Histo-molecular differentiation of renal cancer subtypes by mass spectrometry imaging and rapid proteome profiling of formalin-fixed paraffin-embedded tumor tissue sections

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Abstract

Pathology differentiation of renal cancer types is challenging due to tissue similarities or overlapping histological features of various tumor (sub)types. As assessment is often manually conducted outcomes can be prone to human error and therefore require high-level expertise and experience. Mass spectrometry can provide detailed histo-molecular information on tissue and is becoming increasingly popular in clinical settings. Spatially resolving technologies such as mass spectrometry imaging and quantitative microproteomics profiling in combination with machine learning approaches provide promising tools for automated tumor classification of clinical tissue sections.

In this proof of concept study we used MALDI-MS imaging (MSI) and rapid LC-MS/MS-based microproteomics technologies (15 min/sample) to analyze formalin-fixed paraffin embedded (FFPE) tissue sections and classify renal oncocytoma (RO, n=11), clear cell renal cell carcinoma (ccRCC, n=12) and chromophobe renal cell carcinoma (ChRCC, n=5). Both methods were able to distinguish ccRCC, RO and ChRCC in cross-validation experiments. MSI correctly classified 87% of the patients whereas the rapid LC-MS/MS-based microproteomics approach correctly classified 100% of the patients.

This strategy involving MSI and rapid proteome profiling by LC-MS/MS reveals molecular features of tumor sections and enables cancer subtype classification. Mass spectrometry provides a promising complementary approach to current pathological technologies for precise digitized diagnosis of diseases.

43 **Introduction**

44 Kidney cancer (renal cell carcinoma, RCC) accounts for 2.2% of all diagnosed cancers and is
45 the 13th most common cause of cancer deaths worldwide [1]. Clear cell renal cell carcinoma
46 (ccRCC) constitutes 70% of all kidney cancers [2] and exhibits the highest rate of metastasis
47 among renal carcinomas. Two other common but less aggressive subtypes of renal
48 carcinoma are chromophobe renal cell carcinoma (ChRCC) and the essentially benign renal
49 oncocytoma (RO), which account for 5% and 3-7 % of all cases, respectively [3, 4]. The ability
50 to distinguish between the malignant cancer types ccRCC and ChRCC and the benign RO is
51 crucial for a patient in terms of prognosis, progression and intervention strategies as severe
52 as total nephrectomy. Histopathological kidney cancer diagnostics faces many challenges in
53 daily routine. Typically, test panels consisting of a combination of different chemical and
54 immuno-histochemical staining methods are used to systematically obtain a diagnosis [5].
55 Overlapping histological features can make it difficult to differentiate tumor types. Analysis,
56 interpretation and diagnosis/prognosis greatly rely on visual inspection and the experience of
57 the involved clinical pathologists. Complementary techniques such as magnet resonance
58 imaging (MRI) and electron microscopy involve costly instrumentation. Moreover, specific
59 antibodies for staining can be expensive or unavailable for certain molecular targets. Mass
60 spectrometry is emerging as a promising new tool in translational research, from molecular
61 imaging of tissue sections to deep protein profiling of tissue samples [6]. The digital data
62 readout provided by high mass accuracy mass spectrometry and feasibility of molecular
63 quantification makes it a very attractive technology in translational research for investigating
64 human diseases and for diagnostics and prognostics purposes in the clinic. Improvements in

65 mass spectrometry instrument performance and computational analysis paved the way for
66 applications in clinical microbiology [7] and clinical genetics analysis [8]. The fact that mass
67 spectrometry can be applied to a variety of different bio-molecules such as peptides, lipids,
68 nucleic acid makes it extremely versatile and expands the translational and diagnostic
69 possibilities greatly [8-11].

70 Molecular imaging of tissue sections by MALDI mass spectrometry (MSI) was introduced
71 more than 20 years ago [12, 13] and it has been applied in translational research and clinical
72 applications, to study injuries, diseases, or distinguish between different cancer types such as
73 Pancreatic Ductal Adenocarcinoma or Epithelial Ovarian Cancer Histotypes [14-18].

74 Mass spectrometry-based proteomics relies on advanced LC-ESI-MS/MS technology, where
75 peptide mixtures are separated by liquid chromatography (LC) prior analysis by electrospray
76 ionization tandem mass spectrometry (ESI MS/MS) and protein identification by protein
77 database searching [19, 20]. Current LC-MS/MS strategies enable comprehensive
78 quantitative protein profiling from tissues and body fluids [21, 22]. While having been used to
79 identify potential biomarkers or new candidate cancer targets and molecular signaling
80 networks the relatively long LC gradients (hours) and extensive sample preparation protocols
81 make it difficult to apply in a routine clinical setting. Modern mass spectrometers are steadily
82 increasing in sensitivity and scanning speed [23]. In addition, improved chromatographic
83 systems that enable rapid solid phase extraction integrated with reproducible separations are
84 emerging [24-27], enabling fast (minutes) and sensitive (nanogram) analysis of complex
85 biological samples.

86 We hypothesized that histo-molecular information from both MALDI MS imaging (MSI), *in situ*
87 protein digestion and LC-MS/MS applied to detailed characterization of 5 µm cancer formalin
88 fixed paraffin embedded (FFPE) tissue sections will provide spatial molecular maps and
89 sufficiently deep proteome profiles to characterize and classify tumor subtypes. We
90 investigated this by testing a series of malignant and benign renal carcinomas, including clear
91 cell renal cell carcinoma (ccRCC), chromophobe renal cell carcinoma (ChRCC) and renal
92 oncocytoma (RO). We obtained histo-molecular images at a resolution of 150µm x 150µm
93 that sufficed to spatially resolve features to distinguish tumor subtype areas from surrounding
94 tissue. Miniaturized sample preparation by *in situ* protein digestion was used to recover
95 peptides from distinct areas of the FFPE tumor sections for rapid proteome profiling by LC-
96 MS/MS.

97

98 **Material and Methods:**

99 **Materials**

100 Xylene (analytical grade), ammonium bicarbonate, Sodium citrate, trifluor-acetic acid (TFA),
101 formic acid (FA), acetic acid (AcOH), acetonitrile (ACN), methanol and α-Cyano-4-
102 hydroxycinnamic acid (CHCA) were purchased from Sigma. Polyimide coated fused silica
103 capillary (75 µm ID) was from PostNova, C_{18} Reprosil Pur reversed phase material was from
104 Dr. Maisch (Ammerbuch-Entringe, Germany), recombinant Trypsin was purchased from
105 Promega (WI. USA), Indium-tin-oxide (ITO) glass slides were purchased from Bruker
106 (Bremen, Germany), water was Milli-Q filtered.

107 **Formalin fixed paraffin embedded samples:**

108 Patient samples were collected at Odense University Hospital, Denmark. All samples were
109 obtained upon patient's consent. Formalin fixed paraffin embedded (FFPE) tissues from 11
110 RO patients, 12 ccRCC patients and 5 ChRCC patients were used for LC-MSMS analysis (for
111 ChRCC due to the lower number of patients 2 subsequent slides were used from 2 patients
112 adding up to a total of 7 sections). Out of the patient cohort 9 RO, 9 ccRCC and 5 ChRCC
113 were used for mass spectrometry imaging analysis.

114 **Tissue preparation:**

115 *Preparation of formalin fixed paraffin embedded samples*

116 FFPE blocks were cut into 5 µm thick sections and mounted onto indium tin oxide (ITO)
117 covered glass slides (for MSI) or regular microscopy glass slides (for LC-MS/MS). Before
118 deparaffination slides were left on a heated block at 65° C for 1 hour to improve adhesion (an
119 overview on the used FFPE samples can be found in supplementary table S1 and S2).

120 *Deparaffination*

121 FFPE section slides were incubated in Xylene for an initial 10 min. and then another 5 min.
122 using fresh solution each time. Slides were shortly dipped into 96% EtOH before they were
123 washed for 2 min in a mixture of chloroform/Ethanol/AcOH (3:6:1; v:v:v). The slides were then
124 washed in 96% EtOH, 70% EtOH, 50% EtOH and Water for 30 sec. each.

125 *Antigen retrieval*

126 Tissue slides were heated in 10mM citric acid buffer pH 6 for 10 min in a microwave oven at
127 400 Watt (just below the boiling point) before left for further 60 min incubation at 98°C on a
128 heating plate. Slides were cooled down to room temperature and incubated for 5 minutes in
129 25 mM ammonium bicarbonate (ABC) buffer. Slides were allowed to dry before application of
130 trypsin protease.

131 *Tryptic digest*

132 For MALDI MS imaging:

133 20µg of Trypsin (Promega) was used per slide and was dissolved at a concentration of
134 100ng/µl in 25mM ABC /10% ACN before being deposited on the tissue using the
135 iMatrixSpray [28] device equipped with a heating bed (Tardo Gmbh, Subingen, Switzerland)
136 using the following settings: sprayer height = 70mm, speed = 70mm/s, density = 1µL/cm^2,
137 line distance= 1 mm , gas pressure= 2.5 bar, heat bed temperature= 25°C . After trypsin
138 deposition the slides were incubated in a humid chamber containing 10mM ABC/ 50% MeOH
139 at 37°C over night.

140 For *on-tissue* digest intended for LC-MS/MS proteome profiling:

141 Droplets of 2µl Trypsin solution (50ng/µL in 25mM ABC /10%ACN, 0.02%SDS) were
142 deposited using a gel loading pipet tip. Droplets were placed on 2-6 different tumor areas of
143 each FFPE tissue section. The extraction positions were chosen randomly within the defined
144 tumor margins which were defined by HE-stain and MSI clustering. The droplets were quickly
145 allowed to dry to prevent spreading across the tissue. Slides were transferred to a closed
146 humidity chamber (10mM ABC /50% MeOH) for overnight digestion at 37°C. The digestion

147 spots were extracted twice with 2µL of 0.1% FA and twice with 1.5µL of 30%ACN. Fractions
148 were combined for each sample and speedvac dried. Samples were reconstituted in
149 0.05%TFA and shortly spun down prior injection into the LC-MS system.

