Renal Graft Fibrosis and Inflammation Quantification by Automated FTIR-imaging Technique

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Supplementary Data

Second Harmonic Generation (SHG) analysis

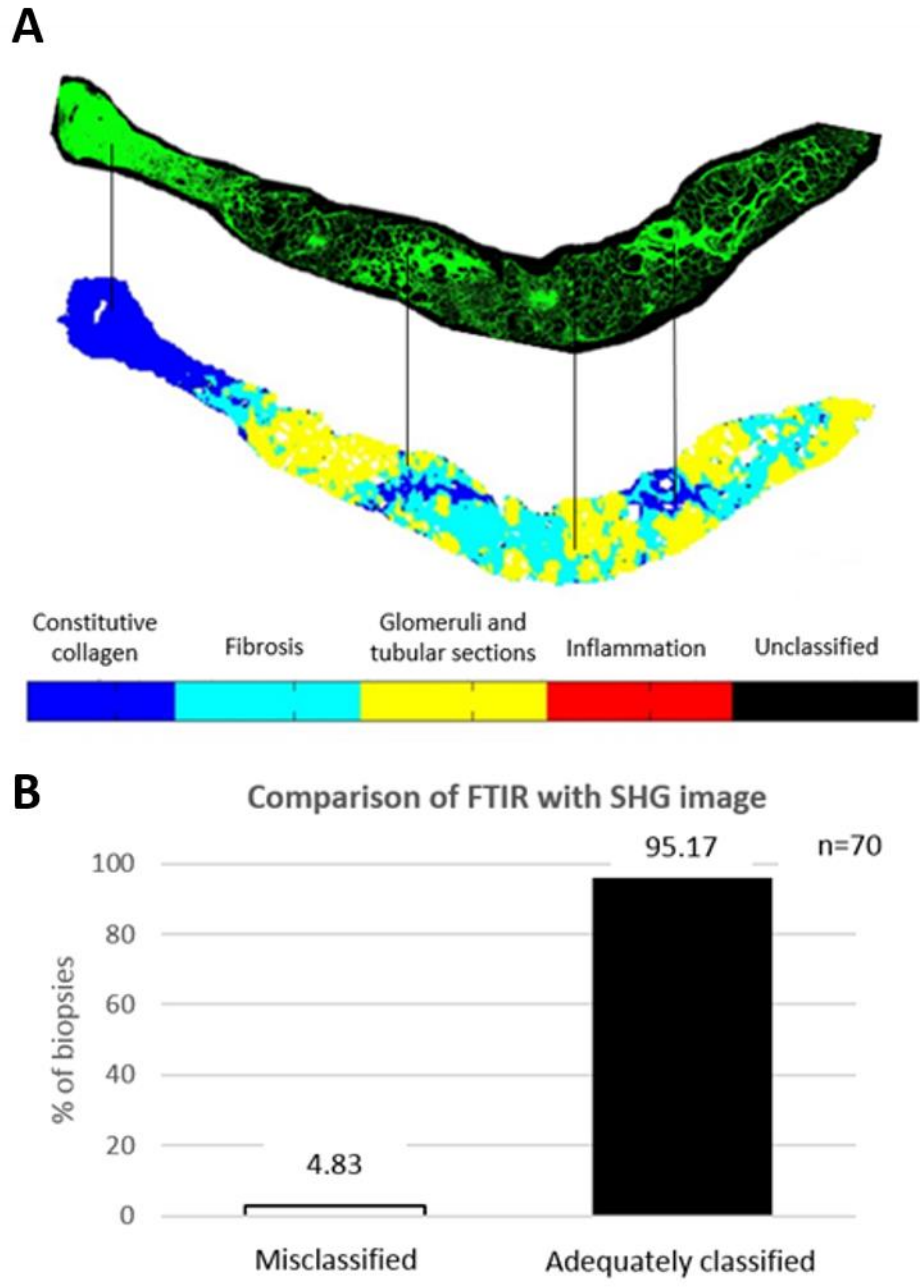
In order to improve the qualitative assessment of fibrosis classification by Fourier-transform infrared (FTIR) imaging, we performed a comparison between FTIR images and Second Harmonic Generation (SHG) signal.

