorporate clinical parameters to determine the quality of deceased donor kidneys.

As a transplant center located within Asia, we previously sought to clarify if clinical evaluation by KDPI was superior to histopathological assessment of donor kidneys.\(^5\) It was concluded that KDPI per se was insufficient to stratify single versus dual implants, and that Remuzzi scoring demonstrated higher sensitivity, specificity, and positive predictive value in selecting kidneys for dual implants. Patients with KDPI > 1.1 were recommended still have routine histopathological biopsies to optimize stratification of ECD kidneys.

With the advancements of in-vivo histological techniques, there has been growing interest in multiphoton microscopy (MPM) as a step up from conventional histological staining methods.\(^6\) It combines Second Harmonic Generation (SHG) and Two Photon Excitation (TPE) Fluorescence to image extracellular matrix architecture in intricate detail.\(^7\) MPM has been previously utilized for deep optical sectioning of live human tissue and is exceptionally accurate at demonstrating specific contrast in tissue exhibiting fibrillar collagenous qualities.\(^8\)

Within the context of donor kidney classification, MPM may be used for the quantification of renal interstitial fibrosis. Since interstitial fibrosis comprises part of Remuzzi’s variables, the aim of the present study was to employ MPM as a novel imaging technique to characterize and quantify interstitial fibrosis in preimplantation kidney biopsies.

2  |  MATERIALS AND METHODS

2.1  |  Sample retrieval

The study protocol was approved by the National Healthcare Group Domain Specific Review Board, Reference Number: 2017/01003. Specific to our objectives, all core biopsy samples were extracted from deceased donors that fulfilled the Extended Criteria Donor (ECD) standards, as delineated by the Scientific Registry of Transplant Recipients. This is defined as (1) kidneys retrieved from donors aged 60 years or above, or (2) donors aged 50–59 years old with at least two of the following: (3) serum creatinine > 1.5 mg/dL, (4) history of hypertension, or (5) death resulting from a cerebrovascular accident. From the Ethics Board-approved tissue repository, 20 core kidney transplant biopsy specimen tissue blocks from our Centre (National University Hospital, Singapore) were selected and retrieved from 7 donors. The Max-Core Disposable Biopsy Gun was used for the procurement of all specimens. It utilizes an 18G needle with a depth of 22 mm. All 20 specimens were taken at the time of preimplantation. Qualities of all deceased-donor kidneys were evaluated by an experienced pathologist, classifying the samples according to the Remuzzi scoring system. The biopsy samples were allocated an Interstitial Fibrosis score for the purposes of analysis. Nonidentifiable clinical data from our kidney transplant center database was retrieved.

2.2  |  Sample preparation and multi-photon microscopy

The unstained de-paraffinized Formalin-Fixed Paraffin-Embedded tissues of 5-micron thickness were imaged using the commercially available laser-based Genesis200 Multi-Photon Microscopy system (Histoindex Pte. Ltd, Singapore). Image acquisition was performed at 2X and 20X objective with 512 × 512-pixel resolution. MPM is an imaging modality comprising SHG and TPE microscopy. SHG signals are detected when a laser of femtosecond range interacts with tissues that have unique, noncentrosymmetric structure within the ECM such as collagen fibers and elastin. When this SHG signal is combined with auto-fluorescence signal from the cells using TPE, MPM is able to penetrate deep into tissues to generate a high-resolution image that reflects both the architecture of the ECM and surrounding cellular structure. On SHG microscopy, the fibrosis within the renal interstitium was readily identified, with fluorescent green signals on SHG analysis corresponding to collagen fibers. In contrast, collagen-devoid tumor tissues and collagen-poor normal renal parenchyma demonstrated minimal SHG signals.

Embedded within the proprietary stain-free imaging technology and analysis software has the capability to detect sensitively and quantify fine collagen dynamics that are otherwise unobserved with traditional staining techniques. The analysis is able to identify and characterize previously described SHG collagen characteristics that correspond with the collagen profiles of the tissues. The percentage of collagen in relation to the total region of interest is defined as the Collagen Area Ratio (CAR). The percentage of collagen within the total tissue sample was expressed as the Collagen Area Ratio in Total Tissue (CART). Both CAR and CART quantify the collagen content of the tissues. After the collagen area was defined, a skeletonization of the collagen was applied, providing a schematic representation of the collagen fibers and their intersection points. The Collagen Reticulation Index (CRI) was defined as the total number of intersection points in the given collagen area, representing the degree of reticulation and interconnection of collagen fibers.