150 *Matrix application*

151 Matrix solutions were freshly prepared from recrystallized α-cyano-4-hydroxycinnamic acid
152 (CHCA) matrix (10mg/mL in 50% Acetonitrile 1% TFA). Matrix was sprayed using the
153 iMatrixSpray (Tardo, Switzerland). Temperature of the heatbed was set at 25°C. The sprayer
154 distance was set to 70mm. Spray speed was set to 100 mm/s. Matrix was sprayed in 3
155 rounds: 8 cycles with a flowrate of 0.5µl/cm^2 line distance of 1mm, 8 cycles of 1µl/cm^2 line
156 distance of 1mm, 8 cycles of 1µl/cm^2 and a line distance of 2mm.

157 *MALDI MS Imaging data acquisition*

158 Optical images of the tissue were obtained before matrix application using a flatbed scanner
159 (Epson) at resolutions of 2400dpi. The imaging data was acquired via FlexImaging software
160 (Bruker, Daltonics, Bremen, version 3.1) with 500 shots/ pixel on a Ultraflextreme MALDI-
161 TOF/TOF MS (Bruker Daltonics, Bremen) equipped with a SmartBeam laser (Nd:YAG 355
162 nm). External mass calibration was performed with a tryptic digest of bovine serum albumin
163 (Sigma). Spatial resolution was set to 150µm in x- and y-direction. Mass spectra were
164 acquired in positive ion reflector mode in the range *m/z* 600-3500. (An average sum spectrum
165 of each cancer condition can be found in Supplementary material 1: Figure S1)

166

167 *LC-MS/MS analysis*

168 LC-MS/MS data was acquired by an Orbitrap Q-Exactive HF-X (Thermo, Bremen) coupled to
169 an Ultimate 3000 capillary flow LC-system. Setup was modified from Thermo Scientific
170 Technical note: 72827. Peptide samples were loaded at 150µl/min (2% ACN, 0.05% TFA) for
171 30 sec onto a 5µm, 0.3 x 5 mm, Acclaim PepMap trapping cartridge (Thermo Scientific).
172 Samples were then eluted onto a pulled emitter analytical column (75µm ID, 15cm). The
173 analytical column was "flash-packed" [29] with C_{18} Reprosil Pur resin (3µm) and connected by
174 Nanoviper fittings and a reducing metal union (Valco, Houston, TX). The flowrate of the 15
175 min gradient was 1.2 µL/min with solvent A: 0.1% formic acid (FA) and solvent B: 0.1% FA in
176 80% ACN. Gradient conditions for solvent B were as followed: 8% to 25% in 10 min, 25% to
177 45% in 1.7 min. The trapping cartridge and the analytical column were washed for 1 min at
178 99%B before returning to initial conditions. The column was equilibrated for 2 min. MS
179 settings: ESI spray voltage 2kV, cap temp=275°C, Resolution: 60k, micro scans =1, max IT
180 =100 ms, AGC =$3x10^6$, MSMS resolution 15k, n= top 5, max IT =100 ms, AGC = $1x10^5$.

181

182 *Data Processing of MALDI MS imaging data*

183 The data was baseline subtracted, TIC normalized and statistically recalibrated and then
184 exported into imzML format [30] using the export function of FlexImaging software (Bruker).
185 The exported mass range was m/z 600-3000 with a binning size of 9600 data points. The
186 imzML files were imported into the R environment (version: 3.4.1) and further processed and
187 analyzed using the R MSI package: Cardinal (version: 2.0.3 & 2.4) [31]. In order to extract
188 pixels of tumor tissue each sample was preprocessed as follows: peaklist was generated by

189 peak picking in every 10th spectrum and subsequent peak alignment. The whole data was
190 then resampled using the “height” option and the previous created peaklist as spectrum
191 reference. PCA scores were plotted using car-package (version 3.0.6). Samples were
192 clustered using spatial shrunken centroid clustering [32]. Subsequently, clusters were
193 compared to tumor regions in HE-stained tissue sections (supplementary material 1: Figure
194 S2). The respective clusters containing tumor areas were extracted, so that result files
195 predominantly contained data from tumor areas. The obtained coordinates were then used to
196 extract the corresponding pixel from the unprocessed imzML file. Each tumor type was
197 assigned with a diagnosis factor (ccRCC, RO or ChRCC), which was later used as y-
198 argument in the cross-validation. All extracted imaging acquisition files were further restricted
199 to a mass range of m/z 700-2500. Data was resampled with step size 0.25 Da to allow
200 combining them into one file for further processing. Classification and cross-validation were
201 performed using partial least square discriminant analysis (PLS-DA) [33]. PLS components
202 were tested for optimum with 34 components (**supplementary material 1: Figure S3**).
203 Classification diagnosis was based on the highest scoring condition. Differences between 2
204 conditions had to be higher than 10% of the highest score to be considered distinguishable)

205

206 *LC-MS/MS data processing*

207 The MaxQuant [34] software package (version 1.5.7.0) was used for protein identification and
208 label-free protein quantitation. LC-MS/MS data was searched against the Swissprot human
209 proteome database, using standard settings and “match between runs” enabled.

210 Data filtering, processing and statistical analysis of the MaxQuant output files was performed
211 using the Perseus [35] framework (version 1.6.1.3). Data was filtered excluding the following
212 hits: only identified by site, contaminants and reversed. The log-transformed data was filtered
213 for proteins present in at least 70% of all experiments. Significance filtering was based on
214 ANOVA testing, using FDR threshold of 0.01 with Benjamini Hochberg correction. In order to
215 perform PCA analysis and classification missing values were imputed by normal distribution
216 (separately for each column/sample). Data shown in heatmap was Z-score normalized.
217 Perseus output tables were transferred into ClustVis [36] for visualization of hierarchical
218 clustering and principle compound analysis (PCA). Gene Ontology and functional analysis
219 was performed via String DB (version 11.0.0) [37] and Panther DB (version 14.1) [38]. For
220 Panther DB analysis background genome was the human genome and the total of identified
221 proteins from all LC-MSMS runs in the experiments (supplementary material 4-8). Feature
222 optimization cross-validation type was “n-fold” with n = 5. Kernel was either linear or RGF. All
223 other settings were left on their default value.

224

225 **Results:**

226 In this study we investigated the utility of mass spectrometry-based methods for histo-
227 molecular profiling applications in clinical renal cancer pathology. We analyzed thin
228 tissue/tumor sections from three different renal cancer types (ccRCC, RO, ChRCC) by MALDI
229 MS imaging and by an optimized rapid LC-MS/MS workflow adjusted to suit the demands for
230 clinical settings.

231

232 *Imaging by MALDI mass spectrometry*

233 All samples were prepared as 5 µm thin FFPE tissue/tumor sections. The entire FFPE tissue
234 section was analyzed by imaging MALDI MS imaging (MSI). The data was subsequently
235 processed by unsupervised clustering (spatial shrunken centroid clustering [32]). The
236 clustering results **(Figure 1A and 1B)** illustrate the heterogeneity of the tissue sections
237 coming from various tissue types such as stroma, fibrotic, fatty or healthy tissue and the
238 capabilities of imaging MSI for the delineation of cancerous and non-cancerous tissue.
239 Furthermore, when comparing the tumor area of the HE-stain/microscopy with the results
240 from the mass spectrometry imaging based clustering, spectral differences even within the
241 tumor tissue itself can be observed (**Figure 1A and 1C**).

242 Guided by the unsupervised clustering outcome and the corresponding image obtained by
243 HE-staining, pixels from non-relevant surrounding tissue were discarded and only pixel
244 clusters containing actual tumor tissue were used for subsequent comparative analyses
245 (schematic workflow overview can be found in supplementary material 1 : Figure S4).

246 In mass spectrometry imaging, principal component analysis (PCA) is often used for initial
247 analysis of a given data set. Variance and similarities within the image sample set were
248 estimated by PCA over the first 3 components. From a pathology viewpoint RO and ChRCC
249 are more difficult to distinguish than ccRCC and ChRCC. As the sample holder for the
250 imaging experiments can only hold 2 slides at a time, we first compared two conditions in a
251 pairwise manner: 9 ccRCC vs. 9 RO **(Figure 2A)** and 5 RO vs. 5 ChRCC **(Figure 2B)**. Then
252 the data set was combined to compare all three cancer conditions (9 ccRCC, 9 RO, 5
253 ChRCC) to each other **(Figure 2C).** PCA using the first three principle components separate
254 ccRCC well from RO and ChRCC (**Figure 2A, 2C).** Data points from ccRCC showed a wide
255 spread and were splitting into 2 sub-populations. In contrast, the data from RO and ChRCC
256 samples cluster in a much tighter manner and with some overlap **(Figure 2A, 2B, 2C)**. This is
257 particularly the case when considering all three cancer types together (**Figure 2C)**. When
258 compared in a pairwise manner RO and ChRCC show slight separation **(Figure 2B)**
259 suggesting at least some degree of histo-molecular differences between these cancer types.
260 Some overlapping data points in the different cancer type datasets can be observed indicating
261 histo-molecular spectral similarity in parts of the patient tumor tissues. The spread of ccRCC
262 data points in PCA, as compared to the RO and ChRCC subtypes, suggests a greater
263 heterogeneity among the ccRCC patient samples (also observed by LC-MS/MS, see later
264 section).

265 Next, we assessed the ability of the MSI data to distinguish and classify renal cancer
266 subtypes. We generated a classifier based on partial least squares discriminant analysis
267 (PLS-DA) that can then be applied to a given MSI sample set. Due to the limited number of

268 FFPE kidney tumor samples we chose to use a cross-validation strategy that maximizes the
269 use of a sample set for model generation and testing. In this approach a classifier is trained
270 with imaging data from all samples, except for one sample that is set aside. As this sample is
271 not part of the classifier model it can then be used for testing purposes. This was repeated as
272 many times as there are samples ultimately allowing for testing the complete dataset (for n
273 samples we obtain n classifiers and n tested samples).