A confocal microscope (LSM 710-NLO, Zeiss Microsystems, Gennevilliers, France) coupled with CHAMELEON femtosecond Titanium-Sapphire Laser (Coherent Inc., Santa Clara, CA, USA) was used to collect the SHG signal using 860 nm excitation wavelength with 420-440 band-pass filters. All acquisition settings were constant between specimens. Total area of biopsies was acquired by several acquisition fields using a 20x, 0.95-NA objective lens (Zeiss). Images from the same biopsy were then associated by concatenation to rebuild the total area of biopsy. In order to quantify the SHG signal associated with type I collagen, positive and negative signal thresholds defined by the operator were input to binarize the image. Then, the ratio of positive pixels to the total number of pixels was calculated to determine the collagen content used for the quantification of fibrosis.

We observed that 95.17% (63) of renal graft biopsies were adequately classified by FTIR imaging as compared to SHG analysis (**Supplementary Figure 1**). Comparison between FTIR and SHG images confirmed the adequate classification of fibrosis by our model. We did not use SHG for the quantitative assessment of FTIR methods because of the lack of reproducibility of SHG methods. Indeed, quantification by SHG requires the determination of positive and negative thresholds by a human operator, which introduces high variability

Supplementary figure 1: **Assessment of the robustness of classification by FTIR using the LDA model**

Fibrosis and constitutive collagen classes on the FTIR image of each biopsy from the test dataset was blindly compared to the SHG signal on the same sections, evaluated by three observers for 70 biopsies (A). Over 95% of biopsies were adequately classified by the LDA model, whereas only 4.83% were misclassified (B).



Class discrimination based on class centroid Spectral differences

The ability to discriminate fibrosis and constitutive collagen is based on the expertise of the renal pathologist, who must be able to recognize the histological structure by morphological analysis of the tissue. Indeed, constitutive collagen is exclusively located in the capsula, walls of vessels, Bowman capsule, and vessel support-blades. In Masson's trichrome, the presence of interstitial green staining elsewhere indicates the presence of pathological collagen, meaning fibrosis.

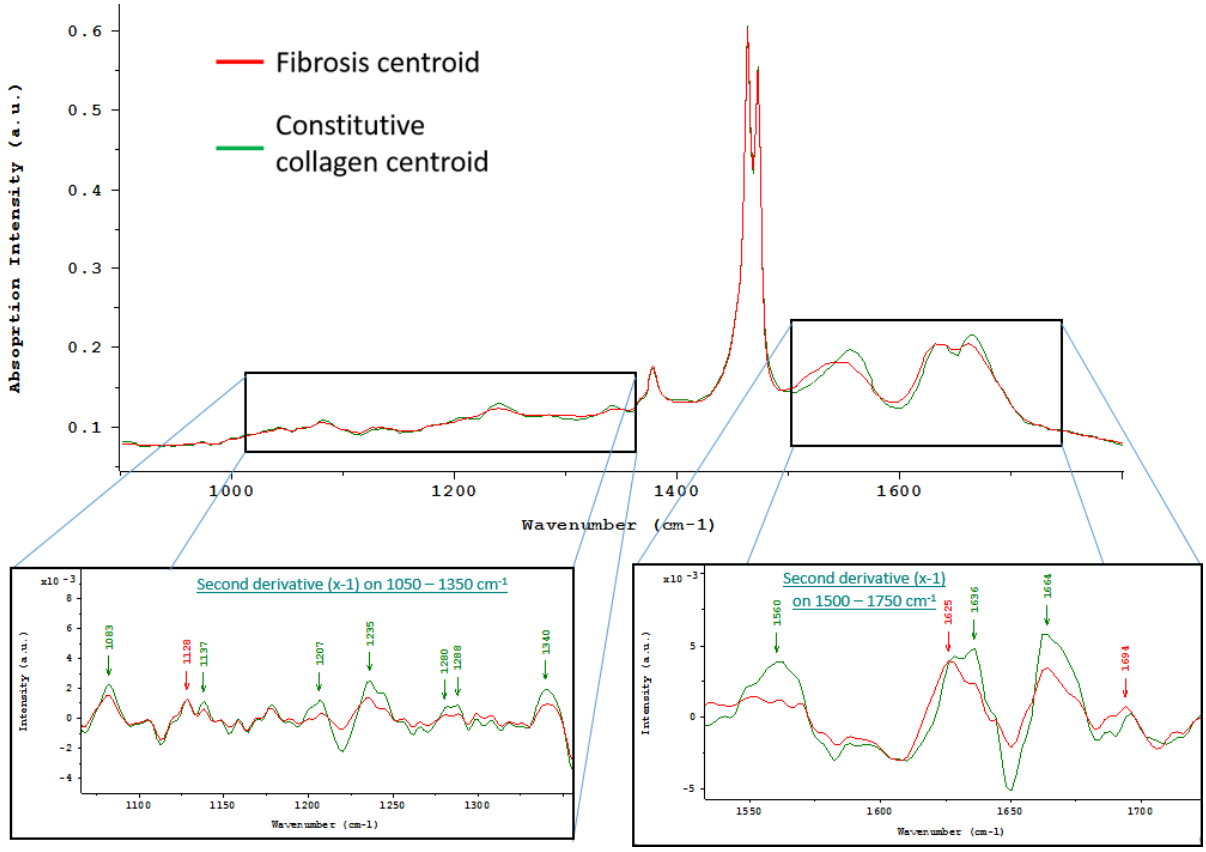
FTIR imaging is able to discriminate fibrosis and constitutive collagen. This ability is due to an intrinsic discrimination of these two histological structures based on comparison of the centroid (spectra) of these two classes. Supplementary Figure 2 displays the centroid spectra associated with fibrosis (red) and with constitutive collagen (green). Some spectral differences appear between these two centroids (arrows) in the 1550 cm^{-1} and 1770 cm^{-1} areas. These spectral differences, based on a different histological structure, explain the ability of FTIR imaging to discriminate these two classes.

