Counting glomeruli in a human transplant kidney using MRI

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INTRODUCTION - The goal of this work was to count the number (Nglom) and measure the volume (Vglom) of every glomerulus in human kidneys with MRI. Changes in the number and size of glomeruli have been linked to ~90% of all chronic renal diseases and many systemic diseases, and nephron number is an early predictor of susceptibility to renal and cardiovascular disease (1, 2). Current histological methods to assess Nglom and Vglom require resection and destruction of the entire kidney and merely extrapolate measurements from small numbers of glomeruli (3). These techniques are neither clinically viable due to their destructive nature nor comprehensive in their assessment of Nglom and Vglom. A method to assess Nglom and Vglom in humans that is non-invasive and measures every glomerulus could be important for clinical diagnostic screening, transplant characterization, and large-scale epidemiological studies. The parameters Nglom and Vglom may be measured in the rat kidney by injecting the superparamagnetic nanoparticle, cationic ferritin (CF), and imaging with MRI (4 - 6). CF binds to anionic proteoglycans in the glomerular basement membrane, and is thus glomerulus-specific in the kidney. This technique does not require destruction of the kidney and measures every CF-labeled glomerulus in the kidney. Here we adapted this same technique to count and measure the size of every glomerulus in human donor kidneys as a first proof-of-concept study of MRI with CF to measure human kidney morphology.

METHODS - *Sample preparation.* Cationized ferritin (CF) was synthesized according to Danon, et al. (7). Four human kidneys were acquired though a donation network (The International Institute for the Advancement of Medicine) with institutional approval and informed consent. Kidneys were received in University of Wisconsin preservative within 24 hours of donor death. Kidneys were viable organs throughout the procedure, up until formalin fixation. The renal artery of three of the kidneys (one from a male, two from females) was catheterized and the kidney was perfused with phosphate buffered saline (PBS), then a 44 mg of CF in PBS, then PBS, then formalin. The fourth kidney was prepared in the same way but remained naive to CF (control). ~ 1 mm^3 tissue biopsies were taken from the cortex of each kidney and prepared for immunofluorescence microscopy (detailed below). All kidneys were stored in 10% neutral buffered formalin. *MRI.* Prior to imaging, kidneys were removed from formalin storage and washed three times in 500 ml PBS. The kidneys were then imaged using a Bruker 7T/35 MRI scanner and a 72-mm quadrature transmit/receive radio frequency coil (Bruker, Billerica, MA). A T_2^*-weighted (TE/TR = 20/39 ms) three-dimensional gradient echo fast low angle shot (FLASH) sequence was used to image the entire kidney. MRI volumes were acquired with a 117x117x117 μm resolution (field of view = 6x6x10.5 cm, matrix size = 512x512x896, 5 averages, total scan time = 10 hr 39 min). *Immunofluorescence.* We took ~1 mm^3 tissue biopsies during preparation. We placed biopsies in 10% neutral buffered formalin for four hours and washed in PBS overnight. Samples were then cryoprotected in sucrose, rapidly frozen, and sectioned at 35 μm. Sections were washed in PBS, permeablized, incubated in rabbit anti-horse spleen ferritin, an Alexa594 goat anti-rabbit secondary antibody and 4',6-diamidino-2-phenylindole (Invitrogen), and imaged on a Zeiss 710 laser scanning confocal microscope. *Image processing.* Nglom and Vglom were measured from the MRI volumes using custom software written in MATLAB (The Mathworks, Natick, MA). Briefly, we derived the Hessian matrix for each voxel in the volume to detect the centroid voxels of glomeruli their surrounding 'glomerular' voxels. Voxels that constitute a glomerulus are the clustered using a gaussian mixture model. It was assumed that human glomeruli would be no less than $1.60x10^6$ μm^3 and no more than $25.6x10^6$ μm^3.

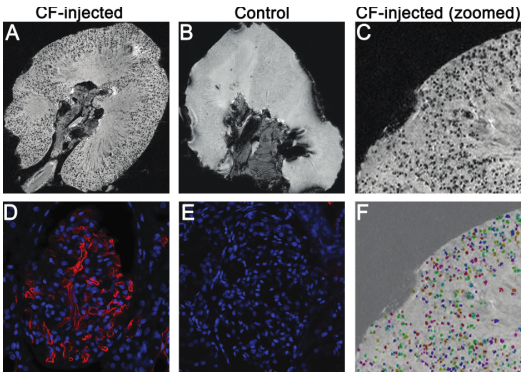

Fig. 1 - MRI of a CF-labeled human kidney reveals punctate dark spots throughout the organ. Each spot represents a single glomerulus (A and C). A control kidney showed no such spots (B). Immunofluorescence confirmed the accumulation of CF (red) in glomeruli of CF inoculated kidneys (D). Naive control glomeruli remained clear of CF-related immunofluorescence (E). Each glomerulus may be identified and counted in the entire kidney (F).

