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Revised

Intact cell MALDI-TOF Mass Spectrometry, a promising proteomic profiling method
in farm animal clinical and reproduction research

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Abstract

The objective of this review is to provide new insights into the possible use of a proteomic method known as Intact Cell Matrix-Assisted Laser Desorption-ionization Time-Of-Flight Mass Spectrometry (ICM-MS) in animal clinical research. Here, we give an overview of the basics of this technique, its advantages and disadvantages compared with other proteomic approaches, past applications and future perspectives. A special emphasis on its implementation in animal reproduction science is given, including examples of the reliable use of ICM-MS on fertility screening. In mammals, the ICM-MS profiles from pig epididymal spermatozoa reflect the proteome changes that they undergo during epididymal maturation and could be associated with the acquisition of fertilizing ability. In chicken, using adequate pre-processing and bioinformatics analysis tools, sperm ICM-MS profiles showed characteristic spectral features that allowed their classification according to their actual

31 fertilizing ability. The association of ICM-MS and Top-down proteomic strategies allowed the
32 identification of chicken fertility biomarkers candidates such as protein vitelline membrane
33 outer layer protein 1 (MVO-1) and avian beta-defensin 10 (AVB10). In female reproduction, a
34 similar approach on ovarian follicular cells allowed the identification of specific markers of
35 oocyte maturation in the oocyte and surrounding cumulus cells. Altogether, these results
36 indicate that ICM-MS profiling could be a suitable approach for molecular phenotyping of
37 male and female gametes.

38

39 **1. Clinical proteomics in animal sciences**

40 Clinical proteomics is a field of proteomics whose objective is the identification of
41 biomolecules showing differential abundance in a certain body fluid, cell and/or tissue associated
42 with a defined clinical condition. These molecules, usually denominated “biomarkers”, can be
43 thereafter used in clinical applications for diagnosis, stratification of patients for specific treatment,
44 or therapy monitoring. A combination of different proteomic biomarkers or “biomarker profile” can
45 also be employed, giving a combined measure associated with the studied condition [1,2]. Biomarker
46 profiles are potentially better at defining a complex patho-physiological condition than single
47 biomarkers, and clinical proteomics is moving its focus to the identification of such fingerprints
48 panels that can define a diagnostic phenotype [1]. In animal sciences, clinical proteomics has not
49 been as widely used as in human medicine, but there is growing interest in identifying and
50 implementing the use of disease biomarker profiles [3]. However, applications of veterinary clinical
51 proteomics go beyond, and also include food security, quality assessment of animal products (meat,
52 milk, eggs, wool), generation of model organisms and phenotyping of production traits [4,5]. One
53 good example of the latter is the application of clinical proteomics in animal reproduction science,
54 and more specifically in searching for gamete quality biomarkers. Optimization of reproduction
55 management is actually essential in farming since keeping high productivity often relies on adequate
56 selection of breeders as well as the use of optimal gamete handling protocols (sperm

57 cryopreservation, oocyte in vitro maturation, in vitro fertilization, etc) [6]. Actually, one of the main
58 problems in animal breeding is that the selection for production traits, such as fast growth, egg-
59 laying capacity or milk productivity, usually has a detrimental effect in reproductive traits [7].
60 Breeders are therefore interested in the accurate phenotyping of the fertility of their animals to
61 maintain high productivity [7].

62

63 **2. Technical approaches employed in clinical proteomics: the emergence of protein profiling** 64 **platforms**

65 Traditionally, biomarker research using proteomics starts with an initial discovery step,
66 whose objective is untargeted screening of the whole proteome of a certain biological system in
67 defined conditions, in search of biomarkers [8]. The initial discovery approach is usually based on the
68 orthogonal separation of whole proteins contained in the sample either using gel electrophoresis or
69 liquid chromatography followed by a “bottom-up” protein analysis (Figure 1). In this strategy,
70 isolated proteins or protein mixtures are digested with a protease to obtain peptides. These
71 fragments of proteins can be analyzed directly by mass spectrometry (MS) and identified by a
72 peptide mass fingerprint (PMF) strategy [9]; and/or using tandem mass spectrometry (MS/MS) using
73 a peptide fragment fingerprint (PFF) in order to obtain sequence peptides [10]. However, this
74 method is cumbersome and complicated to be implemented in a clinical setting for routine
75 diagnostics, since it involves complex sample preparation procedures, analytic processing of technical
76 complexity and limited throughput. This workflow typically results in a long list of candidates
77 (peptides/proteins showing differential relative abundance) to be integrated in order to construct a
78 biomarker profile. These need to be verified and validated following a second analysis, this time
79 using large cohorts, and high-throughput targeted analysis through the application of multiplexed
80 assays [11]. One of the most recent and efficient techniques developed for the latter is known as
81 MRM (Multiple Reaction Monitoring), which shows several advantages such as being accurate, fast
82 and very sensitive [12]. However, the applicability of this technology in clinical settings remains to be

83 seen, since the development of such tests depends on the use of pre-existing quality MS data, is
84 expensive, as well as the specific detection and/or quantification of some of the desired targets can
85 be challenging [13].

86 Behind the tremendous progress and possibilities in mass spectrometry, “Bottom-up” proteomics has
87 been and is still the driving force in proteome analysis. However, the proper assignment of
88 proteoforms with their pattern of modifications remains a challenge for bottom-up proteomics. The
89 real goals in proteomic analyses are knowing the functions of the intact proteins in biological
90 processes with or without their different modifications, their locations and interactions. Many of
91 these aspects may get lost when breaking down proteins to their corresponding peptide fragments.
92 Beside this conventional strategy, the top-down mass spectrometry constitutes an alternative
93 (Figure1) [14, 15]. In this approach, intact proteins are analysed by MS and MS/MS in order to obtain
94 structural informations allowing both the identification of the proteins and the characterization of
95 the post-translational modifications. To perform Top-down proteomics, protein fractionation
96 combined to high-resolution mass spectrometry and specific bioinformatics tools are necessary.
97 Despite the technical difficulty of proteome-wide analysis at the intact protein level, Top-down
98 proteomics offers an alternative to digestion-based approaches. For several years, there is growing
99 interest in developing untargeted methodologies based on the use of peptide/protein profiling
100 platforms, of which the most important are based on the use of Surface Enhanced Laser Desorption
101 Ionization Time-Of-Flight Mass Spectrometry (SELDI-TOF MS), Capillary Electrophoresis Mass
102 Spectrometry (CE-MS, [16]) or Matrix-Assisted Laser Desorption/Ionization Time Of Flight Mass
103 Spectrometry (MALDI-TOF MS). One of the first approaches to peptide/protein profiling is SELDI-TOF
104 MS, which employs active coatings/surfaces with affinity for specific biomolecules. Liquid samples
105 (biological fluids or tissue extracts) are incubated with these surfaces, thus reducing sample
106 complexity and allowing the acquisition of readable peptide/protein profiles by applying MS analysis
107 directly to the surface-bound molecules. These profiles are later compared between conditions, in
108 search for diagnostic patterns [16]. Several studies have explored the possible application of SELDI-

109 TOF profiling in veterinary sciences in the past, e.g for the detection of porcine diseases [17] or
110 bovine paratuberculosis [18]. However, it is nowadays generally accepted that its low reproducibility
111 (inter-laboratory CV% values above 40%) and lack of analytical robustness (difficulties with
112 calibration and mass accuracy) impair its routine use in the clinic [13]. Capillary electrophoresis mass
113 spectrometry (CE-MS) appeared later as an alternative to SELDI-TOF MS profiling using biofluids. This
114 technique employs biomolecule separation through capillary electrophoresis as a method to reduce
115 sample complexity. Separated molecules are then directly analyzed by MS to obtain a profile [19].
116 While CE-MS shows improved reproducibility and sensitivity compared to SELDI-TOF MS, it requires
117 laborious sample preparation and shows low throughput, which are negative aspects in clinical
118 proteomics [20]. CE-MS has been successfully applied in animal sciences [21,22], although it is
119 recognized that its application to clinical proteomics is hampered by its low throughput, the need to
120 standardize and simplify the sample preparation, as well as the development of accurate methods
121 for calibration and data extraction/processing .

122 Along with CE-MS, Matrix-assisted laser desorption/ionization time of flight mass
123 spectrometry (MALDI-TOF MS) profiling is an emerging approach in clinical proteomics. This
124 technique has theoretically a higher potential for clinical application than CE-MS due to its high
125 throughput, ease of operation, simple sample preparation and the existence of established data-
126 processing platforms . MALDI-TOF MS has the ability to detect the most intense intact molecular ions
127 present directly in the biological sample over a relatively wide mass range (typically 1,000 – 30,000
128 Da), with a high sensitivity (from the picomolar to the femtomolar concentrations) and a high
129 tolerance for contaminants such as salts [23]. Consequently, MALDI-MS constitutes a powerful tool
130 for the direct analysis of complex peptide/protein mixtures within biological specimens such as crude
131 extracts and intact cells. It is in fact, the possibility of using whole, untreated cells what makes this
132 technique particularly promising for clinical applications. In spite of this, this platform has been rarely
133 used in medicine and even less in animal sciences. In this review, we will focus on the technical
134 aspects of the platform MALDI-TOF MS using intact cells (known as Intact cell MALDI-TO MS or ICM-

135 MS), as well as the past applications and possibilities of this technique especially in animal sciences.
136 Particularly, we will provide with a more in-deep overview on the past use of this platform in animal
137 gametes.