Multiphoton imaging was performed and designated Regions of Interests (ROI) for analysis of collagen parameters in each specimen. At each region, clinically validated algorithms quantify collagen content and structure. These algorithms were validated against conventional stained Hematoxylin & Eosin (H&E) images by pathologists at the National University Hospital, Singapore (Figures 1 and 2). Collagen
content was measured quantitatively by CAR and CART, and collagen structure was measured by CRI.

2.3 Statistical analysis

Parametric continuous variables were presented as Mean ± Standard Deviation (SD). Nonparametric continuous variables were reported as Median and Range. Data There were no missing values from data collected. Chi-squared tests (or Fisher’s exact test, wherever applicable) were utilized to compare categorical variables. The Mann–Whitney U test was used to compare nonparametric continuous variables. Statistical significance in this study was determined as \( p < .05 \). All reported \( p \) values were two-sided, and analyses were performed with IBM SPSS Version 26.0.

3 RESULTS

3.1 Donor baseline characteristics

Twenty preimplantation core biopsies were imaged from 10 donor kidney samples, of which originated from seven donors (Table 1). All samples comprised of more than 10 glomeruli for histopathological evaluation. Banff and Remuzzi scorings of each biopsy specimen are provided in Table 2. Baseline characteristics are detailed in Table 3. Mean donor age was 54.45 years ± 7.95, and 57.1% of the donors were male. Three donors underwent single kidney implantation. The donors had a mean terminal creatinine level of 91.14 µmol/L ± 30.11 prior to transplantation. Sixteen core biopsies were allocated a Remuzzi scoring of 4–6, while the remaining four were categorized into the 0–3 group. Median Kidney Donor Profile Index (KPDI) scores across all
TABLE 1  Clinical, histopathological, and MPM-derived collagen parameters of 20 preimplantation core biopsy samples

| Patient | Biopsy S/N | Donor Age | Donor Gender | Donor Hypertension | Terminal Creatinine (µmol/L) | Kidney Donor Profile Index (KDPI Score) | Collagen Area Ratio (CAR) | Collagen Area Ratio in Tissue (CART) | Collagen Reticulation Index (CRI) |
|---------|------------|-----------|--------------|--------------------|-----------------------------|----------------------------------|---------------------------|----------------------------------|----------------------------------|
| A       | 1          | 54.07     | Female       | Yes                | 58                          | 72%                              | 33.50                     | 50.44                            | 3.90                             |
|         | 2          |           |              |                    |                             |                                  | 37.77                     | 50.29                            | 3.75                             |
| B       | 3          | 57.55     | Female       | No                 | 95                          | 76%                              | 36.35                     | 45.91                            | 4.44                             |
|         | 4          |           |              |                    |                             |                                  | 39.01                     | 51.40                            | 4.40                             |
| C       | 5          | 44.98     | Male         | Yes                | 93                          | 63%                              | 22.13                     | 32.21                            | 4.77                             |
|         | 6          |           |              |                    |                             |                                  | 29.60                     | 38.47                            | 4.89                             |
|         | 7          |           |              |                    |                             |                                  | 30.16                     | 38.85                            | 4.78                             |
|         | 8          |           |              |                    |                             |                                  | 35.57                     | 45.31                            | 5.12                             |
| D       | 9          | 56.91     | Female       | Yes                | 68                          | 92%                              | 35.86                     | 60.90                            | 4.15                             |
|         | 10         |           |              |                    |                             |                                  | 31.33                     | 46.59                            | 4.15                             |
| E       | 11         | 58.55     | Male         | Yes                | 82                          | 86%                              | 35.06                     | 50.62                            | 4.10                             |
|         | 12         |           |              |                    |                             |                                  | 34.46                     | 51.72                            | 4.34                             |
|         | 13         |           |              |                    |                             |                                  | 31.89                     | 47.52                            | 4.30                             |
|         | 14         |           |              |                    |                             |                                  | 36.30                     | 52.15                            | 4.70                             |
| F       | 15         | 43.23     | Male         | No                 | 152                         | 46%                              | 36.78                     | 53.50                            | 4.53                             |
|         | 16         |           |              |                    |                             |                                  | 25.22                     | 34.58                            | 4.29                             |
|         | 17         |           |              |                    |                             |                                  | 39.13                     | 51.01                            | 5.31                             |
|         | 18         |           |              |                    |                             |                                  | 29.11                     | 41.58                            | 4.54                             |
| G       | 19         | 65.88     | Male         | Yes                | 90                          | 90%                              | 34.93                     | 55.74                            | 3.71                             |
|         | 20         |           |              |                    |                             |                                  | 34.97                     | 56.80                            | 3.62                             |