In our study, the comparison between FTIR images and Masson's trichrome stained slides for the 166 renal biopsies found that 96.3% of FTIR images were adequately classified as regards constitutive collagen.

Supplementary Figure 2: Fibrosis and constitutive collagen centroid differences

The following figure displays the centroid spectra associated with fibrosis (red) and constitutive collagen (green). These spectra were calculated by the classification algorithm and are considered as the spectral signatures of each of these two tissue structures. The spectral differences between the two structures are highlighted in the inserts, corresponding to the second derivatives (multiplied by a factor -1 for readability) of the centroids in the spectral ranges 1050 – 1350 cm-1 and 1500 – 1750 cm-1. These spectral regions are associated with vibrations of carbohydrate and protein bonds of the collagen. Indeed, the clearly highlighted spectral differences correspond to vibrations sensitive to molecular alterations in the collagen, as has been reported in the literature by various research teams (including our own), in other biomedical contexts. For example, one can find evidence of the potential of infrared spectroscopy to distinguish modified collagen and native collagen in investigations

focused on the tumour microenvironment[1-4], in alterations of vascular walls [5] or bones[6], or in skin aging [7, 8].

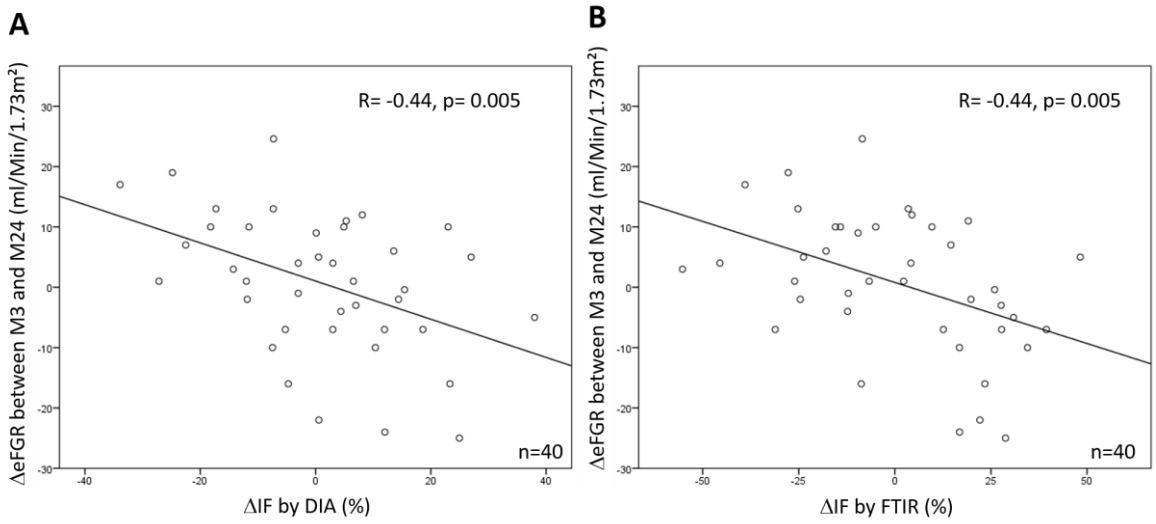


The distinction of fibrosis from constitutive collagen relies on the association of infrared micro-imaging, a non-destructive modality that makes it possible to collect a huge number of spectra, with multivariate statistical data processing. Processing the classification of the spectra that make up the infrared images aims to identify spectral signatures specific to the various histological structures, independently of the variability inherent to the biopsies and individuals. Since classification algorithms are based on statistical comparison between all data, the robustness of the approach increases with the sample size, as is the case in our study.

Correlation between variation of eGFR and interstitial fibrosis between M3 and M24

We found a good correlation between variation in eGFR and interstitial fibrosis between M3 and M24 for both DIA and FTIR techniques (Supplementary figure 3). These results improve the reliability of the two techniques used.