138

139 **3. Intact cell MALDI-TOF MS: the basics**

140 In the following paragraphs we will describe the typical ICM-MS protocol workflow (Figure 2).

141

142 *3.1. Sample preparation*

143 An important application of ICM-MS is to detect endogenous peptides and proteins (intact
144 forms unmodified by any chemical treatments) directly in whole cells using MALDI-TOF profiling
145 without previous sample pre-fractionation, protein extraction or treatment. In this context, “intact
146 cell” means that whole cells are subjected to analysis without any preparatory steps, although they
147 can be cryopreserved or kept frozen prior to analysis. Direct analysis of cells by MALDI-TOF MS thus
148 avoids the problems associated with extraction procedures (lost or degraded biological material),
149 avoids the variability introduced by sample preparation and allows significant increase of a number
150 of conditions to compare (up to several hundred). Samples susceptible of being analyzed through
151 ICM-MS must consist of homogenous cellular populations, thus derived from cell culture or being
152 isolated from a cell mix. Pre-treatment of such cells only requires the removal of the biological matrix
153 where cells are contained, such as biological fluids or culture media. Usually, cells are harvested,
154 rinsed and centrifuged, discarding after the supernatant containing biological matrix proteins. Wash
155 buffers must keep cell integrity, promote crystallization and ionization, and ideally chelate salts.
156 Good examples of the latter are Tris HCl-sucrose buffer (osmolarity at ≈ 300 mOsm/L) [24,25] or
157 ammonium acetate [26,27]. It is important to avoid buffers containing phosphate-buffered saline
158 (PBS), since the salts and the negative charge provided by PBS can interfere with the analysis.

159

160 *3.2. Matrix deposition*

161 Although sample deposition on the MALDI plate is a simple process, the protocol followed at
162 this stage can have a significant effect on the outcome of the analysis [28], and so this has to be
163 optimized for each sample type. A known amount of cells (usually 2×10^3 to 1×10^6 cells) is deposited
164 on the target of a conductive MALDI metallic plate and mixed with an adequate matrix solution. The
165 matrix consists of crystallized molecules, the three most commonly used being 3,5-dimethoxy-4-
166 hydroxycinnamic acid (sinapinic acid) [24-27,29-33], α -cyano-4-hydroxycinnamic acid (CHCA, alpha-
167 cyano or alpha-matrix) [34,35] and 2,5-dihydroxybenzoic acid (DHB) [36]. A solution of one of these
168 molecules is made, often in a mixture of highly purified water and UV-absorbing weak organic acid
169 such as acetonitrile (ACN), which is present in a concentration ranging 40-60%. A counter ion source
170 such as Trifluoroacetic acid (TFA) is usually added to generate the ions, in a concentration ranging
171 0.1-3%. The ratio of analyte to matrix is important, and must be optimized for each sample type.
172 There are multiple ways of performing the mix of samples and matrix. Among these methods, the
173 'dried droplet' method is the most usually employed [24-26, 29, 32-34,36]. It consists of depositing
174 the sample and subsequently the matrix on the plate and then allowing co-crystallization. However,
175 the thin layer [30-31] (a saturated matrix layer + mix of sample/matrix) or the sandwich [27] (a
176 saturated matrix layer + dried droplet) methods can provide better results on a qualitative level. The
177 resulting mixture is then allowed to completely dry at room temperature.

178

179 *3.3. Spectra acquisition*

180 Once the MALDI sample plate is prepared and placed within the mass spectrometer, a laser
181 pulse hits the cells-matrix mixture. The matrix absorbs the laser energy, causing its own desorption
182 and protonation, and subsequent sample protonation and vaporization. The generated ions are then
183 accelerated by an applied high voltage (15-25 kV), separated in a linear field-free tunnel and
184 detected as an electrical signal at the end of the TOF analyzer. Peptides and proteins of different
185 masses and charges (mostly under the single-charged ions form) then fly at different speeds through
186 the TOF analyzer. Their arrival times at the detector are distributed according to the square root of

187 the mass-to-charge ratio ($\sqrt{m/z}$). The result is a spectrum in the range of 1,000-30,000 m/z, whose
188 components (peaks) show a degree of intensity that correlates with their abundance in the original
189 sample. Each spectrum is produced by averaging several hundred to thousand laser shots that are
190 spread over the entire sample spot surface. Several replicate spectra (a minimum of 6, up to 12) are
191 taken for each biological sample under the same experimental conditions and a minimum of three
192 reads per spot are recommended to be performed. Spectral acquisition is performed using an
193 automated MS mode, thus increasing the speed and throughput of this technique. External mass
194 calibration must be performed, using a peptide and protein known mixture whose masses must
195 cover the analytical mass range. A high degree of mass accuracy is desired, and typical calibration
196 error should not be higher than 0.5%. In order to optimize mass accuracy, it is recommended to
197 apply an additional internal calibration (by superposing sample and calibration standard) as well as a
198 lock-mass correction using a high-intensity mass at the middle of the mass range (error of 0.05%).

199

200 *3.4. Spectra pre-processing*

201 Raw MALDI data usually contain a large set of spectra, each containing thousands of
202 measurements composed by m/z-intensity pairs, most of which correspond to noisy signals. In order
203 to distinguish true from noise signals as well as to reduce experimental variance within the data set,
204 several spectral treatments (known as pre-processing) must be carried out. This pre-processing
205 includes the following steps: (1) internal lock-mass calibration using a mass of known or unknown
206 identity, to provide with a higher mass accuracy, (2) baseline correction to remove the effect of noise
207 introduced by the matrix, (3) filtering to smooth the signal, (4) alignment of all spectra using common
208 m/z, (5) automatic peak detection on average spectra (area and/or intensity), and (6) normalization.
209 The criteria followed for this pre-processing can drastically change the outcome of analysis, and so it
210 is essential to pay attention to this stage in order to perform a rigorous study [37].

211

212 *3.5. Statistical analysis and pattern matching algorithms*

213 Once this is accomplished, applying statistical methods and/or machine-learning techniques
214 is often desired in order to identify those spectral features (peaks) associated with a given condition
215 (candidate biomarkers or biomarker profiles), sample grouping (through hierarchical classification or
216 principal component analysis) and classification (through the constructions of class-predictive models
217 based on the use of data-dependent algorithms or through comparison with reference spectra
218 contained in a database). While processing and data analysis were initially based on the use of
219 general tools like R or SPSS (Statistical Package for the Social Sciences) softwares, requiring
220 programming skills and implying a lack of uniformity in the criteria followed for this process, a
221 number of user-friendly tools specific for MALDI profiling data are currently available. In general,
222 there are two kinds of tools regarding the type of post-processing analysis: those based on the use of
223 a reference spectra database to which test spectra are compared, and those in which the user sets
224 examples of the different studied classes. The first group of tools includes commercial software
225 packages containing reference databases, and its use is mainly restricted to microorganism
226 identification (e.g. MALDI Biotyper from Bruker Daltonics or Anagnostec SARAMIS from Shimadzu).
227 The second group entails tools that allow both supervised and unsupervised techniques to analyze
228 data following a clinical approach. In general, the biomarker research-oriented analysis starts with
229 performing a statistical analysis (parametric or non-parametric) using quantitative values (normalized
230 peak height or area). The m/z peaks presenting significant differences between the different studied
231 conditions are selected and relative data are then utilized to perform additional analysis such as
232 Receiver Operating Characteristic curves, cluster analysis, Principal Component Analysis, etc. It is
233 important to set a statistical stringency adequate to the desired statistical power (usually set at 0.8),
234 especially in studies using a reduced sample number. This statistical analysis can be completed with
235 machine-learning techniques. In this case, the diagnostic performance of the ICM-MS-based
236 predictive model for a given condition should be compared with that of traditional diagnostic tests of
237 the same condition. In the recent years, different open-source tools (e.g. Mass-up, MALDIquant) and

238 commercial tools (e.g. ProgenesisMALDI-Nonlinear Dynamics, ClinProTools-Bruker Daltonics.
239 MarkerView™- AB Sciex) specifically designed for MALDI profiling analysis have been released.

240

241 **4. Top-Down MS for identification of proteins detected by ICM-MS**

242 The progress in the general use of MS-based profiling has been somewhat hampered by the
243 difficulties in confidently identifying diagnostic MS signatures [38]. ICM-MS analyses (in general all
244 profiling methods) are ideally completed with the identification of the m/z peaks present in the
245 spectra. As aforementioned, these masses correspond to native, endogenous molecules present in
246 the sample. Of all the mass spectrometry-based identification approaches currently available (Figure
247 3), Top-Down proteomics represents the best approach to identify endogenous peptidofoms and
248 proteofoms, as involves the detection of an intact molecular species and its direct fragmentation,
249 thus providing with a complete description of the primary structure of the protein and of its
250 modifications [39].