274 The optimized PLS-DA model resulted in an accuracy of cancer subtype prediction of 93% for
275 ccRCC and 88% for RO and for ChRCC (pixel based value). Results of the cross-validation
276 study using PLS-DA to classify 23 kidney tumor samples are depicted in **Figure 3**. The PLS-
277 DA prediction scores for each of the three possible tumor type outcomes are shown, i.e.
278 ccRCC, RO and ChRCC. (Median values and boxplot representation of scores are provided
279 in **supplementary material 1: Figure S5 and table S3**). The scores obtained for each pixel
280 are presented by intensity scaled colors plotted over the respective x-y-coordinate of the
281 tissue/tumor sections.

282 Twenty of the 23 patient tumor samples (assignment based on median value) were correctly
283 assigned by the PLS-DA model showing highest intensity and median score for the respective
284 cancer condition **(Figure 3)**. Eight out of nine ccRCC samples were correctly assigned
285 **(Figure 3a-i).** The PLS-DA classification provided high scores for ccRCC samples and clearly
286 distinguished the ccRCC samples from the other two kidney tumor types (Figure 3, left
287 panels). This is in accordance with the PCA results. Likewise, the PLS-DA model provided
288 low scores for ccRCC in the cases of RO and ChRCC samples (Figure 3, middle and right
289 panel). One ccRCC sample was incorrectly classified by PLS-DA as RO **(Figure 3g**).

290 All 5 ChRCC samples (**Figure 3 j-n**) were correctly assigned having the highest score for the
291 ChRCC condition (right panel). PCA indicated mass spectral similarities between the RO and
292 ChRCC samples. Likewise, the PLS-DA model reflects such similarities in the classification
293 outcome. Two ChRCC patient samples received highest scores for ChRCC but only slightly
294 lower scores for RO (**Figure 3k, 3n**). Furthermore, in the case of two RO sample **(Figure 3a,**
295 **3e)** the classification could exclude ccRCC as diagnosis. Although the highest median score
296 was correctly achieved for RO (Supplementary material 1: **table S3**) the difference between
297 RO and ChRCC was considered as too small (<10% of respective max. median score) for a
298 clear distinction of these two tumor types. Notably one kidney tumor sample (Figure 3l)
299 exhibited a unusual scoring pattern as compared to the other tumor samples. This particular
300 sample received high scores for both ccRCC and ChRCC classification (ChRCC being the
301 highest). As mentioned above, we typically observed clear distinction between ccRCC and
302 ChRCC in all the other cases. Upon further pathology and microproteomics analysis this
303 tumor section was re-classified as a sarcomatoid transformation (see below), i.e. a tumor type
304 not included in the PLS-DA model used for classification.

305 The relative importance of individual histo-molecular features of the classifier can be
306 visualized by plotting the PLS coefficients for each condition as a function of m/z values
307 (**supplementary material 1: Figure S6**). A positive coefficient indicates presence or higher
308 abundance of the m/z value in the respective cancer model. A negative coefficient indicates
309 absence or lower abundance in the respective condition. For ccRCC the two highest-ranking
310 m/z values were m/z=723.5 and m/z=704.5. The two highest values for RO were m/z=806.5
311 and m/z=1640.0 whereas the most influential signals for ChRCC comprised m/z= 1169.5 and

312 m/z=1039.5 (top 100 list of the features can be found in **supplementary material 2).**
313 Unfortunately, we were not able to obtain informative MALDI MS/MS fragment ion spectra in
314 order to reveal the identity of these peptide ion signals. Nevertheless, for classification
315 purposes the knowledge of distinct protein/peptide identities (m/z values) is not necessary as
316 long as the signal is characteristic for the tested condition.

317 In conclusion mass spectrometry imaging provided histo-molecular tumor profiles that can be
318 used to distinguish renal cancer subtypes. However, the misclassification of one ccRCC
319 patient and uncertainty of two additional diagnosis outcomes suggested that additional
320 independent test methods would be beneficial for confident classification of renal cancer
321 tumor types.

322

323 *LC-MS/MS based rapid proteome profiling of tumor sections.*

324 MALDI MS imaging provides spatial resolution that is helpful to address molecular
325 heterogeneity in tissue sections. However, MALDI MS imaging lacks analytical depth due to
326 the limited dynamic range of MALDI TOF MS and the poor performance of MALDI MS/MS for
327 protein identification by peptide sequencing directly from tissue sections. Deeper insight into
328 the tissue and tumor histo-molecular profiles and their variance will provide more diagnostic
329 features. We therefore adapted and optimized a microproteomics approach, combining *in situ*
330 protein sample preparation with fast label-free proteome profiling LC-MS/MS. First a
331 miniaturized *in situ* sample preparation method was applied where a small droplet of trypsin
332 solution is placed directly onto the tumor area of interest within a thin tissue section. After

333 overnight incubation the digested protein extract from the tumor area is subsequently
334 recovered and analyzed by mass spectrometry [39]. We reduced the LC-MS/MS analysis time
335 from 90 minutes to 15 min by using short LC gradients and rapid MS/MS functions, allowing
336 for a sample throughput of up to 80 samples per day. A total of 125 *in situ* extracted areas
337 from renal tumor sections were analyzed. Two to six *in situ* extracts were taken from each
338 renal tumor sample (11 RO sections from 11 patients: 47 extraction spots; 12 ccRCC sections
339 from 12 patients: 49 extraction spots; 7 ChRCC sections from 5 patients: 29 extraction spots).
340 Fast label-free LC-MS/MS based microproteomics analysis of all 125 *in situ* digested tumor
341 areas resulted in a total of 2124 identified human proteins. We filtered the data for proteins
342 that were present in at least 70 % of all samples thereby reducing the protein number to 412
343 proteins. Comparative data analysis was performed for proteins that were significantly altered
344 (FDR=0.01) in any of the renal cancer subtypes resulting in a list of 346 differentially
345 regulated proteins. We then used unsupervised hierarchical clustering and PCA to identify
346 similarities and differences between the tumor samples. The x-axis dendrogram of the
347 heatmap shows that the majority of the renal tumor samples grouped according to cancer
348 subtype RO, ccRCC or ChRCC **(Figure 4A)**. Several large clusters of “co-regulated” proteins
349 are evident on the y-axis dendrogram and heatmap for the individual cancer subtypes. This
350 clearly demonstrates that there are renal cancer subtype specific histo-molecular features and
351 patterns in the microproteomics dataset.

352 The protein expression profiles of the three renal cancer subtypes are different based on the
353 heatmap patterns. ccRCC clearly differs from RO and ChRCC (**Figure 4A**: Protein group 2

354 and 4). RO and ChRCC display some differences but generally exhibit a more similar
355 expression pattern (**Figure 4A**: Protein Group 2).

356 These differences and similarities were also revealed by PCA analysis of the microproteomics
357 dataset. RO and ChRCC separate clearly from ccRCC **(Figure 4B)**. RO and ChRCC
358 datapoints are located close together, indicating that differences between the RO and ChRCC
359 cancer subtypes are less dominant. When considering principal components exhibiting less
360 variance (PC3 and PC4), separation of RO and ChRCC sample data is observed (**Figure**
361 **4B**).

362 We observed eight ChRCC proteomics datasets that separated clearly form the other ChRCC
363 datasets, both in hierarchical clustering analysis (**Figure 4A**) and PCA (**Figure 4B**). The
364 protein expression profile of these 8 samples exhibited some similarities to both ChRCC and
365 ccRCC. Interestingly, this data originated from a tumor from a single patient. This was the
366 same patient that also exhibited outlier MSI data with similarities to both ChRCC and ccRCC
367 tumor types, as discussed above (Figure 3I). Further pathology analysis revealed that these
368 samples were sarcomatoid renal cancer, originating from ChRCC and, thus, indeed different
369 from the other ChRCC samples.

370

371 *Protein differences in cancer subtypes*

372 Hierarchical clustering of the proteomics datasets revealed major differences in relative
373 protein abundance between the three renal cancer tumor types. (**Figure 4A**). We investigated
374 the nature of these histo-molecular differences by examining the correlation of these proteins

375 to cellular structures, functions, or biochemical processes. Protein groups that exhibited
376 distinctive abundances for the respective cancer type (**Figure 4:** ccRCC: group 1 & 4, RO:
377 group 2, ChRCC: group 3) were searched for their involvement in protein interaction networks
378 (supplementary material 9) as well as for their functional roles by using gene ontology (GO)
379 enrichment (**Figure 5**, supplementary material 3-8). We compared GO enrichment relative to
380 the experimental gene background as well as to the complete human genome (Figure 5,
381 human genome background: red). The experimental gene background contained all genes
382 corresponding to all 2124 identified proteins in the LC-MSMS experiments (Figure 5,
383 experimental background: blue).

384 RO and ChRCC exhibited a set of upregulated proteins (**Figure 4A**, *protein group 2)* that
385 were enriched for mitochondria associated proteins (GO:0005739), including various ATP
386 synthase subunits. Enriched protein functions comprised *oxidative phosphorylation*
387 (hsa00190), *citrate cycle* (hsa00020), and *fatty acid beta oxidation* (GO:0006635).

388 ChRCC-specific regulated proteins (**Figure 4A**, *protein group 3*) included cytoplasmic
389 proteins (GO:0044444), and proteins associated with *cytoplasmic vesicles* (GO:0031982) and
390 *ribonucleoprotein complexes* (GO:1990904).

