# PROTEOMIC CHARACTERISATION OF RENAL MULTILAMMELAR BODIES INDUCED BY KIDNEY METABOLIC INJURY

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## INTRODUCTION

**Ectopic fat accumulation in organs other than adipose tissue, including the kidney, has been proposed to reflect the severity of obesity-induced end-organ damage. We have recently found extensive accumulation of free cholesterol and polar complex lipids in large multilamellar bodies (MLBs) within proximal tubular epithelium in mice fed with Western-type of diet, an animal model of obesity-induced kidney injury. Here we aim to characterize the protein composition of the multilamellar structures in order to investigate novel potential biomarkers of metabolic renal injury. MLBs were isolated by discontinuous sucrose gradient centrifugation from kidney tissue homogenates and urine of mice fed with Western-type or control of diet for 16 weeks. Composition of the MLB fraction was confirmed by transmission electron microscopy. The proteome complement was characterized using ion mobility separation enabled, data independent label-free LC-MS. Relative within sample abundances were expressed by normalization to the total estimated amount affording comparative proteomes analysis of tissue and body-fluid samples. Orthogonal, confirmatory analysis was conducted of overexpressed species was conducted by immune-histochemistry.**

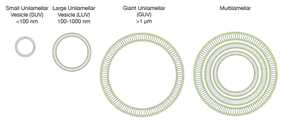


*Figure 1. Spherical liposome structures contain hydrophilic and hydrophobic regions imparted by polar lipid heads and hydrophobic tail groups. Sizes vary from smaller unilamellar vesicles of 20–200 nm to larger vesicles of 200 nm to 1 µm, and upwards of 1 µm for unilamellar vesicles. The latter can also exist as multilamellar vesicles, consisting of multiple lipid bilayers.*

## METHODS

### Animal studies

Wild-type C57BL/6 mice were fed control or Western-type diets (43 energy-% fat, 0.15% cholesterol) *ad libitum*. After 16 weeks, 24-h urine samples were collected, the animals were starved for 4 h and sacrificed by cardiac puncture under anesthesia. Blood and kidneys were collected. All animal procedures were approved by the local Animal Welfare Committee.

### Histology and immunohistochemistry

FFPE kidney sections were stained by PAS-*D* to visualize tubular vacuolisation. The sections were stained for LIMP2 and developed with DAB kit. For visualization of polar lipids and free cholesterol, frozen sections were fixed and stained with Nile Red and filipin. Vacuolized tubuli were quantified in PAS-*D* stained sections.

### Electron microscopy

Fresh renal cortex was fixed in paraformaldehyde/glutaraldehyde and postfixed with 1% osmiumtetroxide. The tissue samples were block-stained with 1% uranyl acetate, dehydrated in dimethoxypropane, and embedded in epoxyresin LX-112. The sections were stained with tannic acid, uranyl acetate, and lead citrate and examined using transmission electron microscopy (TEM).

### Protein analysis and Western blotting

Protein concentrations were measured by BCA. Proteins were resuspended in 5x Laemmli buffer and boiled for 10 min. The proteins were separated by SDS-PAGE and transferred to PVDF membranes by blotting for 2 h. The membranes were blocked in TBST/5% non-fat dry milk or 5% BSA and incubated overnight at 4 °C with the LIMP2 primary antibody. The membranes were washed and incubated with secondary antibody for 1 h at room temperature. Immunoreactivity was visualized using ECL PLUS Western blotting detection reagent.

### Sample preparation and digestion

MLBs were purified from animal renal tissue and urine using gradient centrifugation and subjected to LC-MS analysis. Kidneys with confirmed MLB formation were pooled, subjected to homogenisation and sucrose gradient fractionation. The supernatants were collected, $NH_4HCO_3$ RapiGest solution added and the samples denatured. Next, the extracts were reduced, alkylated and digested with trypsin.

### Sample annotation

$F_{WKM}$, $I_{CKL}$, $C_{WKM}$, $E_{WUM}$, $K_{CUL}$, $A_{TM}$, $H_{TL}$, $O_{WKM}$, and $Q_{WKM}$

W = Western diet; C = control diet; K = kidney; U = urine; T = tubular epithelial cell line; M = MLB positive fraction; L = corresponding lysosomal fraction.