251 As it is generally desired to employ the same platform for both profiling and sequencing,
252 following an on-line approach, there is big interest in the scientific community in advancing in
253 technology and data-processing systems that would allow MALDI Top-Down analysis in complex
254 samples (such as protein extracts, whole cells or tissue slides). Post-Source (PSD) [40] or In-Source
255 Decay (ISD) [41] MS methodologies can be used for de novo sequencing of the whole proteins, with
256 the condition of these being enriched or purified, since their main limitation is the inability of dealing
257 with complex samples, and the MS/MS strategy is routinely restricted to small peptides with $m/z <$
258 5,000 [42,43]. Thus, the *in-situ* targeted identification of peaks of interest observed by MALDI
259 remains challenging. Sample complexity can be decreased through fractionation (e.g. using
260 chromatography, gel separation, depletion of abundant proteins, etc), and fractions are later re-
261 analyzed by MALDI-TOF/TOF. This approach indeed increases the number of positively identified
262 biomolecules, but still shows a low throughput. Alternatively, Top-Down proteomics combining liquid
263 chromatography separations and high resolution tandem mass spectrometry (LC-HR-MS/MS) with

264 electrospray (ESI) source seems to be the most appropriate approach to identify endogenous
265 peptido- and proteoforms [36,41]. ESI generates multiply charged ions and is the preferred MS
266 method for the analysis of both peptides and intact proteins, especially on a proteome-wide scale
267 (global approach). Nowadays, Top-Down proteomic studies have largely been implemented using ESI
268 coupled to either Fourier transform ion cyclotron resonance (FT-ICR) or Orbitrap mass analyzers to
269 obtain high resolution ($R > 100,000$) and mass accuracy (< 5 ppm). Thus, a large number of whole,
270 intact biomolecules can be directly identified, whose masses can be matched with MALDI m/z peaks,
271 with a mass accuracy tolerance $< 0.05\%$. As described in the literature, the combination of ICM-MS
272 and Top-Down HRMS is therefore a convenient analytical strategy [44, 45].

273

274 **5. Intact Cell MALDI-TOF Mass Spectrometry – from bacteria to mammalian cells**

275 The use of ICM-MS was firstly reported in 1996 by Holland et al., and was applied to bacterial
276 chemotaxonomy [46]. In this study, it was described that ICM-MS protein profiles contained species-
277 specific signatures, so they could be used as a taxonomic tool [47]. In effect, the analysis of
278 endogenous peptidoforms and proteoforms can be considered as a functional genomics analysis and
279 therefore useful for phenotyping [48]. ICM-MS has been applied in different studies for bacterial
280 identification, aiding at the development of the field of phyloproteomics [46,49,50]. Identification of
281 the microorganisms was based on the identification of species-specific signatures in the ICM-MS
282 spectra using bioinformatic tools and in the subsequent construction of reference spectral databanks
283 [51]. Thereafter, comprehensive and curated databases were constructed using rigorous
284 standardization procedures and commercialized together with tools that allowed a robust
285 identification analysis [52, 53]. Nowadays, ICM-MS has become a valuable tool for the identification
286 and the classification of microorganisms, including bacteria [47] or yeast [54-56], and it has been
287 introduced in clinical routine microbiological diagnostics. Thus, this approach assists clinicians to
288 provide a fast, accurate diagnosis associated with an adequate antimicrobial treatment in infectious
289 diseases. Furthermore, this approach can also be applied to detect specific bacterial biomarkers, such

290 as antibiotic resistance, and may potentially predict infectious complications or/and being used for
291 the development of novel virulence factor-based therapies [57].

292 Theoretically, ICM-MS could be applied to any cell type including superior eukaryotes, with
293 the sole condition of cells being isolated (not in a tissue). Even if this approach is not yet routinely
294 applied to the analysis of mammalian cells, there is sufficient evidences in the literature showing that
295 ICM-MS spectra from “whole cells” or “intact cells” are sufficiently characteristic to address biological
296 questions in human and animal clinical research (Table 1). In fact, ICM-MS has been applied mostly in
297 cultured cells as a phenotyping tool in order to differentiate similar cell lines including glial [27] or
298 cancer [58] lines. It has also been employed to evaluate the response of established cell lines to
299 different treatments, like toxic chemicals [30]. Another series of studies have isolated different
300 circulating white blood cells like monocytes or neutrophils to evaluate their differential response to
301 different conditions [29, 59-62].

302

303 **6. ICM-MS proteomic analysis in animal sciences**

304 ICM-MS is a promising diagnostic tool, showing several advantages such as being cost-
305 effective, simple and showing a high throughput. As it is based on the comparison of peptide and
306 small proteins profiles rather than the comparison of single biomarkers, it is theoretically more prone
307 to identify phenotypic differences associated with a certain physiological, pathological or
308 experimental condition [5]. Bearing that in mind, multiple applications of ICM-MS in animal sciences
309 could be foreseen. As it could be easily applied in cultured cells, it might be useful in screening
310 studies in toxicology and infectious diseases, thus helping evaluate in a fast way the effect of single
311 and combined substances (toxic compounds, pathogens, immuno-reactive substances, etc) in order
312 to establish similarities, for instance, in their mechanisms of action, evaluating the nature of their
313 response, etc. There are several examples in the literature describing how ICM-MS can be easily
314 implemented in cellular toxicology tests. For instance, it has been shown in a fish cell model that
315 proteome changes induced by copper sulfate, acridine, and β -naphthoflavone were reflected in the

316 ICM-MS profiles [30]. Similar results were obtained in human cell lines treated with hydrogen
317 peroxide and aflatoxin B1 [26].

318 The use of ICM-MS for disease diagnostics in animals could be implemented in blood
319 circulating cells, where the presence of pathognomonic ICM-MS spectra patterns could be
320 researched. This could be particularly interesting in the course of infections often leading to sub-
321 clinical symptoms (e.g. paratuberculosis), and/or those involving intracellular pathogens in blood
322 cells (ehrlichiosis, anaplasmosis, babesiosis etc.). Several studies carried out in human samples have
323 actually evidenced that ICM-MS analysis of human monocytes evidenced proteome changes
324 occurring upon microbial stimulation [29]. ICM-MS spectra from M1 and M2 macrophage subtypes
325 were also significantly different , as well as those obtained from macrophages in contact with
326 extracellular (group B Streptococcus, *Staphylococcus aureus*) and intracellular (*Mycobacterium bovis*,
327 *Orientia tsutsugamushi*, *Coxiella burnetii*) bacteria [60]. The ability of ICM-MS-based analysis to
328 detect molecular differences linked to specific phenotypes has allowed the discovery of several
329 markers. For instance, whole cell MALDI profiling analysis of peripheral chicken cells evidenced that
330 thymosin beta-4 was an abundant peptide present in avian monocytes/macrophages (and not in
331 granulocytes) and that it was regulated by bacterial lipopolysaccharides and peptidoglycans [63]. The
332 same approach helped to put in evidence that β -defensin 2 is an important anti-microbial peptide in
333 chicken and turkey heterophils [63].

334 However, clinical research in animal science is not only focused in finding disease biomarkers,
335 but also in finding molecular tools that could help in animal production. Reproduction management
336 represents one of the most important aspects of animal production, and very few molecular tools are
337 available to monitor the implementation of new management methods in farm animals. In this
338 sense, ICM-MS has been proven useful to follow cell-specific peptidome/proteome changes in the
339 cells related to the animal reproductive system. In the following sections, we will discuss how ICM-
340 MS profiling has been implemented for the analysis of male and female gametes in farm animals.

341

342 **7. ICM-MS proteomic analysis of male gametes**

343 Evaluation of sperm quality is usually based on the assessment of individual sperm
344 parameters (motility, morphology), which is cumbersome and sometimes not well correlated with
345 the actual fertilizing ability of tested sperm. Thus, the development of a fast, accurate tool for
346 fertility screening is largely needed in human and veterinary medicine. From all possible molecular
347 biology techniques applicable, proteomics seems to be one of the most adequate to study sperm
348 biology. Numerous proteomic studies aiming to identify sperm markers of fertility were recently
349 performed in several mammalian species (reviewed in [64-66]). However these approaches are not
350 directly applicable for their routine use and the use of individual biomarkers has not been yet
351 successfully implemented in fertility clinics to stratify sperm according to their quality. Sperm peptide
352 and small protein profiling through ICM-MS has been recently proven useful to identify proteome
353 patterns linked with fertility in chicken [45, 67]. The method included only a few and simple
354 preparatory steps namely cell counting, washing sperm cells to eliminate seminal plasma proteins,
355 and resuspension in the same buffer at a known concentration. The plating procedure was simple:
356 sperm suspension was spotted onto a MALDI plate, overlaid with an adequate matrix and allowed to
357 dry. Further MS analysis can be automated, this increasing the throughput of the method. Using
358 adequate pre-processing and bioinformatics analysis tools, sperm ICM-MS profiles showed
359 characteristic spectral features that allowed their classification according to their actual fertilizing
360 ability [67]. This method was successfully employed in different chicken genetic lines, as well in other
361 livestock species, thus showing a high versatility. Moreover, several peaks of chicken sperm ICM-MS
362 spectra corresponding to peptidoforms (due to specific or non specific proteolytic activities) or
363 proteoforms were identified using HRMS, including some fertility biomarkers candidates, such as
364 protein vitelline membrane outer layer protein 1 or different forms of the avian beta-defensin 10. It
365 has been also shown that the ICM-MS profiles from pig epididymal spermatozoa reflect the
366 proteome changes that they undergo during epididymal maturation, mostly those related with a
367 differential activity of sperm intracellular proteases and peptidases [68]. Moreover, it was observed

368 that the analysis of whole cells allowed a better discrimination of different maturation stages
369 compared with sperm cell extracts as obtained with neutral detergents [68]. In short, these results
370 indicate that ICM-MS profiling could be a suitable approach for molecular phenotyping of male
371 gametes.

