The hidden message of urine: in-depth characterization of the healthy urinary proteome

RENAL CHILD FOUNDATION

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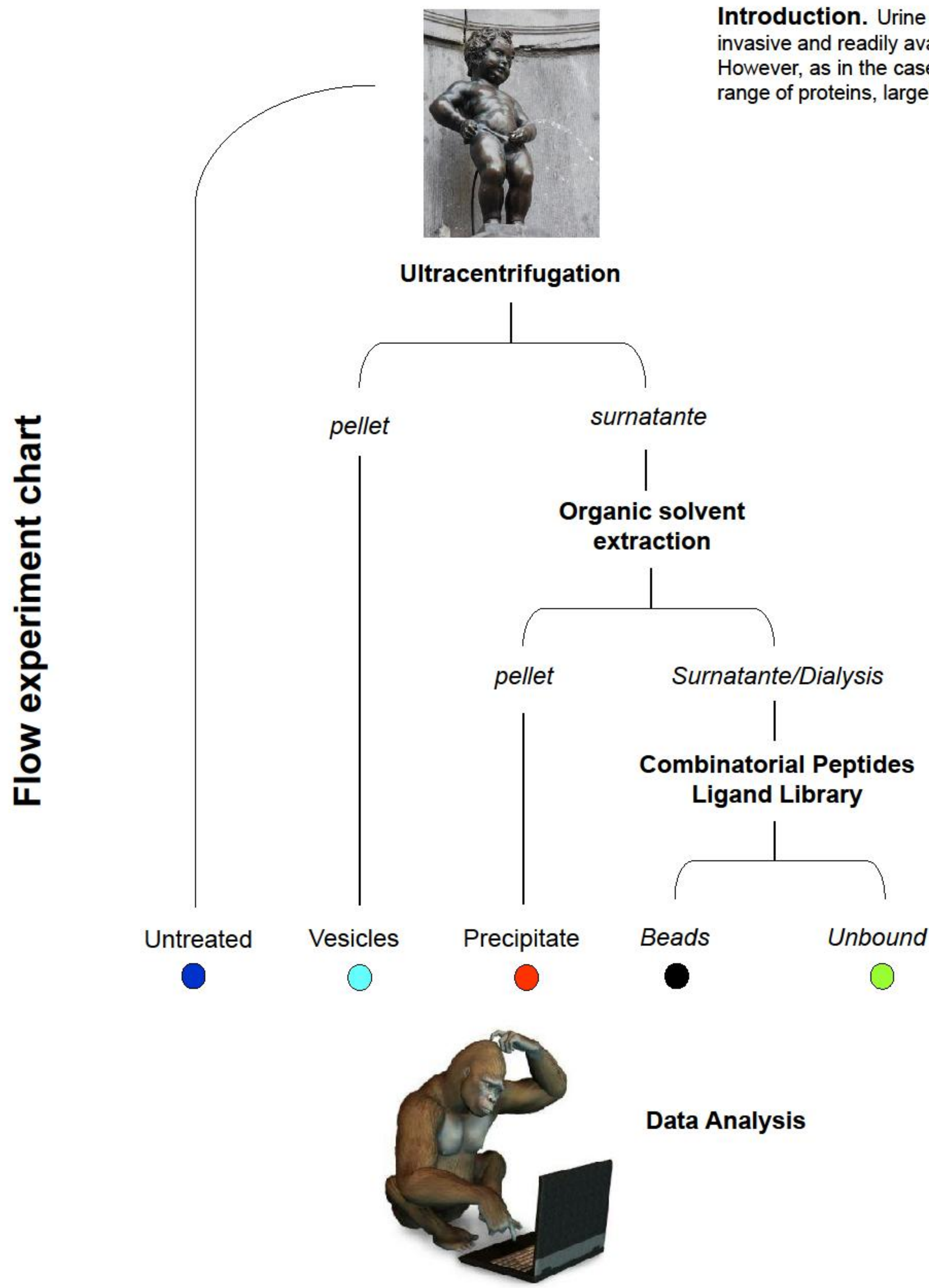
Introduction. Urine is a biological fluid resulting from the filtration of blood, is in close proximity to several organs and tissues, and its collection is non invasive and readily available. Thus, urine has historically represented a particularly interesting source of kidney disease biomarkers.
However, as in the case of other biological fluids, one of the main analytical challenges in the characterization of urinary proteome is the very wide concentration range of proteins, largely exceeding the dynamic range of current analytical approaches.



