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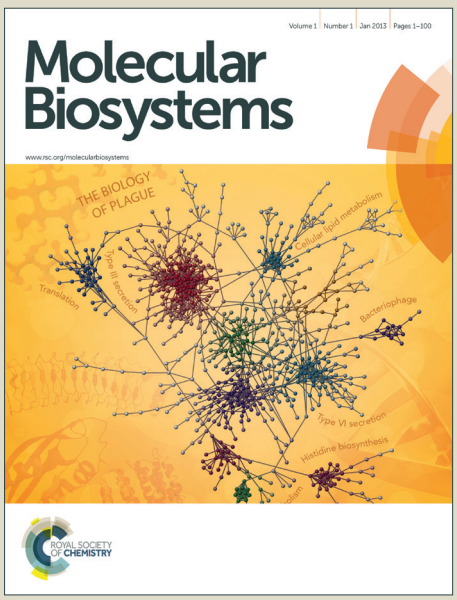

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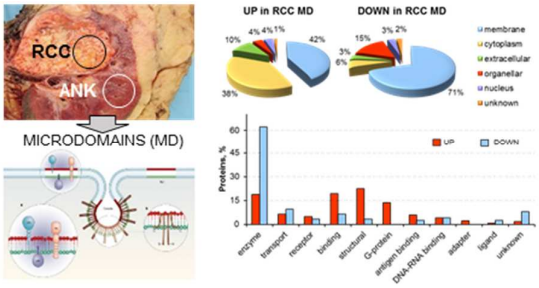
The paper highlights the importance of a specific protocol set-up for the proteome investigation of membrane proteins, applying it to the comparative proteomics of Renal Cell Carcinoma membrane microdomains, by label free quantification.



Comparative membrane proteomics: technical advance in the search of Renal Cell Carcinoma biomarkers

Francesca Raimondo[a*], Samuele Corbetta[a], Andrea Savoia[a], Clizia Chinello[a], Marta Cazzaniga[a], Francesco Rocco[b], Silvano Bosari[c], Marco Grasso[d], Giorgio Bovo[d], Fulvio Magni[a] and Marina Pitto[a]

[a] Department of Health Sciences, Univ. of Milano-Bicocca, Monza, Italy.

[b] Department of Specialistic Surgical Sciences, Urology unit, Ospedale Maggiore Policlinico Foundation, IRCCS, Milano, Italy.

[c] Department of Medicine, Surgery and Dental Sciences, Pathology Unit, Ospedale Maggiore Policlinico Foundation Milano, IRCCS,Italy.

[d] Department of Surgical Pathology, Cytology, Medical Genetics and Nephropathology, Azienda Ospedaliera San Gerardo, Monza, Italy.

*Corresponding author: Francesca Raimondo

Milano-Bicocca University, Via Cadore 48, 20900, Monza, Italy

Tel.: +39 02 6448 8106

Fax: +39 02 6448 8068

Email address: francesca.raimondo@unimib.it

ABSTRACT

Renal Cell Carcinoma (RCC) is the most common kidney cancer, accounting for 3% of adult malignancies, with high metastatic potential and radio-/chemo-resistance. To investigate the protein profile of membrane microdomains (MD), plasma membrane supramolecular structures involved in cell signaling, transport and neoplastic transformation, we set up a proteomic bottom-up approach as a starting point for the identification of potential RCC biomarkers.

We purified MD from RCC and adjacent normal kidney (ANK) tissues, through their resistance to non-ionic detergents followed by ultracentrifugation in sucrose density gradient. MD from 5 RCC/ANK tissues were then pooled and analysed by LC-ESI-MS/MS.

In order to identify the highest number of proteins and to increase the amount of membrane and hydrophobic ones, we first optimized an enzymatic digestion protocol based on Filter Aided Sample Preparation (FASP), coupled to MD delipidation. The MS analysis led to the identification of 742 ANK MD and 721 RCC MD proteins, of which, respectively, 53.1% and 52.6% were membrane-bound. Additionally, we evaluated RCC MD differential proteome by label free quantification; 170 and 126 proteins resulted, respectively, up-regulated and down-regulated in RCC MD. Some differential proteins, namely CA2, CD13 and ANXA2, were subjected to validation through immunodecoration.

These results show the importance of setting up different protocols for the proteomic analysis of membrane proteins, specific to the different molecular features of the samples. Furthermore, the subcellular proteomic approach provided a list of differentially expressed proteins among which RCC biomarkers may be looked for.

INTRODUCTION

Renal Cell Carcinoma (RCC) is the deadliest tumor of the genitourinary tract. Clear Cell Renal Carcinoma represents the most common histotype, accounting for 70–80% of all kidney cancers. Due to its asymptomatic development, approximately 80% of RCCs are discovered incidentally during unrelated diagnostic abdominal imaging, when the disease is already in advanced phase[1,2]. Furthermore, RCC is chemotherapy and immunotherapy resistant, and nephrectomy is the only beneficial therapy. Once metastases develop, the prognosis for long-term survival is poor; the 5-year survival rate for non-treated patients is usually less than 14%. Nethertheless, there are currently no circulating validated biomarkers able to confirm the identity of renal masses, whether benign or malignant[3,4].

Plasma membrane proteomics seems to be a promising tool to address this issue, as membrane proteins perform endocytosis and signaling, and modifications of plasma membrane composition are encountered in several cancers[5,6]. Moreover, about two-thirds of membrane proteins are drug targets, hence crucial for therapy advancement.

In particular, within the membrane, there are specialized microdomains, also known as lipid rafts. Membrane microdomains (MD) are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched lateral assemblies that compartmentalize cellular processes. Caveolae, a subclass of such MD, are flask-like invaginations of the plasma membrane that characteristically display caveolin-1 expression[7].