372

373 **8. ICM-MS proteomic analysis of female gametes**

374 Today, it is possible to initiate early embryonic development of just about any mammalian
375 species by co-incubating oocyte with spermatozoa following a protocol known as *in vitro* fertilization
376 (IVF). The original process includes the administration of high levels of gonadotropins to stimulate
377 oocyte maturation and ovulation [69]. Oocyte maturation is a transition from immature germinal
378 vesicle (GV) transcriptionally silenced oocyte to metaphase-II stage (Meta-II) which is accompanied
379 by polar body extrusion, reorganization of the organelles through the ooplasm and molecular
380 modifications including changes in protein abundance and post-translational modifications. Oocyte
381 maturation plays an important role in the acquisition of oocyte developmental competence, referred
382 as oocyte quality, which, at the same time, is crucial for embryo quality [70]. Maturation can be
383 accomplished *in vitro* starting from the oocytes recovered from small antral follicles at the stage prior
384 to selection and dominance, by using specific culture media, following a protocol known as *in vitro*
385 *maturation* (IVM). IVM requires little or no use of *in vivo*-administered gonadotropins and has been
386 proposed as an alternative to conventional IVF with ovulated oocytes in humans since it reduces the
387 primary adverse effects caused by controlled ovarian stimulation, including the ovarian
388 hyperstimulation syndrome [71]. IVM is actually a routine technique in animal reproduction
389 biotechnologies, used especially in dairy cows of high genetic merit, where the success rate after
390 artificial insemination is often low. In cattle, immature oocytes can be obtained from the ovaries
391 either by ovum pick up or after slaughter, then subjected to IVM, fertilized and developed to
392 transferable embryos. This technique of *in vitro* embryo production allows the use of less semen (this
393 is important in the case of expensive and rare sperm), as well as increasing the progeny of oocyte

394 donors [72]. However, proteomic changes that occur in the oocyte during maturation and that define
395 the quality of *in vitro* matured oocytes are mostly unknown, and so there is no accurate way of
396 evaluating/monitoring how different IVM protocols can affect the process. Classic proteomics has
397 been employed in the past with this objective. In fact, by using 2D-DIGE or 2DE coupled with nano-
398 liquid chromatography and high resolution tandem MS, proteomes were established for bovine,
399 porcine and murine oocytes identifying numerous proteins including putative markers of oocyte
400 developmental competence including proteins needed for fertilization, reprogramming, embryo
401 genome activation and first cleavages during early embryogenesis [73-75]. However, these proteomic
402 approaches require a high amount of oocytes to be performed. In cows, only 8-10 oocytes fully-
403 grown oocytes can be collected per ovary, so the amount of material is limited. The bovine oocyte is
404 about 120 μm in diameter, but it contains a relatively low intra-cellular protein content, about 80-
405 100 ng per oocyte. ICM-MS was recently adapted to bovine ovarian cells from the individual antral
406 follicles to obtain the protein/peptide signatures (<17kDa) of single oocytes, surrounding cumulus
407 cells and granulosa cells [76]. It was demonstrated, that this technology could be applied to single
408 bovine oocytes, and that single-oocyte ICM-MS spectra are characteristic of each stage of oocyte
409 meiotic maturation from prophase-I in immature oocytes to metaphase-II in mature ones [76]. In
410 this study, ICM-MS coupled to an optimized Top-down HR-MS proteomic approach on ovarian
411 follicular cells allowed the identification of specific markers of oocyte meiotic maturation including
412 IGF2 binding protein 3 and hemoglobin B in the oocyte and thymosins beta-4, and beta-10, histone
413 H2B and ubiquitin in surrounding cumulus cells.

414

415 **9. Conclusions**

416 ICM-MS is a peptide/protein profiling platform widely used for phyloproteomics that has been
417 recently revealed as a promising tool for molecular phenotyping in clinical proteomics. The
418 applicability of this technique has been already shown in clinical microbiology and it is starting to
419 bring some attention in biomedicine. Indeed, specific characteristic features present in ICM-MS

420 profiles in a given condition can be considered as biomarker profiles, and individual differential
421 features can be identified following Top-Down proteomics. Although still not widely used, ICM-MS
422 profiling can have multiple applications in animal research, ranging from basic sciences - as for
423 example, in newly established primary cell lines phenotyping or in vitro toxicological studies - to
424 applied sciences for use in disease diagnostics, male fertility classification according to semen
425 profiling or monitoring of oocyte quality, among others.

426

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436 **References**

- 437 [1] V. Thongboonkerd. Clinical proteomics: Towards diagnostics and prognostics. *Blood*, 2007,
438 109, 5075–5076.
- 439 [2] H. Mischak, G. Allmaier, R. Apweiler, T. Attwood, M. Baumann, A. Benigni, S. E. Bennett, R.
440 Bischoff, E. Bongcam-Rudloff, G. Capasso, J. J. Coon, P. D'Haese, A. F. Dominiczak, M. Dakna, H.
441 Dihazi, J. H. Ehrich, P. Fernandez-Llama, D. Fliser, J. Frokiaer, J. Garin, M. Girolami, W. S. Hancock, M.
442 Haubitz, D. Hochstrasser, R. R. Holman, J. P. A. Ioannidis, J. Jankowski, B. A. Julian, J. B. Klein, W.
443 Kolch, T. Luider, Z. Massy, W. B. Mattes, F. Molina, B. Monsarrat, J. Novak, K. Peter, P. Rossing, M.
444 Sánchez-Carbayo, J. P. Schanstra, O. J. Semmes, G. Spasovski, D. Theodorescu, V. Thongboonkerd, R.
445 Vanholder, T. D. Veenstra, E. Weissinger, T. Yamamoto and A. Vlahou. Recommendations for
446 biomarker identification and qualification in clinical proteomics. *Sci. Transl. Med.*, 2010, 2, 46ps42.
- 447 [3] F. Ceciliani, D. Eckersall, R. Burchmore and C. Lecchi. Proteomics in veterinary medicine:
448 applications and trends in disease pathogenesis and diagnostics. *Vet. Pathol.*, 2014, 51, 351–62.
- 449 [4] P. D. Eckersall, A. M. de Almeida and I. Miller, J. Proteomics, a new tool for farm animal
450 science. *Proteomics*, 2012, 75, 4187–9.
- 451 [5] E. Bendixen, M. Danielsen, K. Hollung, E. Gianazza and I. Miller, J. Farm animal proteomics--a
452 review. *Proteomics*, 2011, 74, 282–93.
- 453 [6] A. M. de Almeida and E. Bendixen, J. Pig proteomics: a review of a species in the crossroad
454 between biomedical and food sciences. *Proteomics*, 2012, 75, 4296–314.
- 455 [7] J. L. Williams. Genetic Control of Meat Quality Traits, in *Meat Biotechnology*, ed. F. Toldrá,
456 Springer New York, New York, NY, 2008, pp. 21–60.