Methods. We describe an extensive sub-fractionation method to investigate the characterization of the healthy urinary proteome. After the collection, urine samples were centrifuged to remove cells and debris and then were ultra-centrifuged in order to pellet the micro-vesicles. The supernatant was treated with a mixture of organic solvents to pellet the highly hydrophobic proteins and remove the interference of pigments. This supernatant was dialysed and loaded on Combinatorial Peptides Ligand Library (CPLL) to reduce the dynamic range of protein concentration in urine and at the same time unmask previously undetected proteins. Finally, each urinary fraction was processed using two-dimensional electrophoresis and mass spectrometry techniques, and the qualitative and quantitative data were analyzed using the hierarchical clustering analysis.

Ultracentrifugation. Samples were centrifuged at 200.000 g for 1h

Organic solvent Extraction. Samples were treated with butyl alcohol

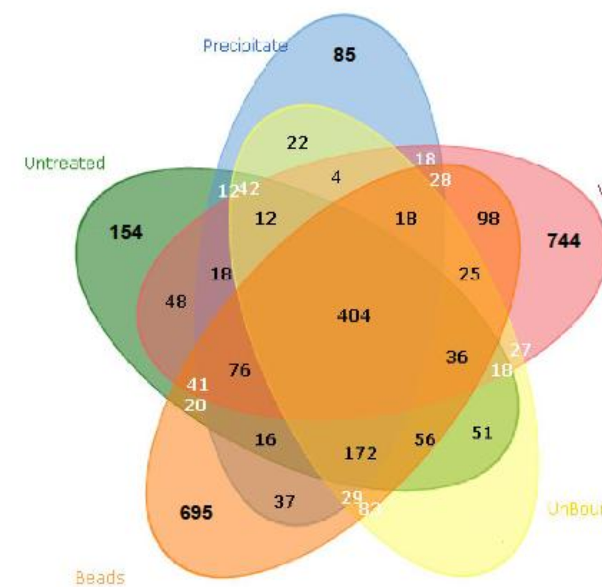
Combinatorial peptide ligand library. Samples were loaded onto a column of 75 µL peptide library beads equilibrated in 25 mM phosphate buffer, pH 7.4. By using a peristaltic pump the samples were left in constant re-circulation overnight at room temperature. The column was then washed with 25 mM phosphate buffer, pH 7.4. Absorbed proteins were eluted with a solution of 2% SDS, 2.5% DTE and 100 mM Tris/HCl pH 7.4 and boiled for 10 min.

Two-Dimensional Electrophoresis. 2D was performed using homemade diluting gel matrix strips in a non linear pH 3-10 interval. The re-swelling of strips was carried out overnight at RT in a focusing solution, *i.e.* 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 15 mM DTE and a 0.6% (v/v) carrier ampholyte cocktail. Samples were cleaned and precipitated and finally dissolved in the focusing solution, i.e. 7 M urea, 2 M thiourea, 4% CHAPS and 65 mM DTE. The second dimension was run on T % 8-16 gels. Image was digitized by means of GS-800 (Bio-Rad). Each experiment was repeated in triplicate.

Protein identification by LC-ESI MS-MS/MS. All mass spectrometric measurements were performed using a Orbitrap mass spectrometer (Thermo Electron, San Jose, USA) coupled to a HPLC.

Statistical analysis. All analysis were performed using R software

Results. The combined use of this proteomic approach has allowed us to create a *"complete"* virtual map of the healthy urinary proteome, identifying a total of 3429 proteins. In addition, the particular chemical and physical characteristics of this sub-fractionation method allowed to highlight: 744 proteins in micro-vesicles, 85 proteins in the extraction with organic solvents, and 695 proteins in CPLL, all unique to each fraction.



Total protein identified

UnTreated	1176
Vesicles	1615
Precipitate	993
Beads	1834
Unbound	999
	3429

Figure 2. Venn diagram shows all possible combinations between proteins identified by mass spectrometry in each urinary fraction (see Figure 1).