391 Subtype-specific protein groups in ccRCC (**Figure 4A**, *protein group 1, 4*) were functionally
392 enriched for *complement activation* (GO:0006956), *regulation of blood coagulation*
393 (GO:0030193) and *platelet degranulation* (GO:0002576). Functions of *protein group 4* were
394 linked with *extra cellular matrix organization* (GO:0043062) and *cytoskeletal binding*
395 (GO:0008092) including proteins *collagen* and *laminin*. We also found several proteins such

396 as *glyceraldehyde-3-phosphate dehydrogenase* associated with the *glycolytic process*
397 (GO:0006096).

398 These functionally important findings can be correlated to known biochemical and
399 morphological features of each of the renal cancer subtypes. It is known that the number of
400 mitochondria is increased in RO and ChRCC tumors (e.g. increased oxidative
401 phosphorylation) [40]. It is also known that ccRCC contains a highly vascularized stroma
402 (complement, coagulation, etc.) and exhibits a strong Warburg effect (glycolysis) [41]. Large
403 intracellular vesicles are found in ChRCC (cytoplasmic proteins, vesicle proteins) [40].

404

405 *Classification*

406 Unsupervised data analysis demonstrated the presence of renal cancer subtype specific
407 differences in the tumor protein profiles. Next, we investigated the feasibility of tumor
408 classification by using the microproteomics data to train a prediction algorithm. We
409 implemented the tumor classification model by using a support vector machine (SVM)
410 approach. The sarcomatoid sample was excluded from the classification. We chose the k-fold
411 cross validation strategy [42] ("n-fold" in Perseus). Here the data is randomly distributed in k
412 groups. The model was then trained with data from k-1 groups and the prediction was applied
413 to the samples in the remaining group. This was repeated k times. Low k-values tend to
414 overestimate error rates. In our study 2-6 extraction spots (samples) were derived from an
415 FFPE section from each patient so too high k-values could underestimate the true error rate.
416 We therefore tested the prediction error rate over several k-values (**Figure 6 A**) applying

417 Radial Basis Function (RBF) and linear kernel functions [43]. As imputation could have an
418 effect on the classification outcome we compared performance to a classification without
419 imputation using the proteins that were present in all sample (100% valid values=27 proteins).
420 For 70% valid values the tested error rates were in the range 0-3.4% for linear (4 wrong
421 predictions at k=2, linear kernel) and 0-2.6% (3 wrong predictions at k=2, RBF kernel).
422 However, k=2 is a very low k-value (excluding half of the samples from the training set) and
423 the error rate is most likely overestimated in this case. For more commonly used k-values
424 (k=3-10) the error rate was 1.7% (2 incorrect predictions) at the highest for k=3 and 0% for
425 any other k-value. Incorrectly predicted outcomes included samples from one RO patient that
426 was predicted as ccRCC. Classification with the data set using 100% valid values without
427 imputation showed error rates of 0-6% (linear kernel) and 0-3.5% (RBF kernel). Over higher
428 k-values the error rate was 0-3.4% and 0-0.8%, respectively. Error rates were slightly higher
429 than for 70% valid values. Given the low protein number used for the classification the
430 outcome was surprisingly positive. In both cases 70% and 100% valid values we observed
431 RBF performing overall slightly better than linear kernel. As error rates for both valid values
432 were quite comparable, we concluded that in our case the imputation did not heavily bias the
433 outcome.

434 Figure 6B exemplifies the outcome of the cross-validation resulted for RBF and k=5 (around
435 23 samples per group equivalent to 4-5 patients excluded from the training set). Each sample
436 was scored for the three tested conditions (ccRCC, RO, ChRCC). The highest scoring
437 condition was used to classify a given sample. Results are shown in a radar plot (**Figure 6B**)
438 and demonstrate 100% accuracy in prediction of renal cancer subtypes.

439 We initially used all 346 differentially abundant histo-molecular features (proteins) to classify
440 the tumor subtypes. Next, we sought to estimate the minimum number of features that suffice
441 to correctly classify all the renal tumor samples (for k=5 and RBF). We used the feature
442 optimization function in the Perseus software, which first ranks the features and then tests the
443 error rate for a decreasing number of features (**Figure 6C**). The minimal number out of the
444 346 features was found to be 30 features (list of the ranked proteins can be found in
445 supplementary material 9). Further reducing the number to 21 features resulted in an error
446 rate of 0.8% and as little as 4 features lead to an error rate of 7.7%. Conclusively only a
447 portion of the dataset, would suffice to successfully classify all the kidney tumor samples,
448 which reflects also in the low error rates of the 100% valid values (**Figure 6A)** using only 27
449 proteins (**supplementary material 3**). However, keeping an excess of quantified protein
450 features would be beneficial as "safety margin" assuring a high enough number of quantified
451 protein features for robust classification of tumors.

452

453 *Data integration from MSI and rapid proteome profiling*

454 Having both MSI and microproteomics sets of data at hand provides several advantages for
455 classification of cancer tumor FFPE samples. Using the MSI approach for tumor classification
456 we observed a higher error rate than with the rapid micro-proteomics approach. In two cases
457 MSI could exclude one cancer type but was not providing clear results towards an RO or a
458 ChRCC diagnosis. Another case where one ccRCC sample was misclassified as RO is
459 particularly problematic as ccRCC might need surgery whereas RO does not. Therefore, by

460 integrating the micro-proteomics classification data the outcome of the MSI classification can
461 be further confirmed, clarified or rejected (**Table 1**). This allows more confidence in diagnosis
462 or could possibly even provide further information on cancer stage or treatment strategies. In
463 a case where the classification model does not cover the cancer condition such as in the
464 patient sample with sarcomatoid transformation we have demonstrated how irregularities and
465 inconsistencies are detected by both MSI and rapid LC-MS/MS based microproteomics
466 (**Figure 3I, 5A, 5B**). This provides an opportunity to further investigate, refine and expand the
467 range of computational and statistical classification models.

468 An additional application making use of the combined data set includes the investigation of
469 histo-molecular properties observed in MSI (e.g. intra- tumor heterogeneity) by correlation to
470 information from the rapid microproteomics approach. Usually a detailed investigation of MSI
471 feature data is achieved by either microproteomics "in situ protein digestion" or laser
472 microdissection based approaches [44] using LC-MS/MS based proteomics analysis with long
473 LC gradient times (1-4 hours). Despite the shorter gradient times and thus lower protein
474 coverage in the present work the information can nevertheless be used to investigate histo-
475 molecular properties observed in MSI (e.g. intra- tumor heterogeneity). We exemplified this
476 in figure 7, using the RO MSI data set previously shown (**Figure 1,** top). Unsupervised spatial
477 shrunken centroid clustering of MSI data [32] revealed two distinct regions within the tumor
478 area (**Figure 7A**: cluster 1 and cluster 2). Correlating the LC-MSMS data from the respective
479 extraction spots within these distinct regions in deed reveals significant differential
480 abundances in 80 proteins (**Figure 7B**, the list of proteins can be found in **supplementary**
481 **material 11**). Hierarchical clustering (**Figure 7C**) of these 80 proteins with regard to their

482 extraction position correlates well with the distinct regions depicted by the MSI clustering (MSI
483 Cluster 2 correlates with extraction spots e-f, MSI Cluster 2 correlates with extraction spots a-
484 d; **Figure 7A**). The proteomics data suggests a lower abundance of mitochondrial associated
485 proteins and a higher abundance in some cytoskeletal protein binding proteins in cluster 2
486 (**Figure 7D**). The area comprising Cluster 2 located on the edge of the tumor and might
487 indicate the differences that can be encountered between the inner and outer tumor regions
488 [45].

489

490 **Discussion**

491 The increasing incidence of renal cancer in western countries calls for improved technologies
492 for detection, diagnosis, treatment and prognosis. Innovative mass spectrometry-based
493 applications are beginning to address challenges in clinics and the healthcare sector, such as
494 the use of targeted proteomics to characterize noninvasive liquid biopsies [46] or the so called
495 iKnife, enabling surgeons to identify cancerous tissue in real time [47, 48]. Mass spectrometry
496 is becoming increasingly applicable in a clinical setting [49, 50]. FFPE sections are a valuable
497 source for mass spectrometry-based diagnosis. As many of the sample preparation steps for
498 MS analysis overlap with the preparation steps for (immuno)histochemical staining, they can
499 be seamlessly fit into the high-throughput sample preparation pipeline for FFPE sections
500 (deparaffination, antigen retrieval) already existing in many hospitals.

501 Our proof of concept study demonstrates the potential and benefits of mass spectrometry
502 techniques for detailed characterization of clinical specimen. Specifically, we demonstrate

503 that mass spectrometry provides valuable results in the diagnosis of different renal cancer
504 subtypes (ccRCC, RO and ChRCC). The imaging mass spectrometry (MSI) approach allows
505 to collect spatially resolved spectra without *a priori* knowledge of the tissue, thereby enabling
506 the differentiation between cancerous and noncancerous tissue, as well as subtyping of
507 tumors.

508 Earlier large scale MSI classification studies have demonstrated results with accuracies
509 ranging from 81% to nearly 100% in subtyping non-small cell lung cancer [51], classifying
510 primary lung and pancreatic cancer [52] as well as differentiating between 6 common cancer
511 types (esophagus, breast, colon, liver, stomach, thyroid gland) [53]. In our study MALDI-MSI
512 could diagnose 87% (20 out of 23) of the tested patients correctly. It has to be pointed out that
513 when transferring our study into a larger scale (n>100 samples) misclassification rates are
514 expected to increase. In two of the 3 misclassified cases it was possible to narrow down the
515 diagnosis to either RO or ChRCC. Despite the promising results the misclassification of one
516 ccRCC sample as RO might be problematic since RO may not require surgery but ccRCC
517 does. Both cases stress how using rapid proteome profiling data in parallel provided
518 additional confidence and can help avoid a false negative prognosis.

