Progress Report for the Diabetes Complications Consortium (Diacomp)

Role of Podocyte Lipids in the Prediction of Diabetic Nephropathy

Development of an Unbiased Stereological method for estimation of the absolute volume of lipid droplets *(V(LD/PC)*) in podocyte.

Up to now, we have developed an unbiased methodology to estimate the absolute volume of lipid droplets (*V(LD/PC)*) in the podocytes. The advantage of this method is that podocyte volume changes (hypertrophy or atrophy) may mask the amount of cytoplasmic lipid droplets which is a common error (reference Trap) when known knowledge about the reference volume (i.e. Podocyte cytoplasmic volume) is not available. This method is capable, in conjunction to estimation of lipid droplet volumes, provide estimation of podocyte volume (as a measure of podocyte hypertrophy or atrophy) and numerical density of podocytes per glomerulus [N_V*(PC/glom)]*.

Herein, we provide the protocol for these measurements as follows:

Step 1: Identification and marking lipid droplets in podocytes

Step 2: Estimation of podocyte nuclear volume using a modification of the point sampled intercept (PSI) method

Step 3: Estimation of the fractional volume of lipid droplets per podocytes [V_V*(LD/PC)]*

Step 4: Estimation of the fractional volume of podocytes per glomerulus [V_V*(PC/glom)]* and fractional volume of podocyte nuclei per podocyte [V_V*(PCN/PC)]*

Step 5: calculation of *V(LD/PC)* and N_V(PC/glom) using the data obtained from step 1 through 4

Step 1: identification and marking of lipid droplets in podocytes. Low magnification (about 4000x) transmission electron micrographs (TEM) are obtained from random sections through the glomeruli, to make montages of complete glomerular profile Figure 1A).

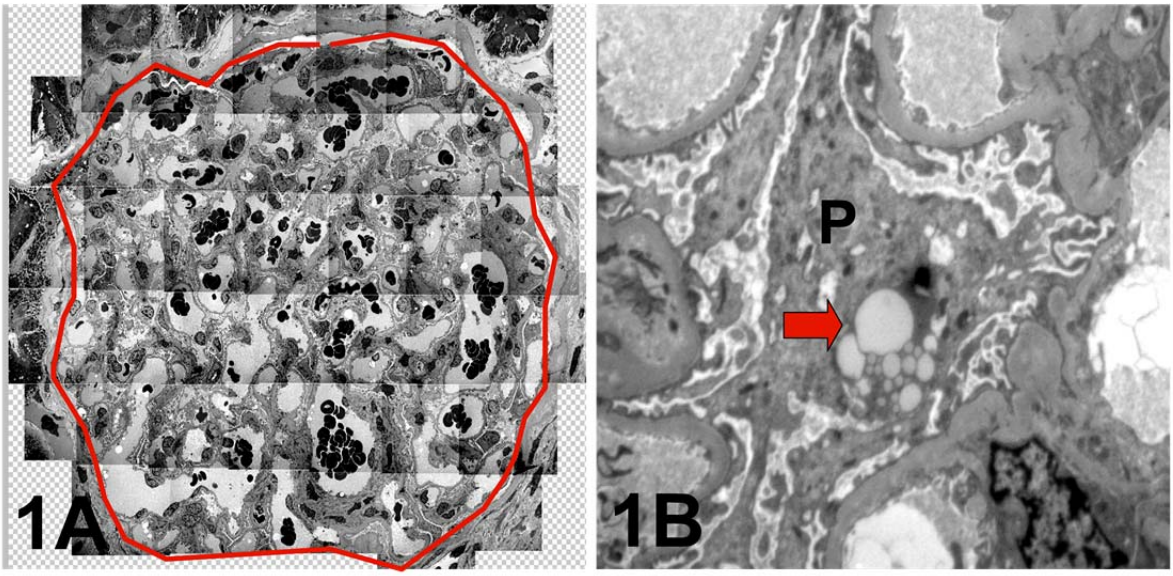

Figure 1A. Montage image of a glomerulus from a patient with type 2 diabetes (TEM, ~4,000) with a red polygon drawn for volume measurements (see the text)**. Figure 1B**. A podocytes (P) within this glomerulus containing multiple lipid droplets (red arrow).

The digital electron micrographs are opened and characterized in the Adobe Photoshop software to identify lipid droplets in podocytes. Lipid droplets are characterized by membrane bound round structures with homogeneous content which is generally semi-electron dense; however, these can also be darkly electron dense or alternatively completely clear. Following identification, these droplets are painted (Figure 1B).