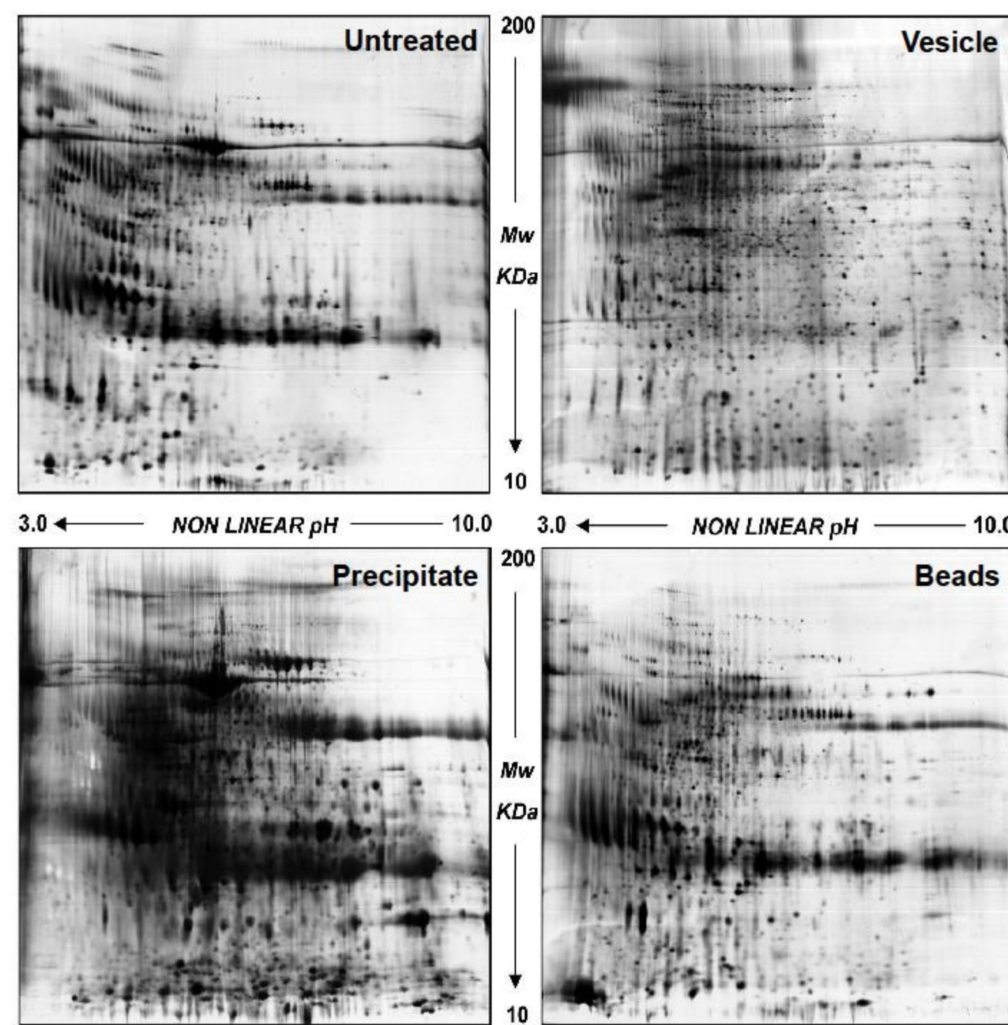


Figure 1. Representative silver staining of two-dimensional proteomic profile of different urinary fractions showing the complexity of urine protein composition.