MD are highly dynamic structures and act as selective signal transduction mediators, enabling interactions between the intra- and extra-cellular compartments. Furthermore, they play a key role in drug resistance, cell migration, cell adhesion, and cell survival, as well as in metastasis, and tumor progression[8-11]. Since membrane microdomains represent a minor and highly selected subset of the cellular proteome, they are particularly well suited to investigation using MS techniques. However, their peculiar lipid composition and the enrichment of highly hydrophobic membrane proteins make the assessment of microdomain proteome a hard issue to address, therefore requiring the optimization of specific protocols. In fact, membrane proteins are usually underrepresented in classical proteomic approaches relying on 2D gel electrophoresis (2DE) and MS analysis. This is due to them being generally not very abundant, their isoelectric points (pIs) being generally alkaline and their solubility in the aqueous media used for isoelectrofocusing (IEF) being poor[11-13]. Recent data on the quantitative proteomics of MD, - lipid rafts or caveolae - have underlined the puzzling role of various signaling proteins in cancer development, but these studies were mainly performed on neoplastic cell lines[14-17]. However, procedures commonly used for cell cultures, such as

metabolic labelling, are not suitable for the study of human tissue MD, making label free quantification a valid choice to achieve differential expression profiles. Moreover, the protocol needs to be adapted to small amount of samples, as feasible when working with human surgical specimens.

For these reasons, in the present study we focused on the pre-analytical phase of human surgical sample preparation using a gel-free approach, through liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), in order to improve the identification of proteins, particularly membrane proteins, in MD. We evaluated three different digestion protocols based on Filter Aided Sample Preparation (FASP) technique, and, after optimization, we assessed differential RCC MD proteome by label free quantification, as a starting point for the identification of potential RCC biomarkers.

MATERIALS AND METHODS

Chemicals

Solutions were prepared with Milli-Q water. Water of HPLC grade, trifluoroacetic acid (TFA), isopropyl alcohol, tributyl phosphate (TBP), ammonium bicarbonate (ABC), dithiothreitol (DTT), iodoacetamide (IAA), urea, Triton X-100, Tween-20, Trizma-base, MES, sucrose, BCA protein assay and BSA were from SIGMA Chemical Co. (St. Louis, MO, USA). ACN, methanol, glycerol were from Merck (Darmstadt, Germany). Porcine trypsin was from Promega (Madison, WI, USA). Bradford protein-binding colorimetric assay was purchased from Bio-Rad (Hercules, CA, USA). Anti-protease inhibitor cocktail (Complete) was from Roche (Monza, Italy). NuPAGE® SDS-PAGE Gel Electrophoresis System components (mini gels, running and loading buffer, molecular weight markers and Coomassie brilliant blue staining, CBB) were supplied by Life Technologies (Paisley, Renfrewshire, UK). Hybond-ECL nitrocellulose membrane was from GE (Little Chalfont, Buckinghamshire, United Kingdom). Anti-aminopeptidse N (CD13) monoclonal antibody, anti-annexin A2 (ANXA2) and anti-carbonic anhydrase (CA2) polyclonal antibodies were from Abcam (Cambridge, UK). Species-specific secondary peroxidase conjugated antibodies and ECL reagents were from Pierce (Rockford, IL, USA).

Patients and specimens

Patients with suspected RCC, not receiving any previous chemotherapy, were submitted to radical nephrectomy, after their informed consent and the local research ethics committee approval (U.O. Comitato di Etica e Sperimentazione Farmaci Direzione Scientifica Fondazione IRCCS Ca'Granda

Ospedale Maggiore Policlinico, Milano). RCC was classified according to the WHO recommendations[18] and the 2009 TNM (tumor-node-metastasis) system classification also using immunohistochemical techniques: only samples diagnosed as conventional clear cell RCC were included in the study. Tumor grading were performed according to the Fuhrman grading system[19].

Immediately after removal, the pathologist collected samples of primary RCC, selected inside homogeneous areas and avoiding grossly necrotic or fibrotic parts, and homologous normal cortical tissue (adjacent normal kidney, ANK) contiguous to the tumoral mass. A minimum of one cm^3 of tumor and normal cortex was placed in sucrose buffered solution (250 mM sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA), kept on ice and immediately transferred to the laboratory.

The clinical and personal features of the studied patients are shown in Table 1. Their neoplasms had quite homogeneous characteristics, being in early phases, and no metastasis nor positive lymph nodes were present at diagnosis.

Table 1 Clear cell renal cell carcinoma samples analyzed in this study and corresponding clinical data.

Patients	Age	Sex	pT	G	Membrane microdomains		
					ANK	ANK vs RCC	
					FASP Set up[a]	FASP[b]	1DE[c]
45DM	53	F	1b	2		X	X
46SA	67	F	2	2	X		
47CA	78	F	1a	2	X		
48GA	54	M	1b	2		X	X
49CA	70	M	3b	3		X	X
50PC	51	M	1b	2			
51MI	61	F	1b	2	X		X
52CA	59	F	2a	2			X
60CC	78	M	3a	3		X	X
61FG	56	M	1b	2		X	X
66SML	48	F	2a	2	X		
70LS	71	M	1b	2	X		
72FG	59	M	1a	2	X		
75CPL	71	M	1b	2	X		
77FG	70	M	1b	2	X		
80MLA	78	F	3b	2-3	X		
91BE	58	M	3b	1	X		
117VLF	43	M	2	2	X		

pT, Tumor stage; G, nuclear grade; [a] samples used for the preparation of ANK MD pool in order to optimize FASP protocol; [b] samples used for the label-free quantitation; [c] samples analyzed by 1DE[20].