Donors was 76% (IQR: 65.25 – 89%), with three out of seven donors obtaining a KDPI of more than 85%.

3.2 Comparison of MPM-evaluated collagen characteristics by KDPI

Although CAR values were identical across all samples, biopsies classified with > 85% KDPI demonstrated a significantly higher CART (51.94 vs. 45.61; \( p = .011 \)) compared to biopsies with 20–85% KDPI percentages (Table 4). Conversely, they had lower CRI compared to biopsies with 20–85% KDPI scores (4.15 vs. 4.53; \( p = .025 \)).

3.3 Comparison of recipient clinical outcomes post-transplant by KDPI

Recipient creatinine and eGFR levels at 1 and 3-years post-transplant did not differ significantly between 20–85% and > 85% KDPI scores (Table 5). However, it is worth noting that single versus dual implantations may potentially contribute to further disparities in clinical outcomes – dual implant kidneys may have overcome the underlying deficiencies of donor kidneys with > 85% KDPI scores. This precludes a definite comparison from being made.

3.4 MPM-evaluated collagen parameters and predictors of long-term recipient outcomes

Upon univariable analysis, CAR and CART were not observed to be independent predictors of recipient renal function at 1 and 3-years post-transplant. Interestingly, an increase in CRI correlated significantly with rise in recipient creatinine levels at 1-year post-transplant (\( p = .027; \) 95% CI: 4.635–66.797).

4 Discussion

This proof-of-concept study demonstrated the use of commercially available MPM-enabled detailed imaging of renal interstitial fibrosis in preimplantation core biopsies of donor kidneys stratified by KDPI scores. CART and CRI values differed significantly between biopsies of 20–85% and > 85% KDPI scores. CRI was demonstrated to be a statistically significant predictor of recipient creatinine levels 1-year post-transplant.

Multiphoton technology flaunts in its ability to precisely quantify the extent of collagen tissue present in tissue, going beyond to analyze collagen organization from the molecular up till the tissue architectural scale. This relies on its nonlinear microscopic method of imaging, as well as the involvement of the Second Harmonic Generation
### TABLE 2 Banff and Remuzzi components of 20 preimplantation core biopsy samples

| Patient | Biopsy S/N | Number of Glomeruli | Glomerular Global Sclerosis | Tubular Atrophy | Interstitial Fibrosis | Arterial and Arteriolar Narrowing |
|---------|------------|---------------------|----------------------------|-----------------|---------------------|----------------------------------|
| A       |            |                     |                            |                 |                     |                                  |
|         | 1          | 52                  | 1                          | 1               | 1                   | 2                                |
|         | 2          | 52                  | 1                          | 1               | 1                   | 2                                |
| B       |            |                     |                            |                 |                     |                                  |
|         | 3          | 26                  | 1                          | 1               | 1                   | 0                                |
|         | 4          | 26                  | 1                          | 1               | 1                   | 0                                |
| C       |            |                     |                            |                 |                     |                                  |
|         | 5          | 63                  | 1                          | 1               | 1                   | 1                                |
|         | 6          | 63                  | 1                          | 1               | 1                   | 1                                |
|         | 7          | 20                  | 0                          | 1               | 1                   | 1                                |
|         | 8          | 20                  | 0                          | 1               | 1                   | 1                                |
| D       |            |                     |                            |                 |                     |                                  |
|         | 9          | 48                  | 1                          | 1               | 1                   | 2                                |
|         | 10         | 48                  | 1                          | 1               | 1                   | 2                                |
| E       |            |                     |                            |                 |                     |                                  |
|         | 11         | 73                  | 1                          | 1               | 1                   | 1                                |
|         | 12         | 73                  | 1                          | 1               | 1                   | 1                                |
|         | 13         | 53                  | 1                          | 1               | 1                   | 1                                |
|         | 14         | 53                  | 1                          | 1               | 1                   | 1                                |
| F       |            |                     |                            |                 |                     |                                  |
|         | 15         | 40                  | 1                          | 1               | 1                   | 2                                |
|         | 16         | 40                  | 1                          | 1               | 1                   | 2                                |
|         | 17         | 34                  | 1                          | 1               | 1                   | 1                                |
|         | 18         | 34                  | 1                          | 1               | 1                   | 1                                |
| G       |            |                     |                            |                 |                     |                                  |
|         | 19         | 15                  | 0                          | 1               | 1                   | 2                                |
|         | 20         | 15                  | 0                          | 1               | 1                   | 2                                |