### LC-MS conditions

Nanoscale LC separation of tryptic peptides was conducted with a trap column configuration using an M-class system and a 90 min gradient from 5-40% ACN (0.1% FA) at 300 nl/min using a BEH 1.7 µm C18 reversed phase 75 µm x 20 cm column. MS data were acquired in triplicate in ion mobility enabled data independent analysis mode (LC-IM-DIA-MS) using a Synapt G2-S*i* instrument.

### Informatics

The LC-MS peptide data were processed and searched with ProteinLynx GlobalSERVER. ISOQuant and Progenesis QI for proteomics were applied for integrated quantitative analysis of data derived from multiple LC-MS runs. Reactome, PANTHER, and BLAST2GO were used for pathway/gene ontology annotation and Graphpad Prism for statistical analysis.

## RESULTS

### Metabolic overload

Metabolic overload in results in accumulation of MLBs inside the proximal tubular epithelial cells. MLBs are observed as lipid-containing vacuoles as illustrated in Figure 2A. These vacuoles are more prominent in Western-diet, shown in Figure 2B. Vacuoles display concentric multilamellar structure and massively fill up the tubular cells exposed to metabolic overload, shown in Figure 2C. Vacuoles/MLBs stain positively for phospholipids and free cholesterol, Figures 2D and 2E, suggesting accumulation of these species inside the MLBs. Intriguingly, MLBs could be isolated from murine urine, shown in Figure 3.

### Qualitative and quantitative MLB proteome

In MLBs isolated from renal murine tissue and urine, a total of 1235 and 526 proteins were identified and quantified, respectively, with an experiment–wide protein and peptide identification FDR<1%. Enriched pathways, shown in Figure 4 included metabolism and cellular processes, an unsurprising finding in structures exposed to metabolic stress/overload. Among other relevant biological processes, MLB proteins are strongly involved in apoptotic processes. These observations are in agreement with the hypothesis that metabolic syndrome-associated kidney injury represents a form of acquired lysosomal storage disease characterized by accumulation of (proteins involved in) intralysosomal phospholipid/cholesterol.

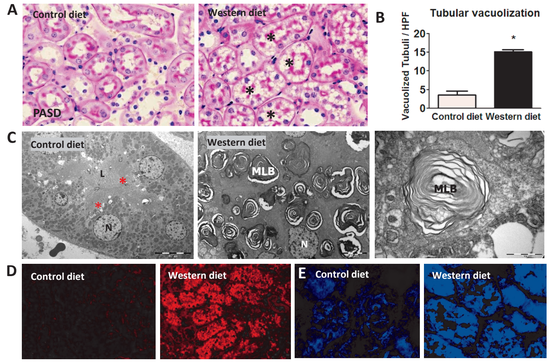


*Figure 2. A) Normal tubular structures in kidneys of mice fed with control diet (left) and prominent tubular vacuolisation (*) in Western-type diet fed mice (right). B) Quantitative analysis of tubular vacuolisation in control and Western-type diet fed mice. C) Small lamellar structures (*) can be observed close to apical membrane and in luminal space L of normal proximal tubular epithelium (left panel). Massive accumulation of MLBs (*) reaching the size of several µm within tubular epithelium of kidneys exposed to Western-diet induced metabolic overload (middle panel, MLB detail - right panel). N = nucleus. D) Presence of phospholipids and E) free cholesterol in tubular vacuoles, respectively, in control (left) and Western-type diet fed mice (right).*

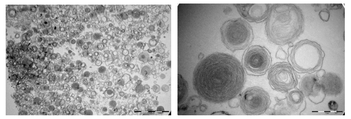

*Figure 3. MLB Structures can be TEM detected and isolated from urine of mice fed Western type of diet.*

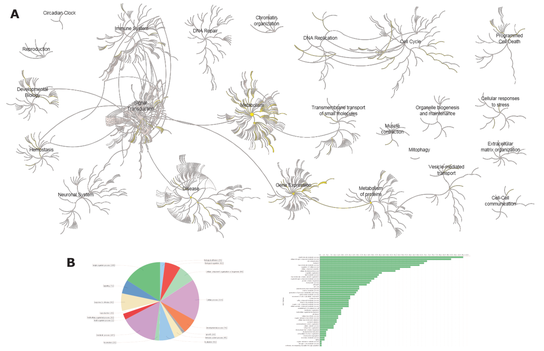


*Figure 4. A) Most significantly enriched pathways (yellow highlighted (projected on human pathway overview map)) and B) biological processes for sample $C_{WKM}$.*