- 457 [8] W. H. McDonald and J. R. Yates. Shotgun proteomics and biomarker discovery. *Dis. Markers*,
458 2002, 18, 99–105.
- 459 [9] Mann M, Hojrup P, Roepstorff P. Use of mass spectrometric molecular weight information to
460 identify proteins in sequence databases. *Biol Mass Spectrom*. 1993 Jun; 22(6):338-45.
- 461 [10] Aebersold R, Mann M. Mass spectrometry-based proteomics. *Nature*. 2003; 422:198–207.
- 462 [11] B. Hernández, A. Parnell and S. R. Pennington. Why have so few proteomic biomarkers
463 “survived” validation?(Sample size and independent validation considerations). *Proteomics*, 2014, 14,
464 1587–92.
- 465 [12] T. Shi, D. Su, T. Liu, K. Tang, D. G. Camp, W.-J. Qian and R. D. Smith. Advancing the sensitivity
466 of selected reaction monitoring-based targeted quantitative proteomics. *Proteomics*, 2012, 12,
467 1074–92.
- 468 [13] H. Mischak, A. Vlahou and J. P. A. Ioannidis. Technical aspects and inter-laboratory variability
469 in native peptide profiling: the CE–MS experience. *Clin. Biochem.*, 2013, 46, 432–43.
- 470 [14] J. P. Savaryn, A. D. Catherman, P. M. Thomas, M. M. Abecassis and N. L. Kelleher. The
471 emergence of top-down proteomics in clinical research. *Genome Med.*, 2013, 5, 53.
- 472 [15] Kelleher NL. Top-down proteomics. *Anal Chem*. 2004; 76:196 A–203 A.
- 473 [16] A. Albalat, H. Husi, J. Siwy, J. E. Nally, M. McLaughlin, P. D. Eckersall and W. Mullen. Capillary
474 electrophoresis interfaced with a mass spectrometer (CE-MS): technical considerations and
475 applicability for biomarker studies in animals. *Curr. Protein Pept. Sci.*, 2014, 15, 23–35.
- 476 [17] M. G. J. Koene, H. A. Mulder, N. Stockhofe-Zurwieden, L. Kruijt and M. A. Smits. Serum
477 protein profiles as potential biomarkers for infectious disease status in pigs. *BMC Vet. Res.*, 2012, 8,
478 32.
- 479 [18] L. Zhong, D. L. Taylor and R. J. Whittington. Proteomic profiling of ovine serum by SELDI-TOF
480 MS: optimisation, reproducibility and feasibility of biomarker discovery using routinely collected
481 samples. *Comp. Immunol. Microbiol. Infect. Dis.*, 2010, 33, 47–63.
- 482 [19] A. Stalmach, H. Husi, K. Mosbahi, A. Albalat, W. Mullen and H. Mischak. Methods in capillary
483 electrophoresis coupled to mass spectrometry for the identification of clinical proteomic/peptidomic
484 biomarkers in biofluids. *Methods Mol. Biol.*, 2015, 1243, 187–205.
- 485 [20] L. Molin, R. Seraglia, A. Lapolla, E. Ragazzi, J. Gonzalez, A. Vlahou, J. P. Schanstra, A. Albalat,
486 M. Dakna, J. Siwy, J. Jankowski, V. Bitsika, H. Mischak, P. Zürgbig and P. Traldi, A comparison between
487 MALDI-MS and CE-MS data for biomarker assessment in chronic kidney diseases. *J. Proteomics*, 2012,
488 75, 5888–97.
- 489 [21] R. Mansor, W. Mullen, A. Albalat, P. Zerefos, H. Mischak, D. C. Barrett, A. Biggs and P. D.
490 Eckersall. A peptidomic approach to biomarker discovery for bovine mastitis. *J. Proteomics*, 2013, 85,
491 89–98.
- 492 [22] J. E. Nally, W. Mullen, J. J. Callanan, H. Mischak and A. Albala. Detection of urinary
493 biomarkers in reservoir hosts of leptospirosis by capillary electrophoresis-mass spectrometry.
494 *Proteomics. Clin. Appl.*, 2015, 9, 543–51.
- 495 [23] A. Mehta and L. P. Silva. MALDI-TOF MS profiling approach: how much can we get from it?
496 *Front. Plant Sci.*, 2015, 6, 184.

- 497 [24] J. F. Povey, C. J. O'Malley, T. Root, E. B. Martin, G. A. Montague, M. Feary, C. Trim, D. A. Lang,
498 R. Alldread, A. J. Racher and C. M. Smales. Rapid high-throughput characterisation, classification and
499 selection of recombinant mammalian cell line phenotypes using intact cell MALDI-ToF mass
500 spectrometry fingerprinting and PLS-DA modelling. *J. Biotechnol.*, 2014, 184, 84–93.
- 501 [25] V. Labas, L. Spina, C. Belleanne, A.-P. Teixeira-Gomes, A. Gargaros, F. Dacheux and J.-L.
502 Dacheux. Analysis of epididymal sperm maturation by MALDI profiling and top-down mass
503 spectrometry. *J. Proteomics*, 2015, 113, 226–43.
- 504 [26] N. H. L. Chiu, Z. Jia, R. Diaz and P. Wright. Rapid Differentiation of In Vitro Cellular Responses
505 to Toxic Chemicals by Using MALDI-TOF Mass Spectrometry. *Environ. Toxicol. Chem.*, 2015, 34, 161–
506 6.
- 507 [27] J. Hanrieder, G. Wicher, J. Bergquist, M. Andersson and A. Fex-Svenningsen. MALDI mass
508 spectrometry based molecular phenotyping of CNS glial cells for prediction in mammalian brain
509 tissue. *Anal. Bioanal. Chem.*, 2011, 401, 135–47.
- 510 [28] G. M. Toh-Boyo, S. S. Wulff and F. Basile. Comparison of sample preparation methods and
511 evaluation of intra- and intersample reproducibility in bacteria MALDI-MS profiling. *Anal. Chem.*,
512 2012, 84, 9971–80.
- 513 [29] D. Portevin, V. Pfluger, P. Otieno, R. Brunisholz, G. Vogel and C. Daubenberger. Quantitative
514 whole-cell MALDI-TOF MS fingerprints distinguishes human monocyte sub-populations activated by
515 distinct microbial ligands. *BMC Biotechnol*, 2015, 15, 24.
- 516 [30] S. L. Kober, H. Meyer-Alert, D. Grienitz, H. Hollert and M. Frohme. Intact cell mass
517 spectrometry as a rapid and specific tool for the differentiation of toxic effects in cell-based
518 ecotoxicological test systems. *Anal. Bioanal. Chem.*, 2015, 407, 7721–31.
- 519 [31] B. Munteanu, B. Meyer, C. von Reitzenstein, E. Burgermeister, S. Bog, A. Pahl, M. P. Ebert and
520 C. Hopf. Label-free in situ monitoring of histone deacetylase drug target engagement by matrix-
521 assisted laser desorption ionization-mass spectrometry biotyping and imaging. *Anal. Chem.*, 2014, 86,
522 4642–7.
- 523 [32] G. Vogel, A. Strauss, B. Jenni, D. Ziegler, E. Dumermuth, S. Antz, C. Bardouille, B. Wipf, C.
524 Miscenic, G. Schmid and V. Pflüger. Development and validation of a protocol for cell line
525 identification by MALDI-TOF MS. *BMC Proc.*, 2011, 5 Suppl 8, P45.
- 526 [33] H. Feng, L. C. Sim, C. Wan, N. S. C. Wong and Y. Yang. Rapid characterization of protein
527 productivity and production stability of CHO cells by matrix-assisted laser desorption/ionization time-
528 of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.*, 2011, 25, 1407–1412.
- 529 [34] R. Ouedraogo, A. Daumas, E. Ghigo, C. Capo, J. L. Mege and J. Textoris. Whole-cell MALDI-TOF
530 MS: a new tool to assess the multifaceted activation of macrophages. *J Proteomics*, 2012, 75, 5523–
531 5532.
- 532 [35] A. Karger, B. Bettin, M. Lenk and T. C. Mettenleiter. Rapid characterisation of cell cultures by
533 matrix-assisted laser desorption/ionisation mass spectrometric typing. *J. Virol. Methods*, 2010, 164,
534 116–21.
- 535 [36] L. Bai, E. V Romanova and J. V Sweedler. Distinguishing endogenous D-amino acid-containing
536 neuropeptides in individual neurons using tandem mass spectrometry. *Anal Chem*, 2011, 83, 2794–
537 2800.

- 538 [37] H. López-Fernández, H. M. Santos, J. L. Capelo, F. Fdez-Riverola, D. Glez-Peña and M. Reboiro-
539 Jato. Mass-Up: an all-in-one open software application for MALDI-TOF mass spectrometry knowledge
540 discovery. *BMC Bioinformatics*, 2015, 16, 318.
- 541 [38] P. Findeisen and M. Neumaier. Mass spectrometry-based clinical proteomics profiling:
542 current status and future directions. *Expert Rev. Proteomics*, 2009, 6, 457–9.
- 543 [39] D. R. Ahlf, P. M. Thomas and N. L. Kelleher. Developing top down proteomics to maximize
544 proteome and sequence coverage from cells and tissues. *Curr. Opin. Chem. Biol.*, 2013, 17, 787–794.
- 545 [40] P. Chaurand and R. M. Caprioli. Direct profiling and imaging of peptides and proteins from
546 mammalian cells and tissue sections by mass spectrometry. *Electrophoresis*, 2002, 23, 3125–3135.
- 547 [41] D. Debois, V. Bertrand, L. Quinton, M.-C. De Pauw-Gillet and E. De Pauw. MALDI-in source
548 decay applied to mass spectrometry imaging: a new tool for protein identification. *Anal. Chem.*,
549 2010, 82, 4036–45.
- 550 [42] D. Suckau and A. Resemann. MALDI Top-Down sequencing: calling N- and C-terminal protein
551 sequences with high confidence and speed. *J. Biomol. Tech.*, 2009, 20, 258–62.
- 552 [43] Z. Liu and K. L. Schey. Optimization of a MALDI TOF-TOF mass spectrometer for intact protein
553 analysis. *J. Am. Soc. Mass Spectrom.*, 2005, 16, 482–90.
- 554 [44] B. Parks, L. Jiang, P. M. Thomas, C. D. Wenger, M. J. Roth, M. T. Boyne, P. V Burke, K. E.
555 Kwast and N. L. Kelleher. Top-down proteomics on a chromatographic time scale using linear ion trap
556 fourier transform hybrid mass spectrometers. *Anal. Chem.*, 2007, 79, 7984–91.
- 557 [45] V. Labas, I. Grasseau, K. Cahier, A. Gargaros, G. Harichaux, A.-P. Teixeira-Gomes, S. Alves, M.
558 Bourin, N. Gérard and E. Blesbois. Qualitative and quantitative peptidomic and proteomic
559 approaches to phenotyping chicken semen. *J. Proteomics*, 2015, 112, 313–35.
- 560 [46] R. D. Holland, J. G. Wilkes, F. Rafii, J. B. Sutherland, C. C. Persons, K. J. Voorhees and J. O. Lay.
561 Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser
562 desorption/ionization with time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.*, 1996,
563 10, 1227–32.
- 564 [47] M. Welker. Proteomics for routine identification of microorganisms. *Proteomics*, 2011, 11,
565 3143–3153.
- 566 [48] W. P. Blackstock and M. P. Weir. Proteomics: quantitative and physical mapping of cellular
567 proteins. *Trends Biotechnol.*, 1999, 17, 121–7.
- 568 [49] M. A. Claydon, S. N. Davey, V. Edwards-Jones and D. B. Gordon. The rapid identification of
569 intact microorganisms using mass spectrometry. *Nat. Biotechnol.*, 1996, 14, 1584–6.
- 570 [50] T. Krishnamurthy and P. L. Ross. Rapid identification of bacteria by direct matrix-assisted
571 laser desorption/ionization mass spectrometric analysis of whole cells. *Rapid Commun. Mass
572 Spectrom.*, 1996, 10, 1992–1996.
- 573 [51] C. J. Keys, D. J. Dare, H. Sutton, G. Wells, M. Lunt, T. McKenna, M. McDowall and H. N. Shah,
574 Compilation of a MALDI-TOF mass spectral database for the rapid screening and characterisation of
575 bacteria implicated in human infectious diseases. *Infect Genet Evol*, 2004, 4, 221–242.