Step 2: The volume of the podocyte nuclei is estimated by a modification of point sample intercept method (PSI) as follows (Gundersen HJ, JensenEB. Stereological estimation of volume-weighted mean volume of arbitrary particles observed on random sections. J Microsc

138:127-142, 1985. PMID:4020857). PSI is an unbiased efficient shape-independent method for estimation of particle size. We have a developed a modification of PSI through changing the sampling strategies of the originally proposed PSI to reduce the volume weighted property of this method. Through this modification, all nuclear profiles (as opposed to only those sampled by a point grid in the original PSI) are included and each nucleus receives only one set of intercept measurements through a random point (as opposed to multiple measurements if a nucleus is hit by multiple points in the original PSI). A point/lattice grid with points 21 μm apart is super-imposed on the montage images of glomerular profiles. This grid has enough point density to have at least one point fallen on every visible podocyte nucleus. On each podocyte nucleus, one point is randomly selected using a random number generator. A line (intercept line) is passed through this sampling point in a random direction. Nuclear diameter along the intercept line is measured according to PSI using Adobe Photoshop measuring tool (Figure 2).

Figure 2

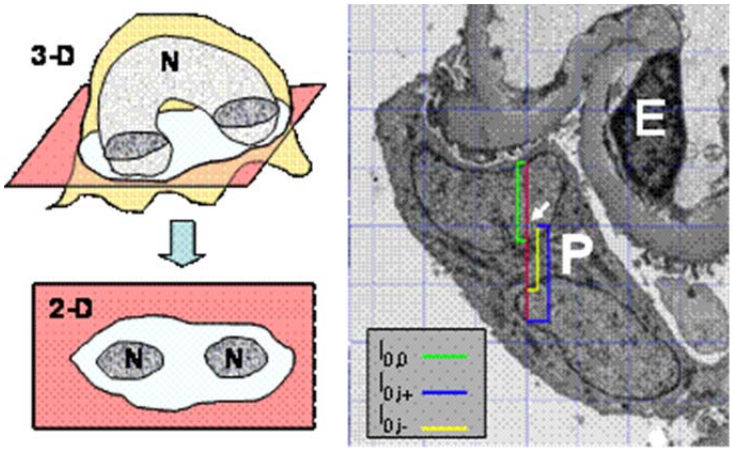

Figure 2. (Left-top) A cartoon showing a podocyte with a complex nucleus (N) sectioned randomly with a plan. (Left-bottom) Two-dimensional (2-D) profile of the same podocytes showing two nuclear profiles. (Right) A podocyte (P) with two nuclear profiles with PSI measurements (see the text below).

The average podocyte nuclear volume ($\overline{V}_{PCN}$) is estimated as $\overline{V}_{PCN} = \frac{\pi}{3}(\overline{l_{0,0}^3} + 2\overline{l_{0,e}^3})$ where $l_{0,0}$ is the nuclear diameter along the intercept line (red line, Figure. 2) passing through the random point (white arrow, Figure. 2) and $l_{0,e}^3 = l_{0,i+}^3 - l_{0,i-}^3$, where $l_{0,i+}^3$ and $l_{0,i-}^3$ are distances from the

sampling point to the closest and farthest nuclear borders if the intercept line passes through concave regions due to complex shape. In fact, it is not rare to observe more than one nuclear profile in podocytes due to complex shape of their nuclei (Figure. 2).

Step 3: Estimation of $V_V(PC/glom)$. In order to estimate $V_V(PC/glom)$, a point grid with coarse points 15 μm apart and 4 fine points per each coarse point is overlaid on the montage images. A polygon which connects the most outer border of podocytes surrounding the glomerulus is drawn (Figure 1A). The coarse points falling inside the polygon and the fine points falling over the podocytes are counted. $V_V(PC/glom)$ is estimated using the following equation:

$$Vv(PC/glom) = \frac{4 \times \sum CP_{glom}}{\sum FP_{PC}}$$

Estimation of $V_V(PCN/PC)$. Using the same point grid described above, and separate the counting the number of fine points falling over podocyte nuclei, $V_V(PCN/PC)$ is estimated using the following equation: $V_V(PCN/PC) = \frac{\sum FP_{PCN}}{\sum FP_{PC}}$

Step 4: **Calculation of V(LD/PC) and N_V(PC/glom)**

Calculation of *V(LD/PC)*. In the select command of Adobe Photoshop software, color range command is chosen to select the color used for marking lipid droplet. Using the measurement log the individual and total areas of lipid droplet will be automatically measured (Figure 3). It is important that the scale be calibrated in Adobe Photoshop using an image from a carbon replica calibration grid taken at the same magnification as the montage image. Subsequently ($V_V(LD/PC)$ is calculated using equation:

$$V_V(LD/PC) = \frac{\sum A_{LD}}{A_{FP} \times (\sum FP_{PC} - \sum FP_{PCN})}$$

The average volume of podocyte is calculated using equation $\overline{V}_{PC} = \frac{\overline{V}_{PCN}}{V_V(PCN/PC)}$; and from that, the *V(LD/PC)* is calculated as $V(LD/PC) = V_V(LD/PC) \times \overline{V}_{PC}$

Also ***N_V(PC/glom)*** is calculated using equation $N_V(PC/glom) = \frac{\overline{V}_{PC}}{V_V(PC/glom)}$