Subcellular fractionation and microdomain isolation

After nephrectomy, fresh RCC and ANK tissue samples were submitted to subcellular fractionation through differential centrifugation; all steps were performed in a cold room (4°C) or on ice. Subcellular and microdomain-enriched fractions were isolated and characterized as described[20].

Electrophoresis and western blotting

Protein separation was performed with the NuPAGE® electrophoresis system (Life Technologies). Equal amounts (10 µg) of subcellular fraction proteins were separated by 4-12% NuPAGE and blotted onto nitrocellulose membranes using a "tank" electrophoretic transfer apparatus (Hoefer). Membranes were developed with the respective primary antibodies (anti-CD13 1:2000, anti-ANXA2 1:2000, anti-CA2 1:1000), followed by peroxidase-conjugated secondary antibodies (Pierce) and enhanced chemiluminescence detection (SuperSignal West-Pico ECL, Pierce). Images were captured with a CCD camera, LAS4000 (GE Healthcare).

Delipidation and Protein Precipitation by Tri-nbutylphosphate/Acetone/Methanol

During the set-up of delipidation experiments, we assessed a loss of MD proteins of about 20%. Accordingly, we performed delipidation on 150 µg of ANK and RCC MD, since 100 µg of proteins were required for FASP protocols. In order to evaluate the reproducibility of protein extraction, we performed protein quantitative assay by BCA on delipidated samples and verified recovery after NuPAGE/CBB stain, by loading equal aliquots of ANK and RCC MD delipidated samples. Aliquots (100 µl) of the MD pools (1 µg/µl) were mixed with 14 volumes of ice-cold TBP/acetone/methanol mixture (1:12:1) and incubated at 4°C for 90 minutes. Proteins were precipitated by centrifugation at 2800 xg for 15 minutes (4°C), washed sequentially with 1 mL of TBP, acetone and methanol, and then air-dried[21].

Filter Aided Sample Preparation: protocol optimization

Due to the peculiar MD lipid composition, we optimized an enzymatic digestion protocol based on Filter Aided Sample Preparation (FASP)[22], comparing three different protocols (Table 2), with and without previous delipidation. For this purpose, equal amounts of MD isolated from ANK tissues of 11 patients (Table 1) were pooled in order to eliminate inter-individual differences.

Protocol 1 was an adapted version of the Wiśniewski et al.[22] FASP digestion, already reported for the MS analysis of urinary exosomes[23]. The lysis of MD, about 100 µg of proteins, was performed through a 30 min incubation in RIPA buffer (50 mM Tris–HCl pH7.4, 1% NP40, 0.25% Sodium deoxycholate, 150 mM NaCl, 1 mM EDTA). MD were then submitted to disulfide bonds reduction with 50 mM DTT in 50 mM ABC (95°C for 5 min). After sample cooling, the lysates were

transferred to the ultrafiltration units (Amicon Ultra-0.5 mL 30 kDa, Millipore) and centrifuged at 14,000 xg for 15 min in order to eliminate lysis buffer and DTT. The filters were washed with 300 µL of 50 mM ABC (14,000 xg for 10 min) and the samples were incubated with 80 µL of 100 mM IAA in darkness for 30 min. IAA was discharged by a centrifugation at 14,000 xg for 5 min, and the filters were washed with 200 µl of 50 mM ABC for five times (14,000 xg for 10 min). Protein was digested overnight at 37°C by 4 µg of trypsin for each sample. After digestion, the filtrated tryptic peptides were collected by centrifugation with two washes of water (40 µL and subsequently 100 µL).

In protocol 2, lysis and reduction steps were the same of protocol 1; alkylation was performed incubating the samples with 100 µL of 50 mM IAA in darkness for 20 min. After IAA elimination, the filters were washed with 100 µl of 50 mM ABC for four times (14,000 xg for 15 min). Protein digestion was performed overnight at 37°C adding 1 µg of trypsin. After a centrifugation at 14,000 xg for 10 min, the filtrated tryptic peptides were collected, and the filters washed with 50 mM ABC and 500 mM NaCl, two times respectively. The eluted peptides were acidified by 0.1% TFA.

In protocol 3 the sample was solubilized by a denaturation buffer containing SDS and DTT (2% SDS, 50 mM DTT, 100 mM Tris-HCl pH 7.6), incubating at 95°C for 5 minutes. Dissolved MD membranes (70 µl) were mixed with 430 µl of 8 M urea in 100 mM Tris-HCl, pH 8.5 (UA 8.5 solution), transferred into the ultrafiltration device and centrifuged at 14,000 xg for 15 minutes. Alkylation was performed as in protocol 2, followed by four washing, two with 8 M urea in 100 mM Tris-HCl, pH 7.9 (UA 7.9 solution) and two with 50mM ABC (14,000 xg for 15 min). Protein digestion and peptide elution were the same as protocol 2.

Table 2 FASP protocols

FASP Protocol	Denaturation buffer	DTT	IAA	Washing buffer	Trypsin digestion	Eluition
1	RIPA	0.4 M	0.1 M	ABC	4 µg	H_20
2	RIPA	0.4 M	0.05 M	ABC	1 µg	NaCl 0.5 M TFA 0.1%
3	SDS	0.1 M	0.05 M	UA	1 µg	NaCl 0.5 M TFA 0.1%

ABC, 50 mM ammonium bicarbonate buffer ; UA, 8 M urea in 100 mM Tris-HCl pH 8.5 and 8 M urea in 100 mM Tris-HCl pH 7.9; TFA, trifluoroacetic acid.