- 576 [52] K. Sogawa, M. Watanabe, K. Sato, S. Segawa, C. Ishii, A. Miyabe, S. Murata, T. Saito and F.
577 Nomura. Use of the MALDI BioTyper system with MALDI-TOF mass spectrometry for rapid
578 identification of microorganisms. *Anal Bioanal Chem*, 2011, 400, 1905–1911.
- 579 [53] A. Zavalin, E. M. Todd, P. D. Rawhouser, J. Yang, J. L. Norris and R. M. Caprioli. Direct imaging
580 of single cells and tissue at sub-cellular spatial resolution using transmission geometry MALDI MS. *J.*
581 *Mass Spectrom.*, 2012, 47, i.
- 582 [54] J. Qian, J. E. Cutler, R. B. Cole and Y. Cai. MALDI-TOF mass signatures for differentiation of
583 yeast species, strain grouping and monitoring of morphogenesis markers. *Anal Bioanal Chem*, 2008,
584 392, 439–449.
- 585 [55] L. G. Stevenson, S. K. Drake, Y. R. Shea, A. M. Zelazny and P. R. Murray. Evaluation of matrix-
586 assisted laser desorption ionization-time of flight mass spectrometry for identification of clinically
587 important yeast species. *J Clin Microbiol*, 2010, 48, 3482–3486.
- 588 [56] N. Fatania, M. Fraser, M. Savage, J. Hart and A. Abdolrasouli. Comparative evaluation of
589 matrix-assisted laser desorption ionisation-time of flight mass spectrometry and conventional
590 phenotypic-based methods for identification of clinically important yeasts in a UK-based medical
591 microbiology laboratory. *J. Clin. Pathol.*, 2015, 68, 1040–2.
- 592 [57] J. Gagnaire, O. Dauwalder, S. Boisset, D. Khau, A.-M. Freydiere, F. Ader, M. Bes, G. Lina, A.
593 Tristan, M.-E. Reverdy, A. Marchand, T. Geissmann, Y. Benito, G. Durand, J.-P. Charrier, J. Etienne, M.
594 Welker, A. Van Belkum and F. Vandenesch, Detection of *Staphylococcus aureus* delta-toxin
595 production by whole-cell MALDI-TOF mass spectrometry. *PLoS One*, 2012, 7, e40660.
- 596 [58]. Povey JF, Saintas E, Aderemi AV, Rothweiler F, Zehner R, Dirks WG, Cinatl J, Racher AJ, Wass
597 MN, Smales CM, Michaelis M. Intact-Cell MALDI-ToF Mass Spectrometry for the Authentication of
598 Drug-Adapted Cancer Cell Lines. *Cells*. 2019 ,2;8(10)
- 599 [59] R. Ouedraogo, C. Flaudrops, A. Ben Amara, C. Capo, D. Raoult and J.-L. Mege. Global analysis
600 of circulating immune cells by matrix-assisted laser desorption ionization time-of-flight mass
601 spectrometry. *PLoS One*, 2010, 5, e13691.
- 602 [60]. Ouedraogo R, Textoris J, Gorvel L, Daumas A, Capo C, Mege JL. Analysis and Characterization of
603 Immune Cells and Their Activation Status by Whole-Cell MALDI-TOF Mass Spectrometry. *Methods*
604 *Mol Biol*. 2019; 2024:339-351
- 605 [61] L. Kannan, N. C. Rath, R. Liyanage and J. O. Lay Jr. Direct screening identifies mature β -
606 defensin 2 in avian heterophils. *Poult Sci*, 2009, 88, 372–379.
- 607 [62] L. Kannan, N. C. Rath, R. Liyanage and J. O. Lay Jr.. Thymosin beta in macrophage. *Ann. N. Y.*
608 *Acad. Sci.*, 2007, 1112, 425–434.
- 609 [63] L. Kannan, R. Liyanage, J. O. Lay Jr. and N. C. Rath. Evaluation of beta defensin 2 production
610 by chicken heterophils using direct MALDI mass spectrometry. *Mol Immunol*, 2009, 46, 3151–3156.
- 611 [64] Druart, X., Rickard, J.P., Tsikis, G., de Graaf, S.P., 2019. Seminal plasma proteins as markers of
612 sperm fertility. *Theriogenology* 31, 30172-30174.
- 613 [65] Dipresa, S., De Toni, L., Foresta, C., Garolla, A., 2018. New Markers for Predicting Fertility of the
614 Male Gametes in the Post Genomic Age. *Protein Pept Lett* 18, 0929866525666180418120635.
- 615 [66] Jodar, M., Soler-Ventura, A., Oliva, R., 2017. Semen proteomics and male infertility. *J Proteomics*
616 162, 125-134.