RESULTS – 3D MRI of the human kidneys perfused with CF revealed punctate spots of signal darkening (Fig. 1 A and C). Each dark spot represents a single glomerulus and is caused by the accumulation of the superparamagnetic CF in the glomerular basement membrane. The control kidney showed no such spots (Fig. 1 B). The specific binding of CF to the glomerulus was confirmed with immunofluorescence microscopy (Fig. 1 D and E) and electron microscopy (data not shown). Our algorithm identified and labeled glomeruli in the MRI datasets of CF-inoculated kidneys (Fig. 1D). In this figure, glomeruli identified by the algorithm are assigned an arbitrary color for the purpose of distinguishing neighboring glomeruli from one another. Counting (Nglom) of these identified spots yielded a count of 772,922 glomeruli for the kidney from the male, and 328,181 and 702,506 for the two kidneys from the females. Based of the number of voxels composing each identified glomerular region we report glomerular volumes of $8.0x10^6 \pm 4.28x10^6$ μm^3 for the kidney from the male, and $6.9x10^6 \pm 3.9x10^6$ μm^3 and $6.8x10^6 \pm 2.9x10^6$ μm^3 for the two kidneys from the females. MRI-based measurements of Nglom and Vglom are consistent with histological measurements reported in the literature (8). This MRI-based technique for measuring Nglom and Vglom in human kidneys also allows for assessment of the intra-renal glomerular volume distribution (an example of which is shown in Fig. 2).

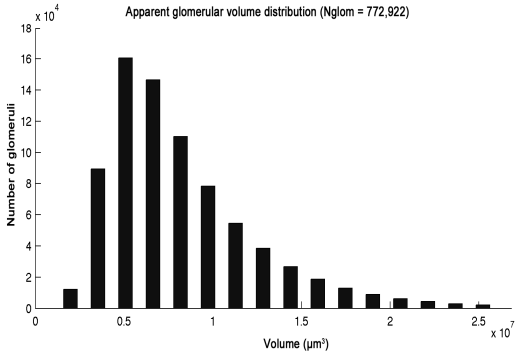

Fig. 2 - Histogram of apparent glomerular volume distribution of a CF-inoculated kidney from a human male. Since the MRI-based method for counting glomeruli measures every labeled glomerulus in the kidney, this new method presents a unique opportunity to assess the distribution of glomeruli defined by their size. The average ± the standard deviation of glomerular sizes measured in this kidney was $8.0x10^6 \pm 4.28x10^6$ μm^3.

DISCUSSION – Here we demonstrated that human glomeruli may be labeled with the CF and subsequently counted and measured with MRI. Immunofluorescence confirmed that CF binds specifically to the glomerulus and T_2^*-weighted MRI confirmed that the labeled glomeruli are MRI-detectable. We further measured the apparent glomerular volume distribution - which is undetectable by other techniques and may be important for intra-renal physiology and diagnostics. We have established the accuracy of the MRI-based method in rats using the standard disector/fractionator method for validation (4), though histological validation of Nglom and Vglom is still pending in this human study. An advantage of CF as a contrast agent is that ferritin can be made recombinantly (9), potentially lowering the toxicity and opening the possibility of use in vivo. **Conclusions - These results, along with previous in vivo detection of glomeruli and counts made in the rat, suggest the possibility of counting glomeruli in the clinic**.

Refs: (1) Brenner et al. *Am J Hypertens* 1988 (2) Puelles et al. *Nephrol Dial Trans* 2012 (3) Bertram et al. *Cell and Tiss Res* 1992 (4) Beeman et al. *AJP Renal* 2011 (5) Bennett, et al. *MRM* 2008 (6) Heilmann et al. *Nephrol Dial Trans* 2012 (7) Danon et al. *J Ultrastruct Res* 1972 (8) Nyengaard et al. *Anat Rec* 1992 (9) Uchida et al. *MRM* 2008