microscopy.\(^7\) SHG allows for accurate identification of noncentrosymmetric molecules such as fibrillar collagens, which are unambiguously implicated in multisystem pathologies that demonstrate fibrosis.\(^10\)–\(^14\) Of which includes fibrillar collagen, that is found within the extracellular matrix of the renal parenchyma. This is also coupled with TPE – fluorescence imaging of the fibrotic regions, undergoing extrinsic fluorescent labeling and auto-fluorescence with two-photon excitation sequence.\(^15\) In fact, MPM is similarly evaluated within the fields of genito-urinary oncology, yielding some promising results. It could potentially serve as a biomarker for prostate tumor aggressiveness,\(^16\) or even as a prognostic indicator of progression of nonmuscle invasive bladder cancer.\(^17\)

Most notably, a distinct advantage that it has over routine histological assessment is the ability to evaluate tissue samples without the need for any staining.\(^7\) Pathologists have conventionally relied upon various methods (light microscopy, immunohistochemistry, and electron microscopy) for the fixation, processing, sectioning, and staining of a said tissue specimen.\(^18\) Glomerulosclerosis, tubular atrophy, interstitial fibrosis, and vascular attenuation are the key points of interest to determine the classification, suitability, and type of transplantation to be performed. With the incorporation of MPM technology, shortcomings of histological assessment can be accounted for.\(^19\)

Ongoing research to establish the utility of MPM and artificial intelligence in assessing the other components of Remuzzi scoring is still underway.\(^20\)

In the same vein, Ranjit et al. incorporated the use of similar technology – SHG and fluorescence lifetime imaging (FLIM) of tissue autofluorescence to quantify renal fibrosis in fixed tissue samples of a mouse UUO model.\(^21\) They reported sensitivity and specificity values that were on-par with standard histological methods, and SHG-FLIM independently predicted the extent of fibrosis in the mouse model. The authors advocated for coexisting usage of SHG-FLIM with pre-existing histological methods to bolster the accuracy of collagen quantification in renal fibrosis.

To holistically validate the utility of MPM, further correlation with other traditional staining methods besides H&E is required. Martin et al. assessed the capability of SHG/TPE in assessing collagen deposition in cardiac tissue, verified with traditional picrosirius red staining.\(^22\) In the context of collagen characterization in intestinal specimens, SHG/TPE was noninferior to Masson’s trichrome staining in differentiating between Crohn’s disease and intestinal tuberculosis.\(^23\) There appears to be promising evidence for the introduction of MPM as a definitive adjunct to histological methods of evaluating collagen features in biopsy samples, even beyond the fields of renal transplantation. However, when extended to the other aspects of preimplantation biopsy assessment (glomerular sclerosis, tubular atrophy, and arteriolar changes), the capability of MPM technology remains unclear. To our knowledge, this is the first study to comparatively analyze MPM (combined SHG-TPE) technology in donor kidneys with existing validated Remuzzi and KDPI criteria. As a proof-of-concept, the MPM-derived
TABLE 3  Donor baseline characteristics