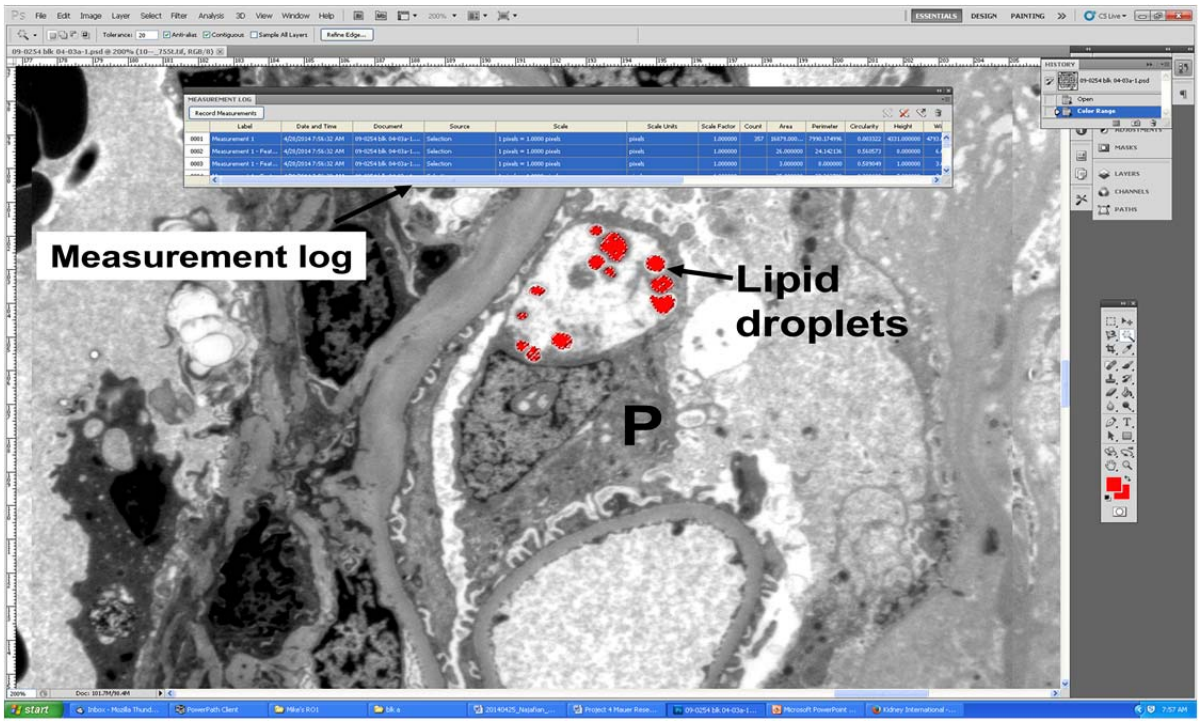

Figure 3. A podocyte (P) with lipid droplets marked with red color and selected. Individual areas of lipid droplets are recorded automatically in the measurement log.

Total N(PC/glom) is calculated as N(Podo/glom)=Nv(Podo/glom) MGV, where MGV is the mean glomerular volume estimated by the Weibel-Gomez method (*Weibel ER: Correction of systematic errors due to finite section thickness. In: Stereological Methods, Vol. 1, Practical*

Methods for Biological Morphometry, edited by Weibel ER, London, New York, Toronto, Sydney, San Francisco, Academic Press, 1979, pp. 139–146).

One of the advantages of this methodology is that an estimation of the maximum deviation of the average volume obtain by the modified PSI from a number-weighted method (such as disector) can be obtained. Through mathematical reasoning it can be shown that the maximum deviation is estimated as $\frac{V_V(PCN/glom)}{1+CV^2_{MPSI}}$, where CV^2_{MPSI} is the squared coefficient of variation of modified PSI calculated as $CV^2_{MPSI}=\frac{4\pi(a_0^2.\nabla_0)}{\overline{V}^2_{PCN}}-1$, where a_0 is the nuclear profile area estimated by point counting and ∇_0 is the area of a triangle made by connecting 3 random points inside podocyte nucleus.

So far, montage images from glomeruli in biopsies obtained from type 2 diabetic patients have been prepared and classical structural parameters of these biopsies have also been measured through another study. Currently we are performing the above explained methodology on biopsies from five normoalbuminuric and five microalbuminuric type 2 diabetic patients. We aim to determine the following;

1: identification of the minimum number of podocytes to provide a robust estimation of *V(LD/PC)*

2: pilot comparison of *V(LD/PC)* in normoalbuminuric and microalbuminuric type 2 diabetic patients and its correlation with N_V*(PC/glom*) and $\overline{V}_{PC}=\frac{\overline{V}_{PCN}}{V_V(PCN/PC)}$ and other histological structural parameters of diabetic nephropathy in these patients.