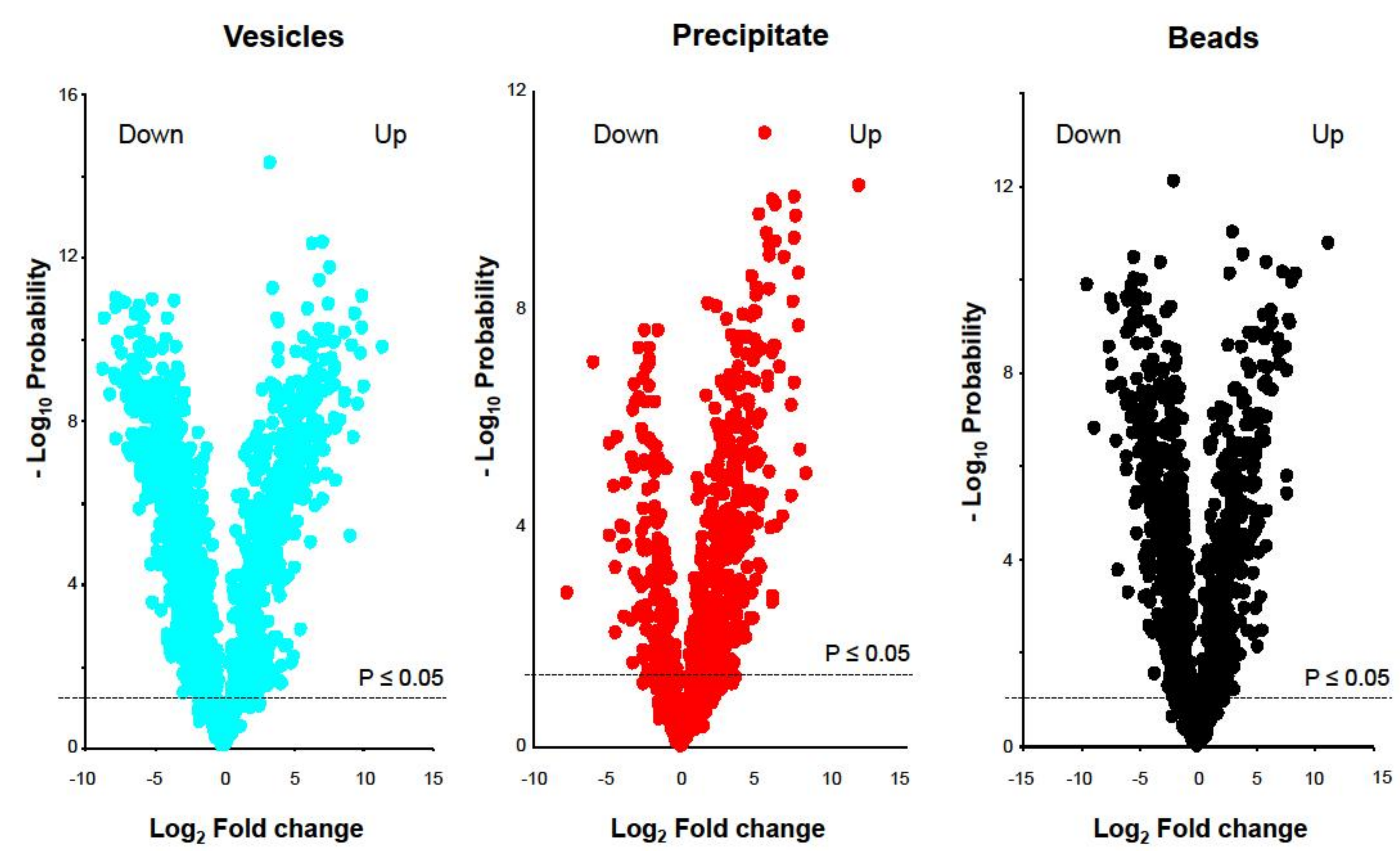


Figure 3. Volcano plots were used to quickly identify the statistical significance of protein expression changes associated with the different urinary fractionation techniques compared to the untreated sample. A $-Log_{10}$ Probability value above 1.3 (y-axis) corresponded to a statistical significance change (p< 0.05) associated with the use of each analytical method.