519 Both MSI and LC-MS/MS PCA data showed that the patient-to-patient tumor variability is
520 significant for ccRCC. Possible reasons might be due to necrotic areas or increased bleeding
521 observed in some of the tissues. Furthermore we did not consider difference in grades, which
522 might have an influence on the data spread. For robust MSI performance inclusion of a larger
523 patient cohort (n>100) will likely provide higher confidence and resolve this issue or even
524 provide differentiation of tumor grades.

525 LC-MS/MS based microproteomics analysis correctly classified all tested renal tumor samples
526 in cross validation experiments. The efficient peptide separation and sequencing capability of
527 LC-MS/MS provided deeper insight into the renal cancer proteome than possible by the MSI
528 approach alone. Remarkably, unsupervised clustering identified data inconsistencies and
529 irregularities in the patient cohort. An unexpected feature pattern revealed a sarcomatoid
530 transformation within the ChRCC cohort, without *a priori* knowledge (**Figure 4A, 4B**). This
531 goes to demonstrate that once the "digital" data is acquired then the computational and
532 statistical applications can uncover relevant and important features of the patient datasets.
533 This sensitivity, specificity and versatility will have major implications for future clinical
534 practices, including histo-molecular pathology technologies.

535 Using short LC runs of only 15 min. we generated a list of 346 significantly altered proteins
536 (p=0.01). The minimum number of proteins determined to be necessary for 100% accurate
537 tumor classification was much lower (30 features). This low number of features enables a
538 targeted proteomics approach aimed at quantifying only a select panel of proteins. Using
539 fewer features would also allow further reduction of LC run time and increase overall sample
540 throughput. Using our fast LC-MS/MS setup we analyzed a total of 125 samples in a series
541 without experiencing blocking of the LC columns, glass capillaries or ESI needles. LC
542 systems such as the EvoSep system [25] that are specifically dedicated for clinical
543 applications and tailored to be used also by non LC-MS experts can add additional
544 robustness to this approach. Furthermore, implementation of image pattern guided pipetting
545 robots may enhance reproducibility and throughput, e.g. using liquid extraction surface
546 analysis (LESA) technology [54, 55]. The latter has been successfully applied in the study of

547 traumatic brain injuries [56] as well as in mouse brain for the identification of proteins and
548 peptides from MSI experiments [57]. The missing value problem is still a common problem in
549 label free quantitative proteomics. Successful implementation of protein identification on MS1
550 level only has been presented recently [58] and could be interesting in the here presented
551 context to follow up in future experiments.

552 Functional protein analysis using bioinformatics tools revealed molecular networks and
553 biochemical processes consistent with previously known macroscopic, morphological and
554 histological features of the renal cancer subtypes. RO and ChRCC exhibited upregulation of
555 mitochondrial associated proteins. Increased numbers of mitochondria are frequently
556 observed in these cancer types by electron microscopy [59] and have been identified in
557 previous proteomics studies [60]. As most cancer rely on glycolysis (Warburg effect) this
558 seems rather unusual. However, those mitochondria are dysfunctional and it has been
559 speculated that the increase in number might be a cellular compensation response [61].

560 In addition increased intracytoplasmic associated proteins were detected in ChRCC
561 distinguishing it from the other cancer types. Microscopically, ChRCC distinguishes from other
562 renal carcinomas by its pale cytoplasm resulting from large intracytoplasmic vesicles
563 explaining the relative increase of intracellular cytoplasm-associated proteins and vesicle
564 proteins.

565 Clear cell renal cell carcinoma frequently contains zones of hemorrhage that are most likely
566 responsible for the increased levels of complement and coagulation cascade associated
567 proteins, as determined by our microproteomics method. ccRCC is also characterized by

568 hypervascular stroma [3], which may account for the enrichment of extracellular matrix
569 proteins. Enhanced glycolysis as a hallmark of many cancer types including ccRCC [41]
570 correlating well with our detection of upregulated glycolysis associated proteins.

571 For classification we applied PLS-DA to MSI data and support vector machine to the LC-
572 MSMS data. These common classification methods have previously been applied to MSI for
573 the differentiation of papillary and renal cell carcinoma based on lipidomics analysis [62] as
574 well as for the classification of epithelial ovarian cancer subtypes [16]. There are, however,
575 numerous other classification methods available. Mascini *et al.* used principal component
576 linear discriminant analysis in order to predict treatment response in xenograft models of
577 triple-negative breast cancer [63]. Recently, deep convolutional networks have been
578 proposed [64].
579 Both MSI and short gradient LC-MS/MS microproteomics methods come with their individual
580 advantages. Applying both approaches in parallel for routine analysis is most beneficial to
581 improve confidence in diagnosis and identify irregularities. In order to create very robust
582 classifiers for use in clinical settings the promising results of this study need to be further
583 supported in the future by analysis of larger patient cohorts.

584 With the enormous progress in sample handling and instrument technology, machine learning
585 [65] and the availability of new databases [66] mass spectrometry is on its way to become a
586 versatile tool in the hospital clinics of the future.

587

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594 **Authors Contributions:**

595 U.M., O.N.J. and N.M. planned and outlined the project. N.M. provided the patient samples
596 and patient diagnosis. U.M. performed all experiments and data analysis. U.M. and O.N.J.
597 wrote the manuscript.