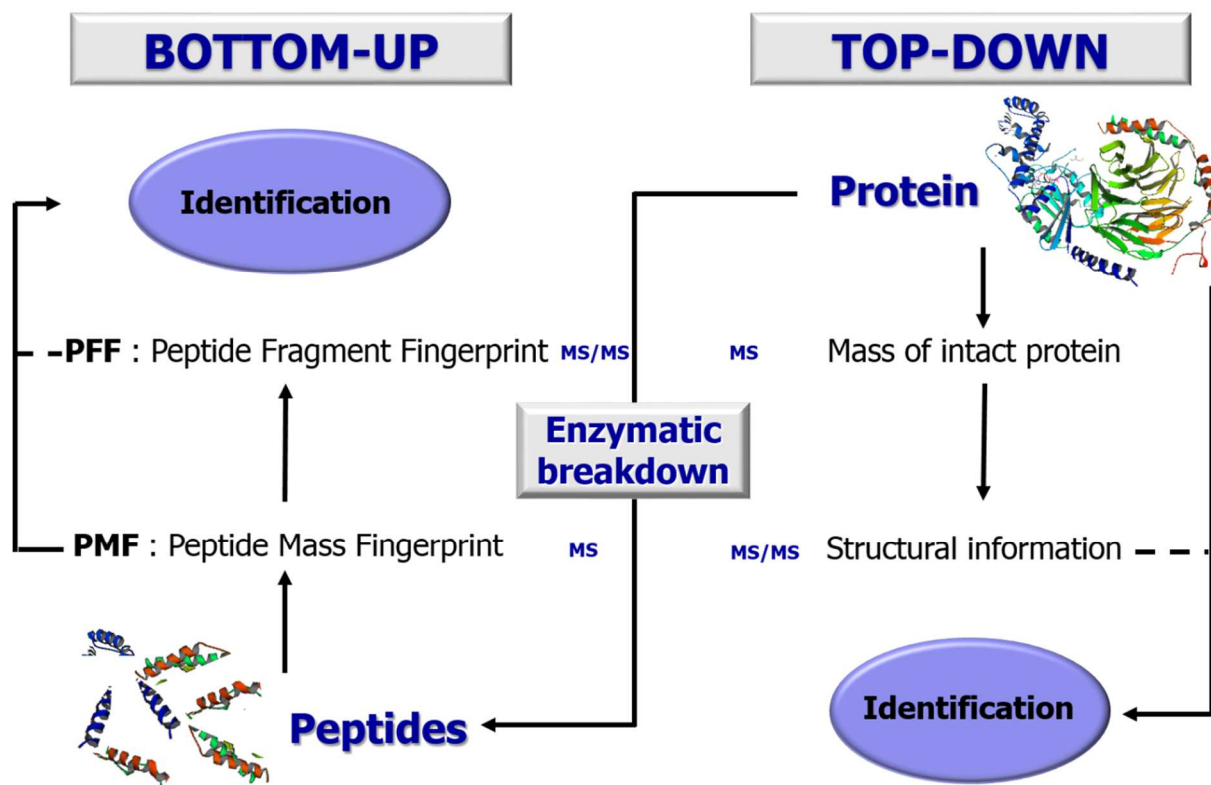
- 617 [67] Soler L, Labas V, Th  lie A, Grasseau I, Teixeira-Gomes AP, Blesbois E. Intact cell MALDI-TOF
618 MS on sperm: a molecular test for male fertility diagnosis. *Mol Cell Proteomics*. 2016, 15(6):1998-
619 2010.
- 620 [68] V. Labas, L. Spina, C. Belleannee, A.-P. Teixeira-Gomes, A. Gargaros, F. Dacheux and J.-L.
621 Dacheux. Analysis of epididymal sperm maturation by MALDI profiling and top-down mass
622 spectrometry. *J. Proteomics*, 2015, 113, 226–43.
- 623 [69] B. G. Brackett, D. P. Wolf and M. Zelinski-Wooten. *Advances in Animal In Vitro Fertilization*.
624 2001, 21–51.
- 625 [70] G. Marteil, L. Richard-Parpaillon and J. Z. Kubiak. Role of oocyte quality in meiotic maturation
626 and embryonic development. *Reprod. Biol.*, 2009, 9, 203–24.
- 627 [71] E. M. Chang, H. S. Song, D. R. Lee, W. S. Lee and T. K. Yoon. In vitro maturation of human
628 oocytes: Its role in infertility treatment and new possibilities. *Clin. Exp. Reprod. Med.*, 2014, 41, 41–6.
- 629 [72] L. Zhang, R. S. Denniston and R. A. Godke. A simple method for in vitro maturation, in vitro
630 fertilization, and co-culture of bovine oocytes. *J. Tissue Cult. Methods*, 1992, 14, 107–112.
- 631 [73] G. J. Arnold and T. Frohlich. Dynamic proteome signatures in gametes, embryos and their
632 maternal environment. *Reprod. Fertil. Dev.* 2011, 23, 81–93.
- 633 [74] I. Virant-Klun and J. Krijgsveld. Proteomes of animal oocytes: what can we learn for human
634 oocytes in the in vitro fertilization programme? *Biomed Res. Int.*, 2014, 2014, 856907.
- 635 [75] Q. Fu, Z.-F. Liu, Y.-L. Huang, Y.-Q. Lu and M. Zhang. Comparative proteomic analysis of mature
636 and immature oocytes of the swamp buffalo (*Bubalus bubalis*). *Int. J. Mol. Sci.*, 2015, 17.
- 637 [76]. Labas V, Teixeira-Gomes AP, Bouguereau L, Gargaros A, Spina L, Marestaing A, Uzbekova S.
638 Intact cell MALDI-TOF mass spectrometry on single bovine oocyte and follicular cells combined with
639 top-down proteomics: A novel approach to characterise markers of oocyte maturation. *J Proteomics*.
640 2018 . 20;175:56-74.
- 641 [77] L. F. Marvin-Guy, P. Duncan, S. Wagniere, N. Antille, N. Porta, M. Affolter and M. Kussmann.
642 Rapid identification of differentiation markers from whole epithelial cells by matrix-assisted laser
643 desorption/ionisation time-of-flight mass spectrometry and statistical analysis. *Rapid Commun. Mass*
644 *Spectrom.*, 2008, 22, 1099–1108.
- 645 [78] L. Kannan, N. C. Rath, R. Liyanage and J. O. Lay Jr.. Thymosin beta in macrophage. *Ann. N. Y.*
646 *Acad. Sci.*, 2007, 1112, 425–434.
- 647 [79] C. M. Buchanan, A. S. Malik and G. J. S. Cooper. C. M. Buchanan, A. S. Malik and G. J. S. Cooper,
648 *Rapid Commun. Mass Spectrom.*, 2007, 21, 3452–3458.
- 649 [80] X. Zhang, M. Scalf, T. W. Berggren, M. S. Westphall and L. M. Smith. Identification of mammalian
650 cell lines using MALDI-TOF and LC-ESI-MS/MS mass spectrometry. *J Am Soc Mass Spectrom*, 2006, 17,
651 490–499.
- 652 [81] J. Bergquist. Cells on the target matrix-assisted laser-desorption/ionization time-of-flight mass-
653 spectrometric analysis of mammalian cells grown on the target. *Chromatographia* 1999, 49, 41–48.
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655 **Figure Legends**

656 Figure 1: Proteomic approaches distinguishing traditional bottom-up strategies and no-classical top-
657 down strategy

658 Figure 2: Typical ICM-MS workflow

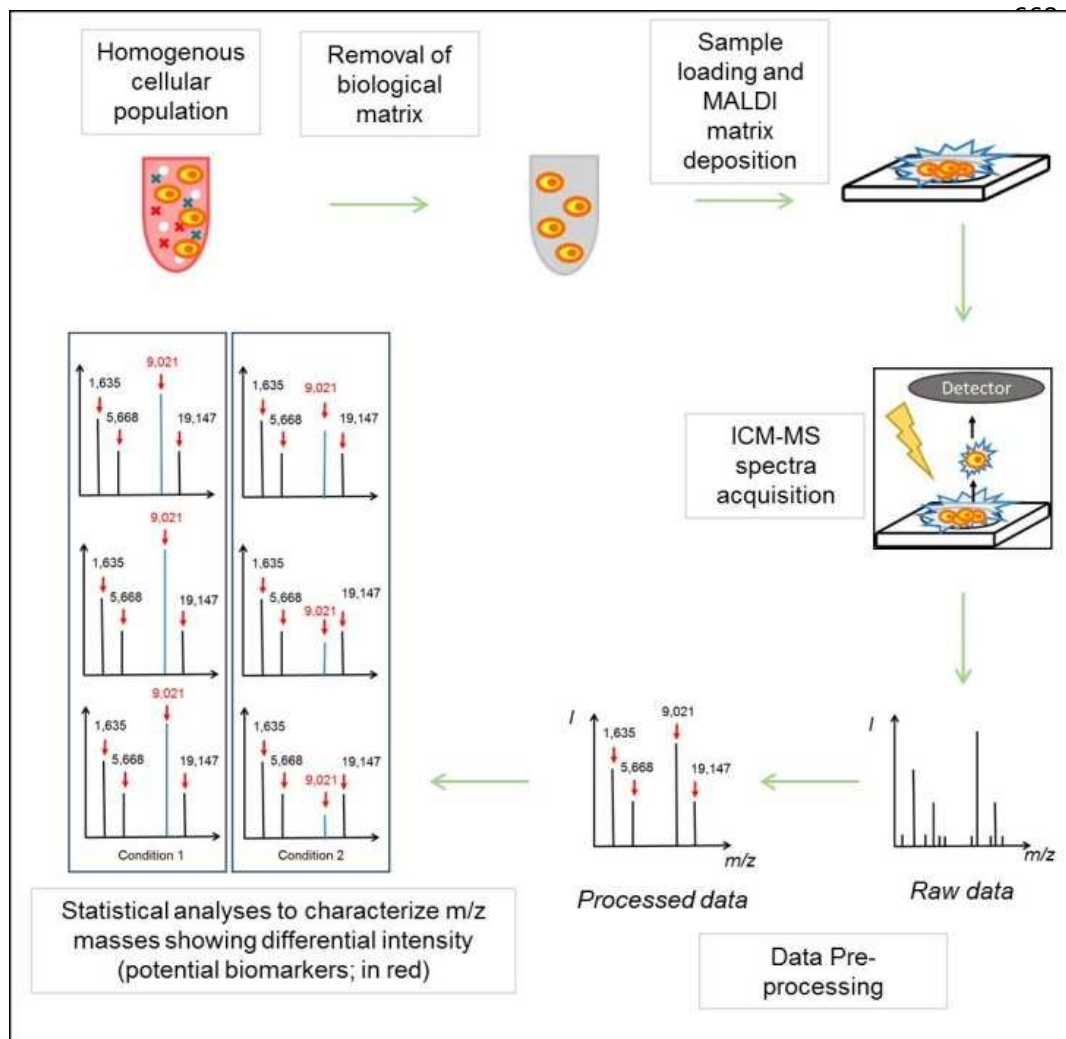
659 Figure 3: Top-Down protein identification workflow adapted for ICM-MS spectra characterization. 1.
660 Fractionation can be done using chromatographic separation, gel separation or any other sample
661 treatment aiming at decreasing sample complexity. 2. Top-Down protein identification can be
662 performed from whole cells, crude protein extracts, or pre-fractionated protein extracts. Analysis can
663 be based on the use of MALDI (A,B) or ESI (C,D) source ionisation m and fragmentation can be
664 performed using PSD/ISD (A,B), or HCD (B,C,D).



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667 Figure 1.