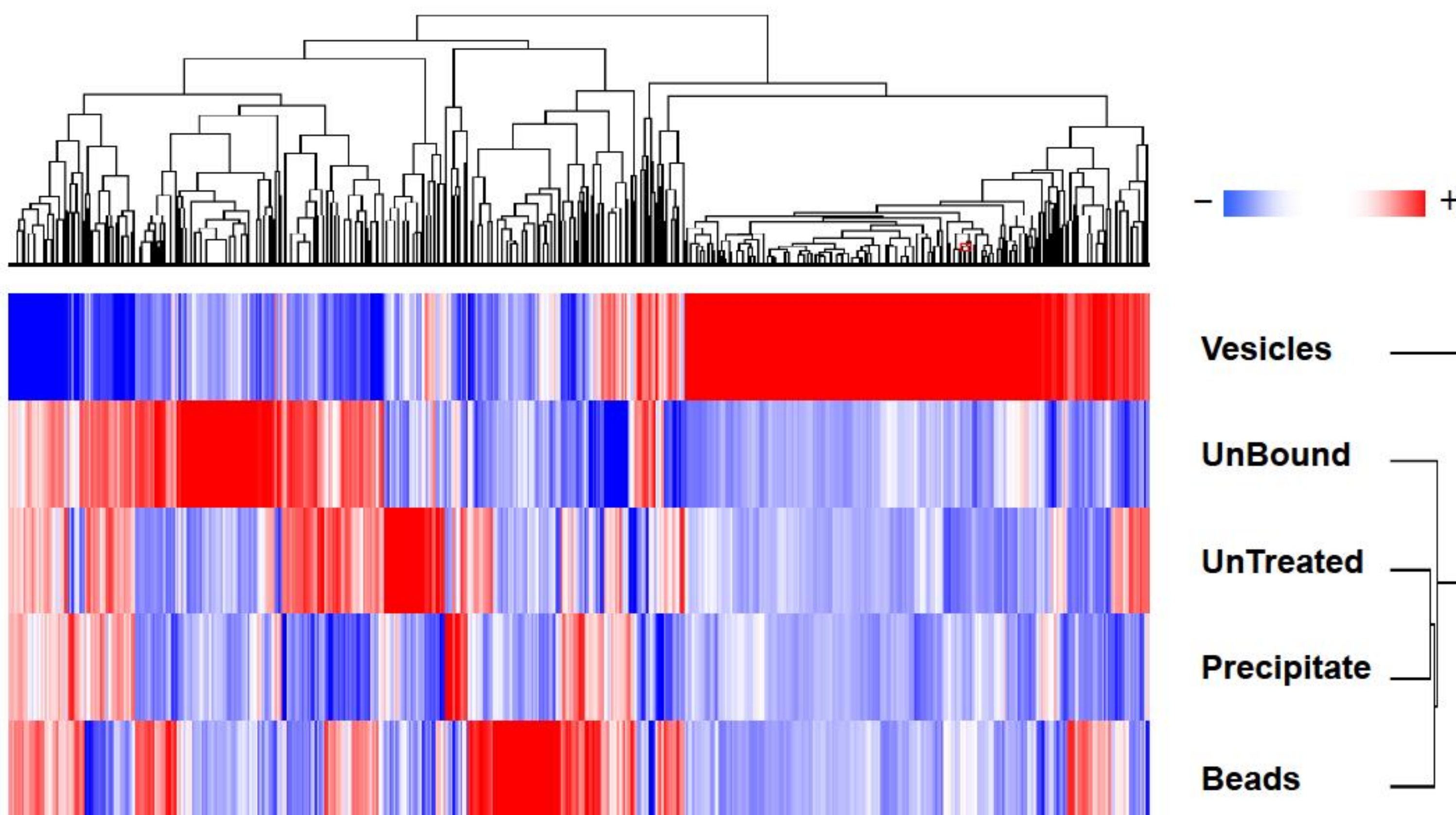


Figure 4. Heat map generated from all proteins identified by mass spectrometry reflects their intensity values in the urinary fractions obtained by means of each analysis method. In heat map analysis, protein expression values were converted in a color-scale. In addition, the column and row tree dendrogram place similar intensity values of different proteins near each other according to the results of cluster analysis.

Conclusion. This complex sub-fraction scheme allowed an extensive coverage of normal human urinary proteome, thus leading to a new perspective in the search of diagnostic biomarkers of renal function and/or disease.
The next step of this study will consist in applying this sub-fractionation method to the characterization of urinary proteome of patients affected by different types of kidney diseases in order to identify specific disease biomarkers of potential diagnostic and prognostic value.

DOI: 10.3252/pso.eu.51era.2014
Poster presented at:

PosterSessionOnline.com