598 **Competing Interests:**

599 The Authors declare no competing interest

600

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793

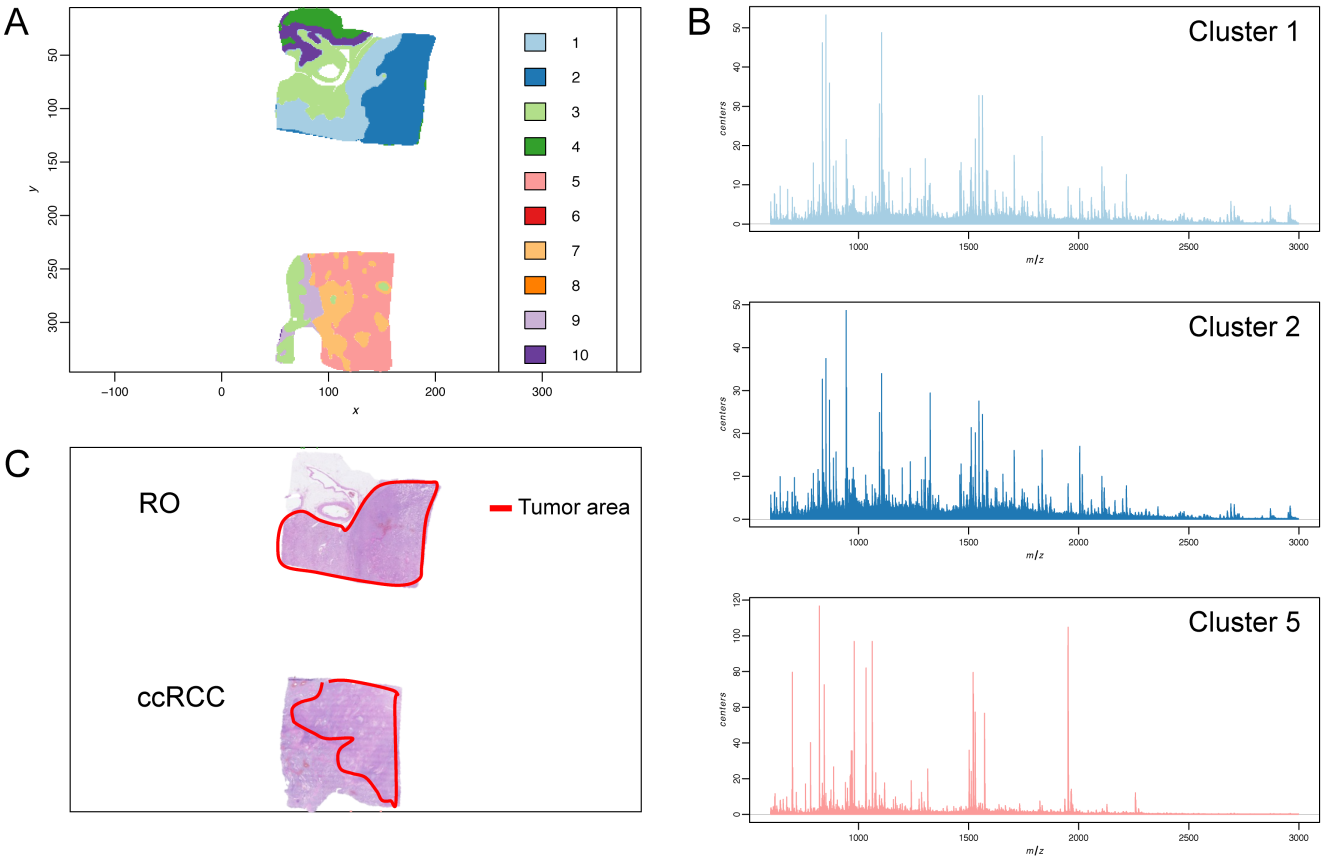
794

795 **Table 1**

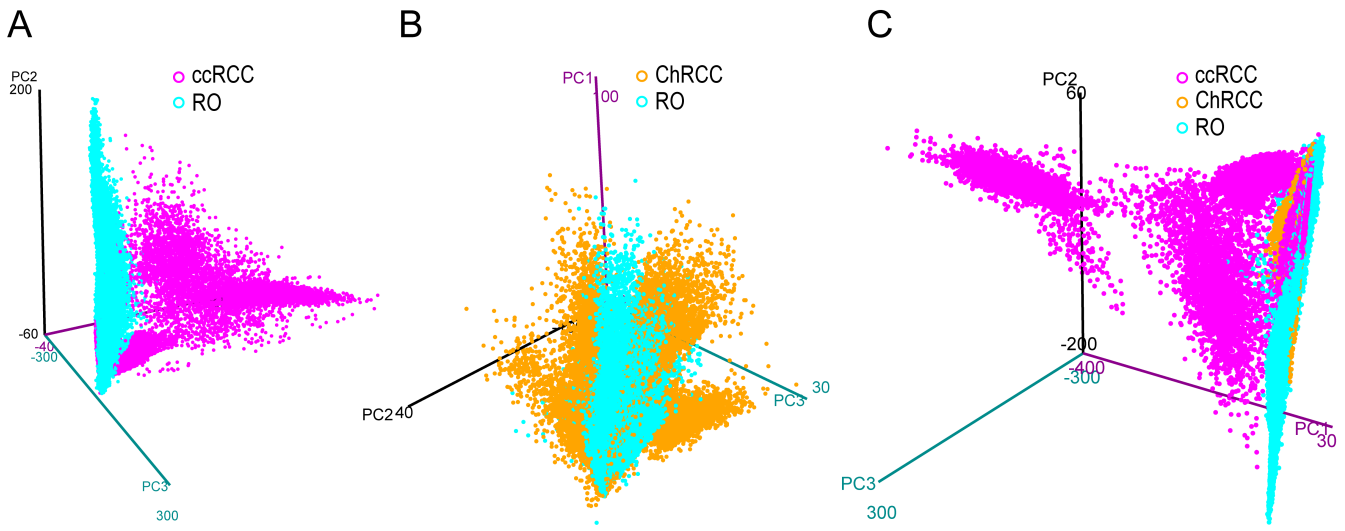
Pathologist diagnosis	Patient	MSI diagnosis	Rapid LC-MSMS diagnosis	Conclusion
RO	839	RO/ChRCC	RO	RO
RO	119	RO	RO	RO
RO	527	RO	RO	RO
RO	270	RO	RO	RO
RO	529	RO/ChRCC	RO	RO
RO	560	RO	RO	RO
RO	940	RO	RO	RO
RO	857	RO	RO	RO
RO	381	RO	RO	RO
ccRCC	427	ccRCC	ccRCC	ccRCC
ccRCC	370	ccRCC	ccRCC	ccRCC
ccRCC	620	ccRCC	ccRCC	ccRCC
ccRCC	73	ccRCC	ccRCC	ccRCC
ccRCC	545	ccRCC	ccRCC	ccRCC
ccRCC	999	ccRCC	ccRCC	ccRCC
ccRCC	797	RO	ccRCC	ccRCC/further validation
ccRCC	601	ccRCC	ccRCC	ccRCC
ccRCC	336	ccRCC	ccRCC	ccRCC
ChRCC	634	ChRCC	ChRCC	ChRCC
ChRCC	835	ChRCC	ChRCC	ChRCC
ChRCC	264*	ChRCC with irregularities	ChRCC with irregularities	further validation -> sarcomatoid transformation
ChRCC	756	ChRCC	ChRCC	ChRCC
ChRCC	925	ChRCC	ChRCC	ChRCC

796

Figure 1


A
B
C
Cluster 1
Cluster 2
Cluster 5
RO
ccRCC
Tumor area
1
2
3
4
5
6
7
8
9
10
x
y
m/z
centers