Digestion and peptide extraction of RCC and ANK MD

MD isolated from RCC and ANK tissues of 5 patients were pooled (Table 1), and the resulting samples were lysed and submitted to protein extraction and digestion by the FASP protocol 3, after delipidation.

MD proteome analysis by nLC-ESI-MS/MS

All the MS analysis and proteins identification were performed in the same conditions. Before LC MS analysis, tryptic digests for each sample were quantitated by NanoDrop spectrophotometer and desalted using *Ziptip*™ μ-C_{18} (Millipore) following manufactured instructions[24]. About 1 ug of digested proteins for each sample run were injected at least 3 times into Dionex UltiMate 3000 rapid separation (RS) LC nano system (Thermo Scientific, Germany) coupled online with nESI Ion Trap AmaZon ETD (Bruker Daltonics GmbH, Germany). After the μ-trapping column desalting (Dionex, Acclaim PepMap 100 C18, cartridge, 300 μm i.d. × 5 mm, 5 μm) and concentration step, peptides were then separated through 360 min multistep gradient on the analytical 50 cm nano column (Dionex, 0.075 mm ID, Acclaim PepMap100, C18, 2 μm) with a flow rate of 300 nl/min as already described[25]. The ion-trap mass spectrometer equipped with an on-line nanospray source was operated in the data-dependent-acquisition mode. For MS generation, enhanced resolution and trap ICC value of 400,000 were used; for MS/MS acquisition, ICC target was increased to 1,000,000; a narrow range for Smart Fragmentation from 50 to 150% was adopted. CID MS/MS fragmentation was set to fragment the ten most abundant MS peaks with strict active exclusion after one spectra and released after 9 seconds. The obtained chromatograms were elaborated with Compass DataAnalysis™, v.4.0 Sp4 (Bruker Daltonics, Germany) and the resulting mass lists were processed using in house Mascot search engine (v.2.4.0), through Mascot Daemon tool. Database searching was restricted to human Swissprot database (accessed Apr 2014, 544,996 sequences; 193,815,432 residues). Trypsin as enzyme and carbamidomethyl (C) as fixed modifications were set in search parameters. Mass tolerances for all identifications were generally fixed at 2 Da for the precursor ions and 0.8 Da for product ions. Automatic decoy database search and built-in Percolator algorithm were applied to calculate posterior error probabilities for each peptide-spectrum match and to rescore search results with a unique significance threshold. Proteins with at least one identical peptide (p-value <0.05) were considered identified[26]. Data was filtered using a global False Discovery Rate <1% and only proteins with at least one unique identical peptide sequence (p-value <0.05) were considered identified[26].

Label free protein abundance evaluation

Protein abundance in MD of RCC and ANK tissues was determined by Progenesis LC-MS software v 4.1 (Non-linear Dynamics, Newcastle, England). The raw LC-MS/MS data were imported and the ion intensity maps of all runs (3 runs for each class) were visually examined for defects and used for the alignment process, performed using the "Automatic Alignment" function. One sample is thus set as the reference run and the retention times of all other samples within the experiment were automatically aligned to create maximal overlay across the data. The default sensitivity and a peak width of 0.2 min were selected as parameters for the feature detection algorithm. Samples were then allocated to their experimental class (RCC vs ANK). The identification of peptides was performed using an in-house Mascot search engine as described above. The filtered MASCOT search results were imported back in Progenesis LC-MS and the identified peptides with a score lower than 13 were discarded (p-value >0.05). Experimental variation affecting the protein expression was minimized by analyzing the same amount of sample (1 µg) and by the normalization process performed by Progenesis-QI software (http://www.nonlinear.com/progenesis/qi-for-proteomics/v2.0). Protein quantification results were exported and used for further analysis.

Bioinformatic analysis

Gene ontology (GO) analysis was approached using the LocDB database (www.rostlab.org/services/locDB/) for investigating the subcellular localization of the identified proteins and the Protein ANalysis THrough Evolutionary Relationship (PANTHER) database (http://www.pantherdb.org/) for the analysis of their molecular functions. In addition, membrane proteins were deeply studied and transmembrane helix prediction was performed using the online TMHMM 2.0 prediction server (http://www.cbs.dtu.dk/services/TMHMM/)[27,28], palmitoylation was predicted by CSS-Palm 2.0[29], GPI-modification site prediction was conducted through the online big-PI predictor server (http://mendel.imp.ac.at/sat/gpi/gpi_server.html)[30] and myristoylation was predicted using the online Expasy Myristoylator prediction server (http://web.expasy.org/myristoylator/)[31]. Protein-protein interactions were predicted using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database v9.0 (http://www.string-db.org/) and proteins were linked based on the following six criteria; neighbourhood, gene fusion, co-occurrence, co-expression, experimental evidences and existing databases[32].

Tissue specificity of the identified proteins was evaluated using different databases available in the Human Protein Atlas (HPA) (http://www.proteinatlas.org/)[33]. In particular, MD proteins were compared with: i) the human specific proteome of 32 tissues, summing up "tissue enriched", "group enriched" and "tissue enhanced" gene categories; ii) the housekeeping proteome (proteins detected

in all tissues); iii) the renal cancer proteome, considering only the proteins detected in at least 80% of the analysed patient tissues.

RESULTS AND DISCUSSION

Protocol setup

For the optimization of the digestion protocol, we pooled MD isolated from ANK tissues of 11 patients in order to eliminate inter-individual differences. Then we subjected aliquots of 100 µg of proteins to the three ~~digestion~~ protocols, and analyzed them through LC-MS/MS.