Supplementary figure 3 shows the correlation between eGFR as estimated by the MDRD formula, and quantification of interstitial fibrosis between M3 and M24 (n=40): A) Quantification of interstitial fibrosis by the DIA technique (R=-0.44, p=0.005) and B) Quantification of interstitial fibrosis by FTIR imaging (R=-0.44, p=0.005).



References:

1. Noreen, R, Chien, CC, Chen, HH, Bobroff, V, Moenner, M, Javerzat, S, Hwu, Y, Petibois, C: FTIR spectro-imaging of collagen scaffold formation during glioma tumor development. *Anal Bioanal Chem,* 405**:** 8729-8736. doi: 8710.1007/s00216-00013-07337-00218. Epub 02013 Sep 00226., 2013.
2. Benard, A, Desmedt, C, Smolina, M, Szternfeld, P, Verdonck, M, Rouas, G, Kheddoumi, N, Rothe, F, Larsimont, D, Sotiriou, C, Goormaghtigh, E: Infrared imaging in breast cancer: automated tissue component recognition and spectral characterization of breast cancer cells as well as the tumor microenvironment. *Analyst,* 139**:** 1044-1056. doi: 1010.1039/c1043an01454a., 2014.
3. Kumar, S, Desmedt, C, Larsimont, D, Sotiriou, C, Goormaghtigh, E: Change in the microenvironment of breast cancer studied by FTIR imaging. *Analyst,* 138**:** 4058-4065. doi: 4010.1039/c4053an00241a., 2013.
4. Nallala, J, Gobinet, C, Diebold, MD, Untereiner, V, Bouche, O, Manfait, M, Sockalingum, GD, Piot, O: Infrared spectral imaging as a novel approach for histopathological recognition in colon cancer diagnosis. *J Biomed Opt,* 17**:** 116013. doi: 116010.111117/116011.JBO.116017.116011.116013., 2012.
5. Cheheltani, R, McGoverin, CM, Rao, J, Vorp, DA, Kiani, MF, Pleshko, N: Fourier transform infrared spectroscopy to quantify collagen and elastin in an in vitro model of extracellular matrix degradation in aorta. *Analyst,* 139**:** 3039-3047. doi: 3010.1039/c3033an02371k. Epub 02014 Apr 02324., 2014.
6. Gamsjaeger, S, Mendelsohn, R, Boskey, AL, Gourion-Arsiquaud, S, Klaushofer, K, Paschalis, EP: Vibrational spectroscopic imaging for the evaluation of matrix and mineral chemistry. *Curr Osteoporos Rep,* 12**:** 454-464. doi: 410.1007/s11914-11014-10238-11918., 2014.
7. Eklouh-Molinier, C, Happillon, T, Bouland, N, Fichel, C, Diebold, MD, Angiboust, JF, Manfait, M, Brassart-Pasco, S, Piot, O: Investigating the relationship between changes in collagen fiber orientation during skin aging and collagen/water interactions by polarized-FTIR microimaging. *Analyst,* 140**:** 6260-6268. doi: 6210.1039/c6265an00278h., 2015.
8. Nguyen, TT, Eklouh-Molinier, C, Sebiskveradze, D, Feru, J, Terryn, C, Manfait, M, Brassart-Pasco, S, Piot, O: Changes of skin collagen orientation associated with chronological aging as probed by polarized-FTIR micro-imaging. *Analyst,* 139**:** 2482-2488. doi: 2410.1039/c2483an00353a., 2014.