Relative within sample abundances were calculated by expressing the intensity of the three most abundant peptides (post homology parsing) identified to a protein as a function of the sum of the intensities for all quantified proteins, providing a µmol/mol (ppm) amount. The latter can be used to contrast non-related samples as shown, as examples, in Figure 5, highlighting some lysosomal membrane proteins of interest that were strongly increased in abundance in Western-diet fed mice.

The left hand pane of Figure 5 is contrasting the amount distributions of two biological replicates, indicating a similar dynamic range and relative abundance for the highlighted proteins. The right hand side of Figure 5 contrasts the relative abundances of four lysosomal protein markers detected and identified within the MLBs isolated from different sample types (kidney and urine).

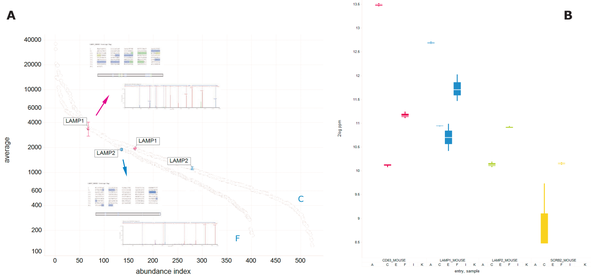


*Figure 5. A) Relative LC-MS abundance (ppm) LAMP1 and LAMP2 detected in samples $C_{WKM}$ and $F_{WKM}$. Shown inset are a coverage map and example DIA product ion spectra, respectively. B) Relative LC-MS abundance ($log_2$ ppm) CD63 (red), LAMP-1 (blue), LAMP-3 (green) and LIMP-2 (yellow)) in samples $F_{WKM}$, $I_{CKL}$, $C_{WKM}$, $E_{WUM}$, $K_{CUL}$, and $A_{TM}$, respectively.*

### Validation

As can be observed from the Western blot analysis results shown in Figure 6, formation of MLBs in proximal tubular epithelial cells of fatty kidney is associated with lysosomal expansion. These observation are in agreement with the LC-MS results, where also increased LIMP-2 detection was observed.

Excreted accumulated lysosomal lipids and proteins therefore might reflect the severity of metabolic syndrome-associate kidney disease and represent a source for urinary biomarkers of chronic renal metabolic injury. Moreover, defective renal tubular lysosomal function, reflected by the presence of renal multilamellar bodies and associated proteins, could represent a novel mechanism involved in the development of metabolic syndrome-associated kidney disease and as such should be considered a novel therapeutic target.

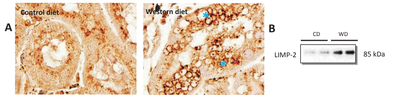


*Figure 6. A) Limiting membranes of the tubular vacuoles stain positive for lysosomal membrane marker LIMP-2, suggesting lysosomal origin of vacuoles/MLB (left panel, asterisks). B) Elevated expression of lysosomal marker LIMP-2 and accumulation in the metabolic overloaded kidneys, CD = control diet, WD = Western diet.*

## CONCLUSION

- **Proteins of mitochondrial and (endo) lysosomal origin were strongly overrepresented among the most abundant proteins of MLB fractions**
- **The proteins largely overlapped between tissue and urinary fractions, suggesting MLBs are excreted in urine**
- **Lysosomal membrane proteins, including LIMP-2, LAMP-1, LAMP-2, CD63 and subunits of lysosomal proton pump were detected in both tissue and urinary MLB-fractions from Western-diet fed animals, but largely absent in corresponding fractions from control animals**
- **Several lysosomal markers LIMP-2, LAMP-1, LAMP-2 and CD63 were overexpressed in kidneys with metabolic overload and localisation of these markers in the limiting membrane of MLBs has been confirmed**
- **MLBs formed in the kidney upon metabolic/obesity-induced renal injury are most likely of lysosomal origin**
- **MLB-derived lysosomal membrane markers, such as LIMP-2 might potentially serve as tissue or urinary markers of obesity-associated renal injury**

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