In order to select the best protocol, we took into consideration: i) the number of total proteins identified by Mascot search engine; ii) the amount of hydrophobic proteins on the basis of the hydrophobicity score, GRAVY index (assigned by ProtParam http://web.expasy.org/protparam/); iii) the percentage of membrane proteins (classification assigned by LocDB and confirmed by TMHMM 2.0).

We first tested an adapted version of FASP digestion, already used for the MS analysis of membrane proteins (hereafter named "Protocol 1") (Table 2)[23]**.** FASP is a method that combines strong detergents for universal solubilization with efficient pre-digestion 'clean up' of the proteome in order to obtain then purified peptides, avoiding the disadvantages of the gel format[22]. The main feature of Protocol 1 is the lysis of MD, performed through a 30 min incubation in lysis RIPA buffer. The resulting analysis with LC-MS/MS led to the identification of only 32 proteins with few signals of low intensity showed by base peak chromatogram (ESI, Fig. S1).

Due to the peculiar lipid composition of MD, we introduced a step of protein delipidation and precipitation with Tri-n-butylphosphate, acetone, and methanol[21]**.** This pretreatment ensured the highest protein recovery, according to Shevchenko and colleagues[34]. By using delipidation followed by the Protocol 1, 103 proteins were identified (Fig. 1), a number still lower than in our previous study (286 proteins)[23]. Then we tested another protocol (Protocol 2), by adding a NaCl washing in order to increase the recovery of hydrophobic peptides, and one acidification step with 0.1% TFA, both on delipidated and non-delipidated samples (Tab 2). These settings determined an increased, yet not fully satisfactory, recovery of proteins, (Fig. 1).

Conditions were then further modified in Protocol 3, which, unlike the other protocols, involves the use of SDS buffer and Urea (Table 2). In particular, we performed all washings with urea 8 M,

which is supposed to fully wash out detergents from membrane proteins[35]. When applied without delipidation, this protocol led to the identification of 170 proteins, of which only 23% were transmembrane proteins (Fig. 1). This could be due to the inefficient removal of lipids-SDS interaction by the filter, interfering in the MS analysis (ESI, Fig. 1). Moreover, the filter could retain some lipid components, together with hydrophobic lipid-bound proteins. Conversely, Protocol 3 coupled to delipidation was the most efficient way to identify proteins in MD. In fact, firstly, the introduction of the delipidation step resulted in a significant increase in the yield of total protein identification (N = 342). Secondly, the total number of membrane proteins greatly increased (N = 222) boosting the percentage of transmembrane proteins to 50%.

These results may depend on the depletion of the lipid components, which likely enables the "release" of transmembrane proteins. Once freed, they become more suitable for tryptic digestion, thus generating peptides that can be eluted through 30-kDa cut-off FASP filter[36].

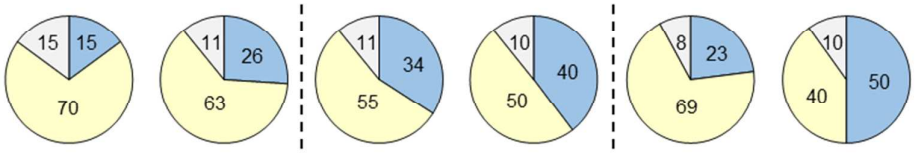
	Protocol #					
	1		2		3	
	ND	D	ND	D	ND	D
Identified proteins, N	32	103	199	292	170	342
Hydrophobic proteins, N (%)	20 (63)	53 (52)	101 (51)	157 (54)	93 (55)	188 (55)
Membrane proteins, N (%)	27 (84)	72 (70)	121 (61)	190 (65)	117 (69)	222 (65)
Transmembrane proteins (%) / Peripheral proteins (%) / Lipid-anchored proteins (%)						

Fig. 1. **Summary of the performance of the three FASP protocols tested in this study**. Total number of identified proteins, hydrophobic and membrane proteins, and the percentage of transmembrane, peripheral and lipid-anchored (GPI- and others) proteins are shown. ND, not-delipidated; D, delipidated.

LC-MS protein identification and label free quantification

The optimized protocol was implemented to perform comparison of RCC with ANK MD. In fact, we speculated that the delipidation step could eliminate some differences in the protein

identification due to their different lipid composition (data not shown), making the comparative proteomic analysis more reliable. We first evaluated the recovery after MD delipidation as shown in ESI figure S2. The extraction of ANK and RCC MD proteins after delipidation resulted reproducible and the protein profiles were comparable with the not-delipidated ones (ESI, Fig. S2). Proteins of microdomain-enriched fractions prepared from 5 paired samples RCC and ANK tissues were pooled and analyzed. Identification was accepted with at least one unique peptide exceeding Mascot score of identity cut-off, in order to ensure a better comprehension of MD proteomes. Exploiting the powerful peak detection of Progenesis QI algorithm (used during the label-free protein expression evaluation) and applying the above detailed criteria, MS/MS analysis after FASP digestion led to the identification of 742 proteins in ANK and 721 in ccRCC MD. Overall, more than 800 protein species were identified, their full details, including scores and number of matched peptides, are shown in ESI, table S1 and S2.