800 **Figure 2**

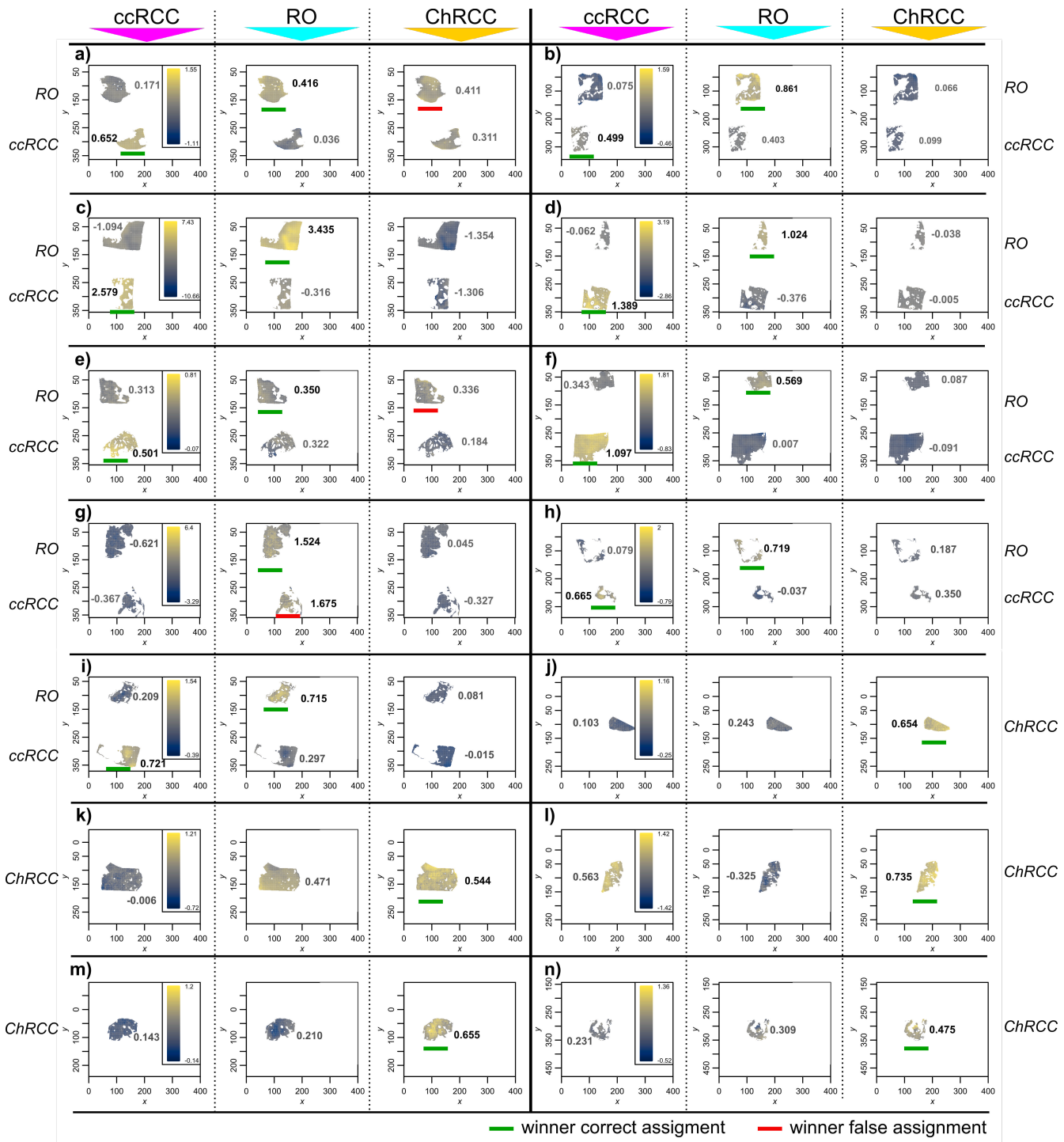


801

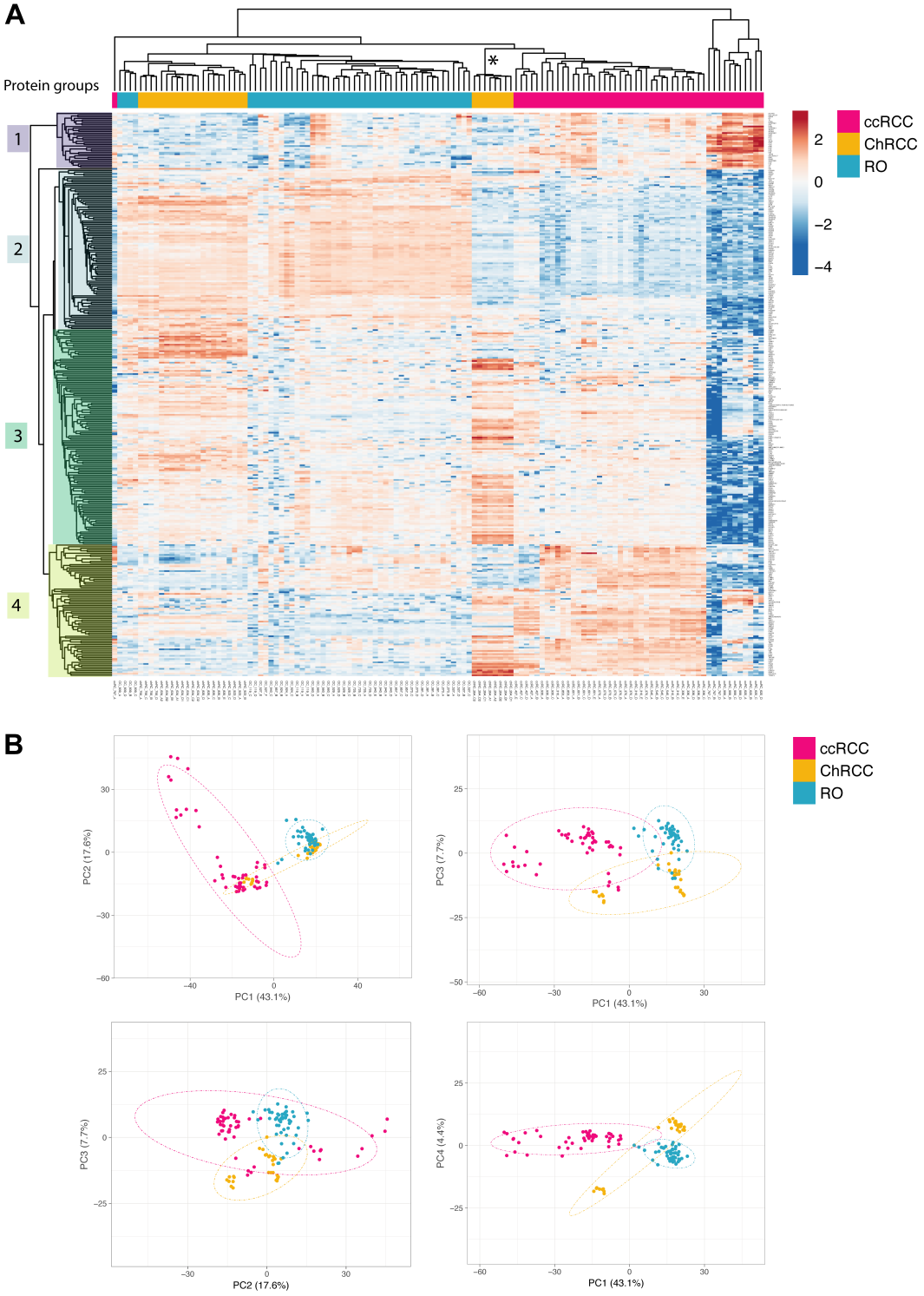
802

803

Figure 3

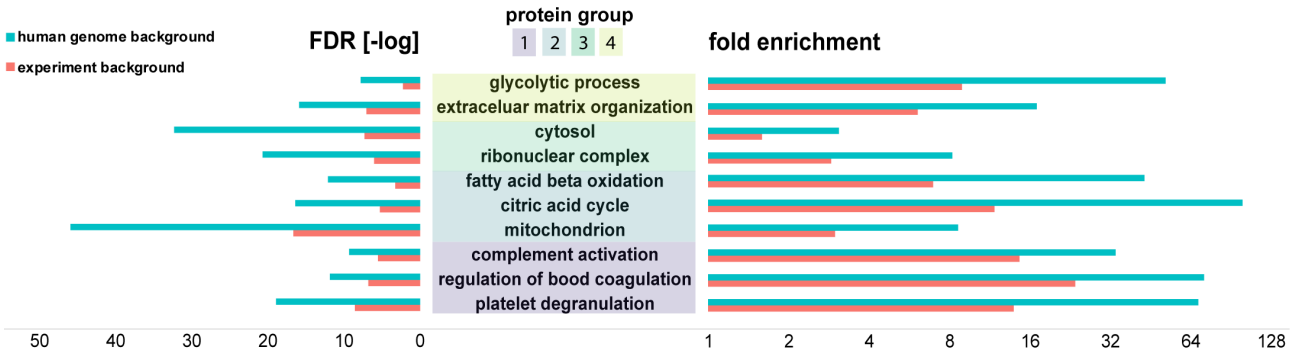

ccRCC
RO
ChRCC
ccRCC
RO
ChRCC
a)
b)
c)
d)
e)
f)
g)
h)
i)
j)
k)
l)
m)
n)
0.171
0.652
0.416
0.036
0.411
0.311
0.075
0.499
0.861
0.403
0.066
0.099
-1.094
2.579
3.435
-0.316
-1.354
-1.306
-0.062
1.389
1.024
-0.376
-0.038
-0.005
0.313
0.501
0.350
0.322
0.336
0.184
0.343
1.097
0.569
0.007
0.087
-0.091
-0.621
-0.367
1.524
1.675
0.045
-0.327
0.079
0.665
0.719
-0.037
0.187
0.350
0.209
0.721
0.715
0.297
0.081
-0.015
0.103
0.243
0.654
-0.006
0.471
0.544
0.563
-0.325
0.735
0.143
0.210
0.655
0.231
0.309
0.475
winner correct assigment
winner false assignment

807 **Figure 4**



808

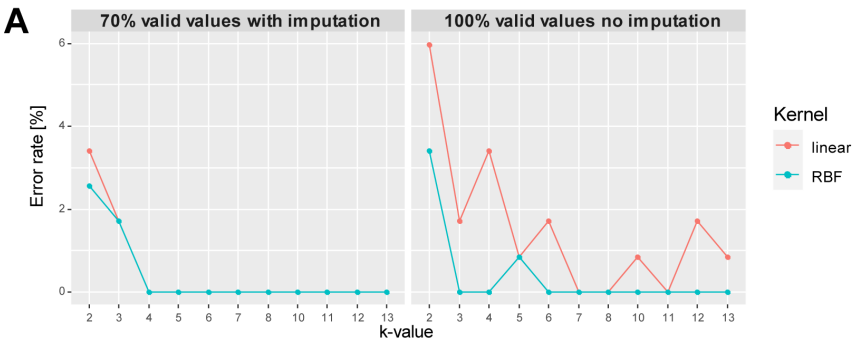
809 **Figure 5**

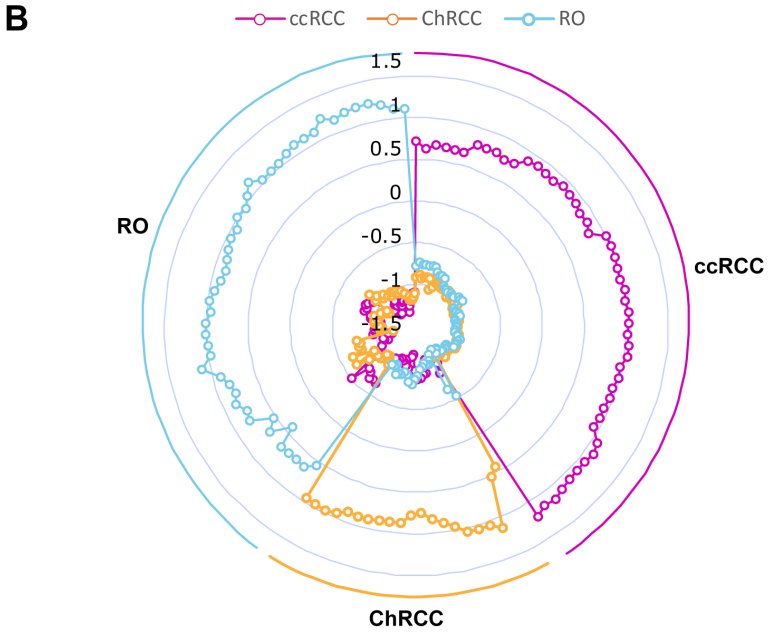


810

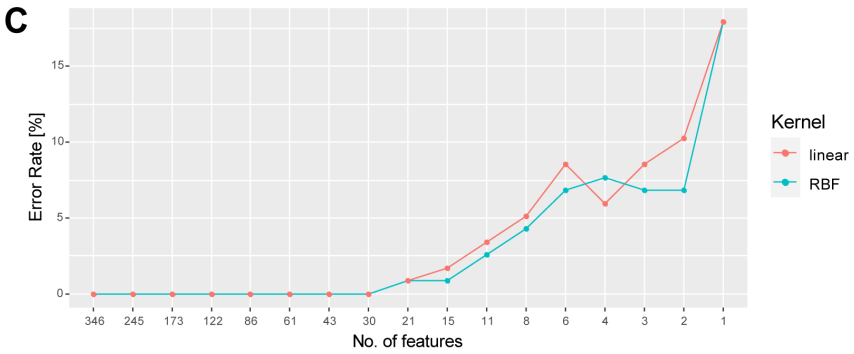
811

Figure 6

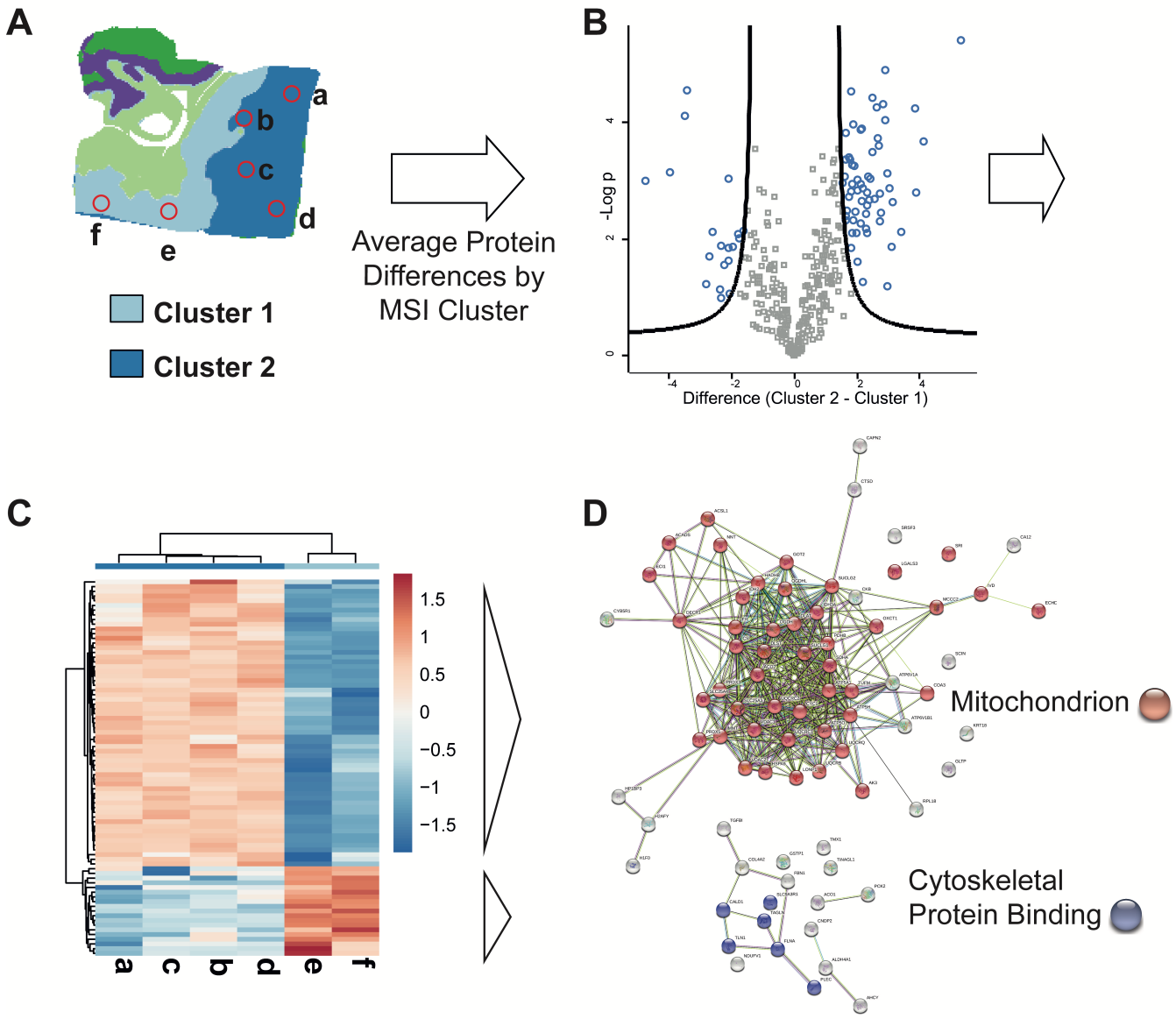




Predicted: ccRCC	Predicted: ChRCC	Predicted: RO	T: True group
49	0	0	True: ccRCC
0	21	0	True: ChRCC
0	0	47	True: RO