| ECDs (n = 7) | Donor age, yr [mean (SD)] | 54.45 (7.95) |
|--------------|---------------------------|--------------|
| Gender [n (%)] | Male | 4 (57.1) |
| Female | 3 (42.9) |
| Diabetes [n (%)] | No | 5 (71.4) |
| Yes | 2 (28.6) |
| Hypertension [n (%)] | No | 2 (28.6) |
| Yes | 5 (71.4) |
| Dialysis [n (%)] | No | 7 (100) |
| Yes | 0 (0) |
| Acute Kidney Injury [n (%)] | No | 5 (71.4) |
| Yes | 2 (28.6) |
| Terminal Creatinine (µmol/L) [mean (SD)] | 91.14 (30.11) |
| Cause of Death [n (%)] | Cerebrovascular Accident (Stroke) | 7 (100) |
| Others | 0 (0) |
| Remuzzi Score [n (%)] | 0-3 | 1 (14.3) |
| 4-6 | 6 (85.7) |
| Remuzzi Score (No. of biopsies) [n (%)] | 0-3 | 4 (20) |
| 4-6 | 16 (80) |
| Kidney Donor Profile Index (KDPI) Score % [mean (SD)] | 75 (16.48) |
| KDPI [n (%)] | 20–85% | 4 (57.1) |
| > 85% | 3 (42.9) |

TABLE 4  Comparison of CAR/CART/CRI by KDPI

| 20–85% (n = 12) | > 85% (n = 8) | p |
|------------------|---------------|---|
| Collagen Area Ratio (CAR) [median (IQR)] | 34.53 (29.23-37.52) | 34.95 (23.53-35.66) | 1.000 |
| Collagen Area Ratio in Tissue (CART) [median (IQR)] | 45.61 (38.56-50.87) | 51.94 (48.29-56.54) | .011 |
| Collagen Reticulation Index (CRI) [median (IQR)] | 4.53 (4.31-4.86) | 4.15 (3.80-4.33) | .025 |

TABLE 5  Comparison of recipient clinical outcomes by KDPI

| 20–85% (n = 12) | > 85% (n = 8) | p |
|------------------|---------------|---|
| eGFR 1 Year (mL/min/1.73 m²) [median (IQR)] | 68.50 (43-105) | 53 (12.75-55) | .160 |
| Creatinine 1 Year (µmol/L) [median (IQR)] | 93.50 (54-168) | 105 (26-160) | .348 |
| eGFR 3 Years (mL/min/1.73 m²) [median (IQR)] | 60 (46-90) | 49.50 (8.25-66) | .086 |
| Creatinine 3 Years (µmol/L) [median (IQR)] | 107.50 (67-157) | 90 (22.50-133.50) | .435 |

collagen quantifiers were observed to have internal homogeneity in its evaluation of interstitial fibrosis across all 20 kidney biopsies. Scores were also consistent between biopsies from different kidneys that originated from the same donor. However, there were no correlations between the collagen parameters and Remuzzi scoring, as well as KDPI values in all biopsy specimens.

4.1  Strengths and limitations

To the best of our knowledge, this is the first study to evaluate the potential of MPM to characterize renal interstitial fibrosis in donor kidneys stratified by KDPI scores. However, several limitations ought to be acknowledged. First, the sample size is small, affecting the validity and increasing the margin of error of the aforementioned results. Second, this study is retrospective in nature and hence prone to selection bias. A larger number over a longer duration of the study period would allow us to correlate MPM-derived collagen characteristics with more confidence. Lastly, although CRI was a statistically significant predictor of recipient creatinine levels 1-year post-transplant, it should be noted that this is accompanied by correspondingly wide confidence intervals. Hence, concluding that CRI was truly indeed an independent predictor should be validated with future studies.

5  CONCLUSION

In this proof-of-concept study, we reported noteworthy differences in MPM-derived collagen parameters between donor kidneys of varying KDPI scores. When pegged against validated histological and clinical frameworks in stratifying donor kidney biopsies, it is still an evolving technology in question. With further advancements in imaging techniques, future observational studies are eagerly awaited to support or challenge the reproducibility of our findings.
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CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS
WWS was involved in data analysis and manuscript writing. RZCT and LYO were involved in the histological preparation of the samples. All authors contributed to the conceptualization, planning, and supervision of the study.

DATA AVAILABILITY STATEMENT
Data is available upon request from the corresponding author.

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