The molecular characteristics of the identified proteins were assessed by bioinformatics and prediction tools[37]. Results showed that the majority of identified proteins (53.1% and 52.6% in ANK and RCC, respectively) were membrane-associated, with about 58% of them being transmembrane proteins (ESI, Table S1 and S2). Many of these were typical raft proteins, such as Caveolins (1 and 2), flotillins, Aquaporin-1, Prohibitins, VDAC1 and Thy-1. Comparing our list of MD total identified proteins with RaftProt, a recently published Mammalian Lipid raft Proteome Database (http://lipid-raft-database.di.uq.edu.au/index.html)[38], 580 (72%) were classified as human lipid raft associated proteins and 394 (49%) as High Confidence lipid raft proteins. Moreover, we investigated whether protein sequences contained sites for post-translational modifications such as glypiation, palmitoylation, and myristoylation, using prediction algorithms. They assigned putative myristoylation sites to about 15% of total identified membrane proteins and GPI anchoring to 5% of them. Much more frequent, about 44%, was the prediction of palmitoylation sites. Additionally, STRING analysis classified 67% of proteins as constituents of protein complexes. These findings are intriguing, because both such post-translational modification and protein-protein interaction are likely to determine a -possibly reversible- association to membrane MD of otherwise soluble proteins, allowing for their dynamic binding to microdomains[39]. After applying these tools for the prediction of -direct or indirect- membrane interactions, only 79 proteins (9.8%) were left out.

These results are in good agreement with a recently published work, concerning chicken inner ear membrane MD proteomics[37]. Mass spectrometry of MD fractions identified over 600 putative raft proteins, and most of them were predicted as membrane-associated and involved in trafficking and metabolism. This highlights the common modularity features of MD, as a quite stable structure that plays comparable roles in different settings[40]. In fact, membrane MD are, on one hand, highly

dynamic, with protein (and lipid) components shuttling rapidly between raft and non-raft membrane and on the other hand, present a fixed and common proteomic core, accounting for their structural and specific characteristics.

Very few other papers approached tissue MD proteomics. In particular, a study by Yu et al., adopted a "tube-gel" protein digestion label-free shotgun proteomic strategy to quantify raft proteins in neonatal mouse brain: about 200 raft proteins were identified from a single sample, thus defining a core proteome qualitatively quite similar to ours and to the previous ones[37,40,41].

In our previous work, we analyzed the same RCC and ANK MD samples by a *gel based* method (Table 1)[20]. The present optimized *gel free* approach seems to be more suitable for the study of membrane microdomains, providing higher number of total identified proteins (742 vs 98 in ANK MD and 721 vs 93 in RCC MD). It is noteworthy that more than 88% of the proteins identified with the gel-based approach were also found using the shotgun proteomic strategy (Fig. 2), confirming their localization in the MD.

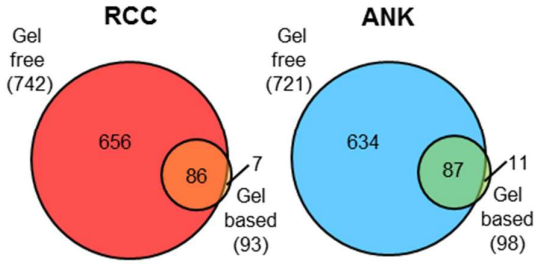


Fig. 2. **Venn diagram of protein species identified in RCC and ANK MD using two different analytical approaches.** Comparison of protein identification data obtained in the present - *gel free* - study and in the previous - *gel based* - one[20].

We assessed the tissue specificity of the MD proteome, comparing the new list of MD proteins with the Human Protein Atlas database (HPA)[33]: the comparison with the human tissue-specific proteomes of 32 different tissues showed a peak matching with kidney-specific ones (Fig. 3). Moreover, the 30.5% of proteins identified in RCC MD were already detected in HPA renal cancer tissue microarrays. It has to be stressed that subcellular fractionation, and in particular MD purification, greatly enhances the possibility to identify low-abundance proteins, undetectable in the whole tissue. Several protein classes (i.e. G proteins, RAS-related proteins, components of the vacuolar ATPase/synthase complex...) cannot indeed result tissue specific because they are typical of the MD proteome, regardless of their tissue localization[40].

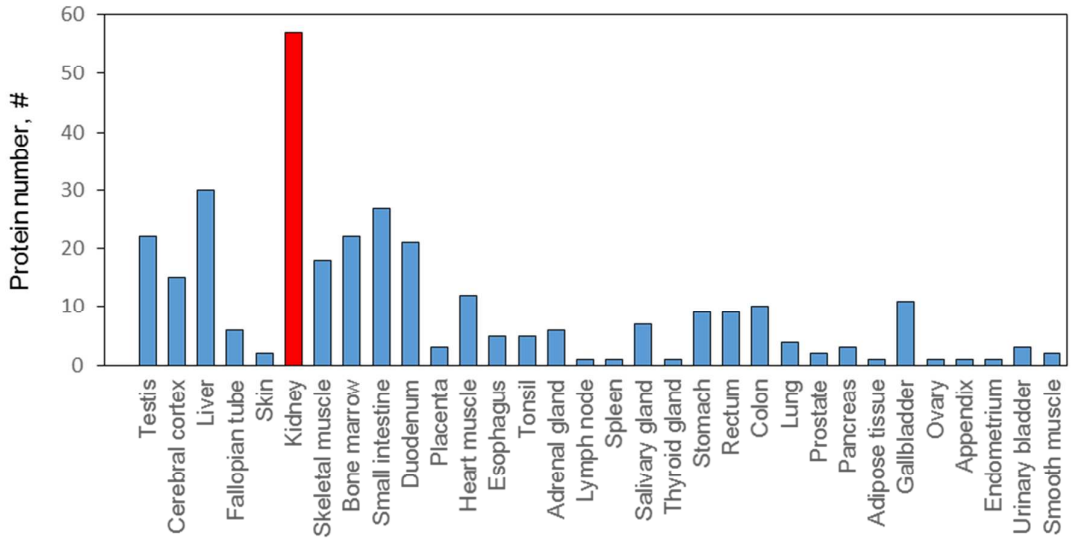


Fig. 3 – **Tissue specificity analysis.** Number of MD proteins matched with the human tissue specific proteomes of 32 different tissues.