814 **Figure 7**



815

816

817 **Table 1**: integrated testing strategy for classification of renal cancer types. Initial pathologist
818 diagnosis and patient number are indicated in the first 2 columns. *Patient sample showed
819 irregularities and after reassessment could be diagnosed as sarcomatoid transformation.
820 Concluding contradictory results would either necessitate further validation or the outcome of
821 the more reliable method (LC-MSMS) could be favored

822

823 **Figure 1** Tumor sample heterogeneity is revealed by mass spectrometry imaging and
824 unsupervised clustering. A) Spatial Shrunken centroid clustering of ccRCC and RO data
825 obtained by imaging mass spectrometry of ccRCC and RO tissue sections. Based on
826 differences and similarities in the spectra each pixel was automatically assigned a certain
827 cluster (indicated by a different color). B) Average MALDI mass spectra of the respective
828 tumor areas (histo-molecular clusters) reveal distinct features and individual variations in the
829 m/z signals. C) HE-stain of tumor tissue section from same FFPE block. Tumor area is
830 indicated in red.

831

832 **Figure 2:** 3D PCA score plot from imaging MALDI MS experiments of kidney tumor tissues.
833 Each plot contains the extracted pixel data from all patients of a given cancer type. Data from
834 ccRCC (A) (magenta) and ChRCC (B) (blue) are compared to RO (yellow). (C) Data from all
835 three cancer types are compared to each other. The graph displays the first 3 principle
836 components (PC1, PC2, PC3) plotted against each other. Clear separation of data points
837 between ccRCC and RO can be observed by pairwise comparison but also in the combined

838 comparison to both RO and ChRCC. In pairwise comparison (B) RO and ChRCC show slight
839 separation but exhibit a great number of overlapping features. ccRCC exhibits the largest
840 differences to RO and ChRCC. RO and ChRCC appear to share more spectral similarities.

841

842 **Figure 3:** Tumor classification by MALDI MS imaging and cross-validation using PLS-DA
843 classification. Unit of x- and y-axis equals step size (150µm). Classification was performed on
844 extracted pixels/spectra from tumor areas only. Classification of 9 ccRCC and 9 RO (a-i)
845 sample as well as 5 RO sample and 5 ChRCC sample (j-n). Pathology diagnosis of the
846 respective patient samples are indicated to the left and right of the images (*RO, ccRCC* or
847 *ChRCC*). Each spectrum-containing pixel is predicted individually. The prediction scores are
848 represented by a color scale. Each patient sample was scored for the 3 cancer conditions
849 resulting in 3 panels for each condition. Each of the panels displays scores for ccRCC (first
850 panel) RO (second panel) and ChRCC (third panel). The respective testing condition is
851 indicated on top above the panels. Median score for the respective condition is indicated in
852 each panel next to the tissue. Classification is based on the condition achieving the highest
853 score within the 3 predictions. Differences below 10% of the respective highest score were
854 considered too close to be distinguishable. A overview table with median values as well as a
855 boxplot representation of scores is provided in supplementary material 1 Figure S5 and table
856 S3. (Cardinal´s smooth.image-function was used for better visibility. Unprocessed image can
857 be found in supplementary material: 1 Figure S7)

858 Each sample is predominantly predicted in the correct diagnosis, achieving accuracies (pixel-
859 based value) of 93% (ccRCC), 88% (RO), 88% (ChRCC). Winner of the classification is
860 marked with a green bar for correct classification and a red bar for incorrect classification.

861

862 **Figure 4:** Unsupervised renal cancer subtype classification by microproteomics using rapid
863 LC-MS/MS protein profiling. A) Heatmap and hierarchical clustering of differential relative
864 protein abundances. Columns indicate samples and rows indicate proteins. The renal cancer
865 subtype of the patient sample is indicated in colored bars on top. The graph shows the large
866 similarities in protein expression profiles among patient samples with the same cancer
867 subtype causing them to cluster together. Furthermore, hierarchical clustering of the protein
868 abundances reveals protein cluster that are detected in a cancer subtype specific manner.
869 Protein groups selected for subsequent network analysis are indicated by color blocks on the
870 y-axis dendrogram (groups 1-4).The asterisk * marks outlier patient from sample figure 3l.

871 B) Principal component analysis of the sample set. Dotted ellipses are such that with a
872 probability of 95% a new observation from the same group will fall inside the area. The first
873 (PC1) and second (PC2) component explain 17.6 % of the total variance whereas the other
874 components lie at 7.7% and 4.4% respectively. There is a clear separation of ccRCC and RO
875 samples already in the first two principal components. Differences between RO and ChRCC
876 are subtle and are only evident when considering components that display lower variance
877 (PC2:PC3 and PC3:PC4). The small group of the eight ChRCC-derived sarcomatoid renal

878 cancers samples cluster relatively far from the other ChRCC samples, thereby identifying
879 these as clear “outliers” that require further attention.

880 **Figure 5:** Bioinformatics analysis (PantherDB) identified enriched biochemical functions in
881 renal tumors. Protein groups were compared against a background of all the 2124 proteins
882 identified in the experiment (blue) and against the background of the human genome (red).
883 Fold enrichment (increase over expected value) as well as -log of the false discovery rate
884 (FDR) are shown.

885

886 **Figure 6:** Microproteomics and SVM model correctly classifies all renal tumor subtypes. A)
887 Development of cross validation classification error rate in relation to increasing k-value
888 (division of sample set in k groups. k-1 groups will be used for training and 1 group for
889 testing). Results of 70%valid values using imputation of missing values (right) as well as
890 100%valid values without imputation (left) are shown. Two classes of algorithms were
891 compared: RBF and linear kernel. RBF performs slightly better than the linear kernel function
892 with lower error rates. Values chosen for k>=3, error rates vary between 0% and 1.6% (2
893 wrong prediction out of 117) for both tested kernel.

894 B) Radar plot of the cross-validated classification (k=5, kernel=RBF) of proteome profiles
895 obtained from each extraction spot sample. Each sample is plotted equi-angular around the
896 center. The pathological diagnosis (ground truth) for each sample at its angular position is
897 indicated on the outside of the radar plot (all ccRCC samples: right, all ChRCC
898 samples:bottom, all RO samples:right). A given sample is represented by 3 datapoints (dots)

899 plotted on a straight axis originating from the center. Each of the 3 datapoints represents the
900 classification score for one of the 3 cancer types (scores for ccRCC: magenta, ChRCC:
901 yellow, RO: blue). Scores range from lowest (center) to highest (outer circle).The highest
902 score indicates highest likelihood for the respective cancertype. The plot shows that for all
903 samples the cancer type with the highest score correlates with the respective pathological
904 diagnosis, indicating the high accuracy of the classification. C) Feature optimization. The error
905 rate for linear kernel and RBF are plotted over the number of ranked features (proteins).
906 Decreasing feature number results in increase of false predictions. Minimum number of
907 features for 0% error rate is at 30 for both RBF and linear kernel (list of ranked proteins can
908 be found in supplementary material 10).

909

910 **Figure 7:** Combined use of MS imaging and rapid LC-MSMS microproteomics provides histo-
911 molecular details of tumor heterogeneity. A) MSI based unsupervised clustering analysis of a
912 RO patient sample. Two clusters (cluster 1 and cluster 2) are detected within the tumor area.
913 Positions used for extraction of LC-MSMS samples are indicated by red circles extractions a-f
914 (2 extractions for cluster 1, 4 extractions in cluster 2).

915 B) Volcano plot of LC-MSMS data derived ratio of protein abundance (Cluster 2 / Cluster1).
916 The –log p values of protein abundances are plotted over the difference of the protein
917 abundance. The black line depicts the chosen significance threshold (p=0.01, 2-fold
918 difference). Proteins above the thresholds are colored in blue and mark significant differences
919 in protein abundance among the 2 compared regions. Proteins with increased abundance are

920 found on the right side of the plot, proteins with decreased abundance are found on the left
921 side of the plot.

922 C) Heatmap display of the significantly different proteins from extraction spots a-f. On the x-
923 axis extraction spots a-d and e-f group together by hierarchical clustering. The grouping is in
924 correlation to the MSI clustering data. On the y axis two protein groups can be observed
925 distinguishing the two x-axis-cluster. One group is upregulated in Cluster 1 the other group is
926 upregulated in Cluster 2.

927 D) StringDB network analysis of upregulated proteins in MSI-Cluster-2 (top, sample: a-d) and
928 upregulated proteins in MSI-Cluster 1 (bottom, sample: e-f). For a given set of proteins the
929 string data base (www.string-db.org) provides information on a dataset in terms of protein
930 attributes such as interaction, function or cellular occurrence. Proteins with higher abundance
931 in Cluster 2 are mainly mitochondrial associated proteins whereas cluster 1 shows increase
932 abundance of cytoskeletal protein binding proteins.

933