We then evaluated the differential proteome by label free quantification, considering proteins "up" or "down-regulated" when the ratio was respectively higher than 1.50 and lower than 0.67[22, 26]. Applying these criteria to 657 quantified protein species, 170 (25.9%) were classified as "up" and 126 (19.2%) as "down" in RCC MD (ESI, Table S1).

The differential proteins ("up" and "down") were correlated with subcellular localization (by LocDB) and molecular function (by Panther) (Fig. 4).

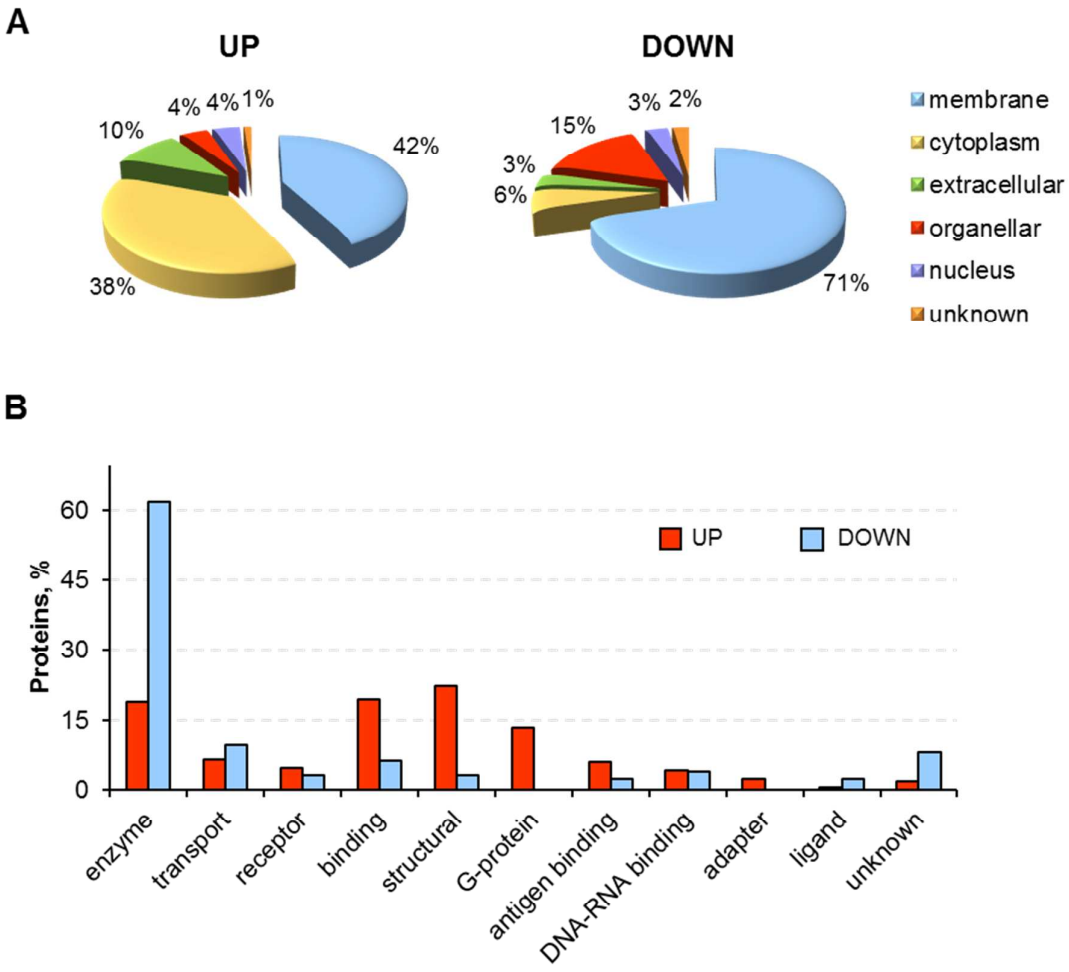


Fig. 4. **Subcellular Localization and Molecular Functions of the RCC differential proteins, divided in "UP" and "DOWN".** A) Pie charts representing the cellular localization of the differential proteins. B) Histogram representation of the percentage of differential in main Molecular Functions.

The majority of "down" proteins were membrane-associated (70.6%) (Fig. 4A); this result could be dependent on the process of de-differentiation typical of cancer cells, which lose their tubular epithelial specialization. In fact, among "down" proteins, we found aquaporin-1 (AQP1), water-channel that is considered a kidney differentiation marker[42], Na+/glucose cotransporters 2 and 5, involved in glucose reabsorption by proximal tubule cells, and renal dipeptidase (DPEP-1), a zinc-dependent membrane metalloprotease[43].

On the contrary, the "up" proteins resulted to be partially membrane (42.3%) and partially cytoplasmic proteins (38.2%) (Fig. 4A). It has to be underlined that some cytosolic proteins may associate to the plasma membrane and MD, through cytoskeletal components[44]. Several cytoskeleton associated-proteins, as Ezrin, Radixin and Moesin, members of the ezrin-radixin-

moesin (ERM) family, involved in cancer progression[45], resulted in fact increased in RCC MD. Interestingly, focusing on post-translational modifications, the predicted palmitoylation sites appeared nearly doubled (from 42,5 to 71%) among "up", compared to "down" proteins. Interestingly, focusing on post-translational modifications, the predicted palmitoylation sites appeared nearly doubled (from 42,5 to 71%) among "up", compared to "down" proteins. It is tempting to speculate that this behavior is driven by a modification of the RCC MD lipid composition, thus allowing cancer cells to address new proteins, perhaps involved in aberrant signaling events, to MD. The elucidation of this hypothesis deserves further investigation.

By analyzing this lists of proteins with Panther, an on-line tool for the classification of genes according to Gene ontology classes, we obtained the picture depicted in Fig. 4B. From a functional point of view, the presence of neoplasm seemed to induce an increase in some peculiar protein classes involved in signaling and adhesion, typical MD-associated processes. In fact, G proteins, structural and binding proteins (e.g.: flotillins, tubulins, annexins, caveolins) were more abundant in RCC MD, compared to ANK.

It has to be underlined that the majority of the proteins were found differential in both our previous *gel-based* study[20] and in the present (after label free quantification): for example, Basigin, DPEP1, AQP1 and Caveolin-1 (ESI, Table S1). This result may indeed represent a preliminary validation.

Validation of differential RCC MD protein content by immunoblotting analysis

Relative quantification results obtained by label-free approach were verified by immunoblotting of three differential proteins, Carbonic Anhydrase 2 (CA2), Annexin A2 (ANXA2) and Aminopeptidase N (CD13), ~~by~~ comparing the signal intensity in different subcellular fractions prepared from ANK and RCC (Fig. 5).

The choice of these three proteins was firstly based on their significant high or low RCC/ANK ratio (CA2, 2.54; ANXA2, 1.97; CD13, 0.37) obtained after label free quantification. Secondly, ANXA2 and CD13 were already reported as MD associated, and listed as High Confidence Raft Proteins in the RaftProt database[38]. On the other hand, CA2 is a typical cytosolic enzyme, responsible for more than 95% of the total kidney carbonic anhydrase activity[46] and it is not usually described as a MD bound protein.

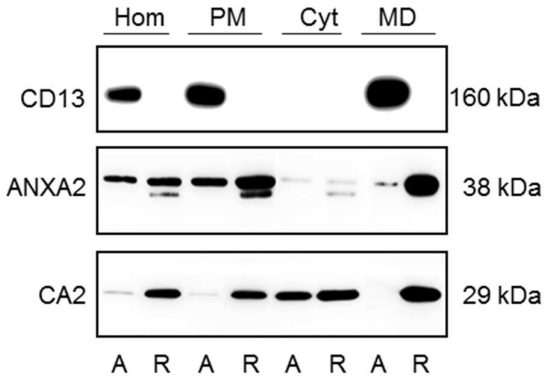


Fig. 5. **Validation of the differential content of Carbonic Anhydrase 2 (CA2) Annexin A2 (ANXA2) and Aminopeptidase N (CD13) in RCC MD, compared to ANK**. A) The abundance of some differential proteins was assessed in ANK (A) and RCC (R) MD, compared with the whole lysate (Hom) and plasma membrane-enriched fractions (PM) from which MD are prepared, and with the cytosolic fractions (Cyt), by immunoblotting with specific antibodies. 7 µg of proteins of Hom, PM and Cyt, and 3 µg of proteins of MD were loaded on SDS-PAGE gels. One (out of 3) representative case is shown.

WB results regarding ANXA2 confirmed label free quantification: its content was higher in RCC samples, compared to ANK. This difference is higher at the MD level than in whole lysates, suggesting the occurrence of an altered subcellular localization in RCC tissue. ANXA2 is actually considered a marker of several cancer cells (reviewed in[47]), and its overexpression in RCC has been already reported[48]. Its key role in the organization of lipid raft signaling domains through binding to membrane phospholipids, in physiological conditions, was also described[49]. A paper published during the writing of the present manuscript[50] outlines the regulatory role of ANXA2 in RCC cell motility and the change in ANXA2 localization from cytoplasm to membranes in RCC tissue (by immunohistochemistry), supporting our findings.

Aminopeptidase N is a transmembrane zinc-dependent ecto-enzyme, expressed by various tissues, including the kidney[51]. Besides its peptidase activity, it is physiologically involved in endocytosis and signaling[52]. CD13 resulted down regulated in RCC MD by label free analysis; accordingly, its signal was undetectable in RCC subfractions, while highly enriched in ANK MD, compared to homogenate and total plasma membrane fractions. Although this protein is usually reported as up-regulated in many cancers, such as mesenchymal tumors, breast, ovarian and colon cancer (reviewed in [53,54]), some studies performed on RCC tissues showed a reduction of CD13 levels,

compared to ANK, both by immunohistochemistry[55] and through the measure of its surface enzymatic activity[56].

CA2 appeared more abundant in all RCC subcellular fractions, and its signal was particularly and specifically strong at the RCC MD level, while undetectable in the corresponding ANK lane, confirming once again data obtained by label free quantification. These data suggest a possible tumor-dependent mis-localization of CA2 in RCC MD. Interestingly, an increased expression of CA2 has been reported in neovessel endothelium of several tumors, such as melanoma and esophageal, renal, and lung cancer, but not in the corresponding healthy vascular cells[57]. Since we prepared fractions from the whole RCC tissue, the signal intensity detected in MD may indeed derive from tumoral neovessels.

Altogether, the validation of these three proteins further supports the potency of the label-free MS approach.

CONCLUSION

In conclusion, our results show the importance of setting up a specific protocol for the proteomic analysis of membrane proteins, specific to the different molecular features of the sample. Moreover, our data confirm that the sensitivity of proteomic profiling can be significantly enhanced by focusing on highly enriched subcellular fractions, as MD. The optimization of all ~~the~~ analytical steps, from sample pre-fractionation and preparation to the MS analysis and *label free* quantification, provided us with a large panel of differential RCC MD proteins, among which tumor biomarkers may be looked for.

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