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Figure 2

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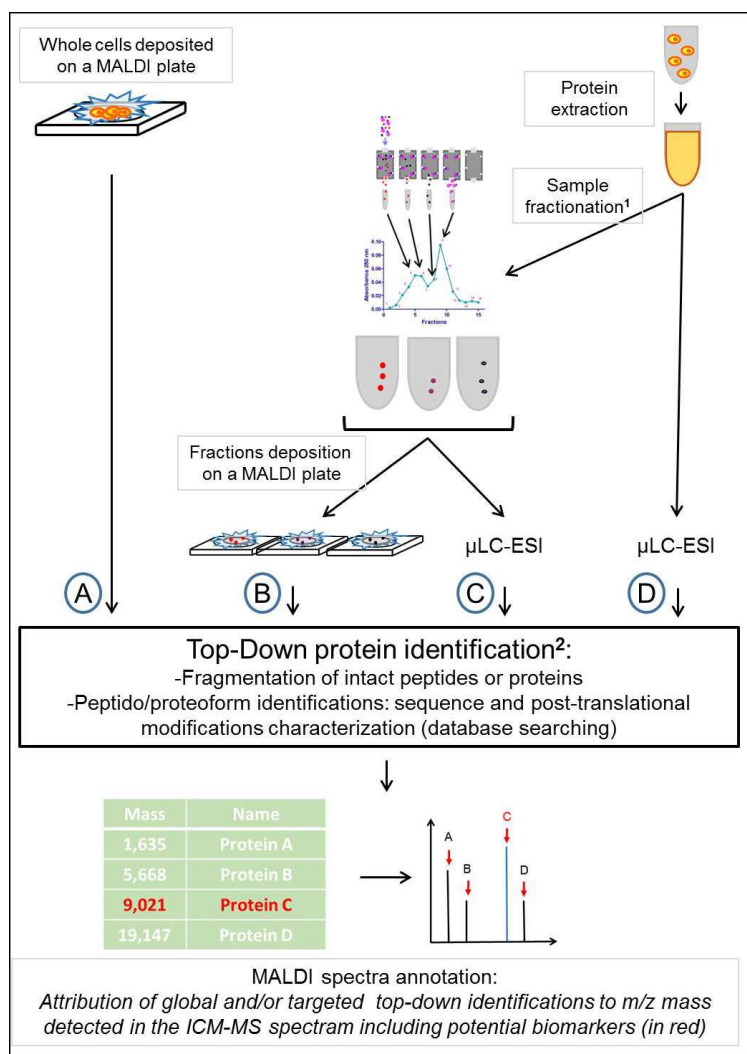


Figure 3

Year	Model	Objectives	Proteomic approach	m/z mass identification approach	Reference
2019	Neuroblastoma cell lines	Authentication of Drug-Adapted Cancer Cell Lines.	Targeted	no	58
2019	macrophages	Characterization of activation of macrophages	Targeted	no	60
2018	Bovine oocytes	Impact of meiotic maturation on single oocytes, cumulus cells and granulosa cells.	Global	Top-Down HRMS	76
2016	Chicken spermatozoa	Diagnosis of avian male fertility	Global	Top-Down HRMS	67
2015	Chicken spermatozoa	Phenotyping of semen to discriminate avian males on their reproductive capacity	Global	Top-Down HRMS	45
2015	Porcine Spermatozoa	Characterization of epididymal sperm maturation process	Global	Top-Down HRMS	68
2015	Human monocytes	Distinction of human monocyte subpopulations and monitorization of activation profiles of monocytes exposed to distinct microbial ligands	Global	No	29
2015	Fish cell line RTL-W1	Characterization of toxic effects and identification of toxins in complex environmental samples	Global	No	30
2015	HepG2 and THP-1 cells	Characterization of <i>in vitro</i> cellular responses to toxic chemicals	Global	No	26
2014	Cancer cells (chronic myelogenous leukemia cell line K562)	Label-free quantification of histone acetylation to measure the tumor-selective pharmacodynamic responses to drug in a mouse model of gastrointestinal cancer	Targeted	No	31
2012	Human macrophages	Monitorization of the various activation patterns of immune cells (macrophage)	Global	No	34
2012	Human monocytes and polymorphonuclear neutrophils	Phenotyping of mammalian primary cells and cell lines (HL-60, THP-1 and Hela S3)	Global	No	62
2011	Mammalian and insect cell lines	Cell line identification	Global	No	29
2011	Rodent glial cells	Brain cells phenotyping (astroglial, microglial, and oligodendroglial cells)	Global	Bottom-up using one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by LC-MS/MS	27
2011	Chinese hamster ovary cells	Selection of stable and high-producing cell lines for monoclonal antibody (mAb) IgG	Global	No	33
2011	Sea slug neurons	Characterization of D-amino acid-containing peptides	Targeted	Top-Down MALDI-TOF-TOF	6

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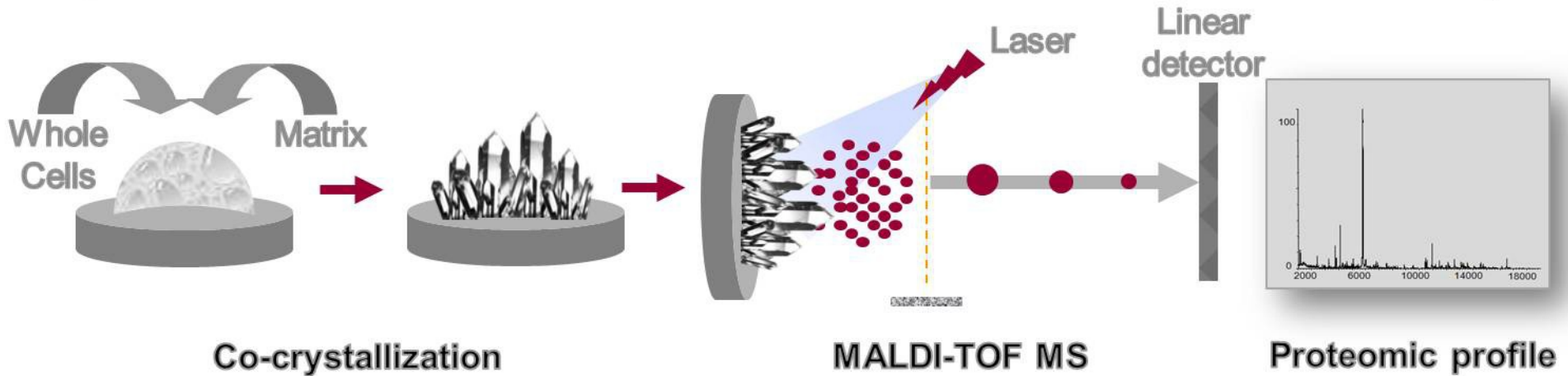
2010	Human and murine immune cells	Immune cells (monocytes, T lymphocytes and polymorphonuclear cells) phenotyping	Global	No	58
2010	Insects and animal cells	Cell lines phyloproteomics	Global	No	35
2010	Chinese hamster ovary cells	Characterization of low/high-producing cell lines for the production of recombinant biotherapeutics	Global	No	33
2009	Avian heterophils	Direct identification of mature β -defensin 2 in avian heterophils	Targeted	Edman sequencing and bottom-up	63
2008	Human colon epithelial carcinoma T84 cell line	Identification of cell differentiation markers	Global	Bottom-up using GeLC-MS/MS	77
2007	Chicken macrophages	Identification and characterization of thymosin beta-4	Target	Purification by HPLC and Bottom-up using MALDI-TOF	78
2007	Mouse pancreatic islet alpha and beta cells	Identification of secretory products (hormones) in endocrine cells	Global	No	79
2006	Human myelomonocytic and lymphoblast, rodent BHK-21	Identification of mammalian cell lines	Global	Top-Down HRMS, MALDI-TOF-TOF and PSD, bottom-up using nanoLC-MS/MS	80
1999	Rat adrenal pheochromocytoma PC 12 cells)	Profiling of mammalian cell line after stimulation with neurotrophic factor (nerve-growth factor)	Global	No	81

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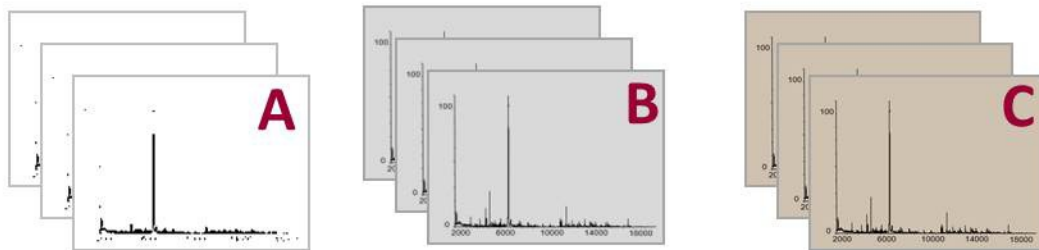
721 Table 1. Literature on superior eukaryotic Intact Cell MALDI-TOF MS studies.

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Intact Cell MALDI-TOF Mass Spectrometry



Differential analysis



Informatic treatments

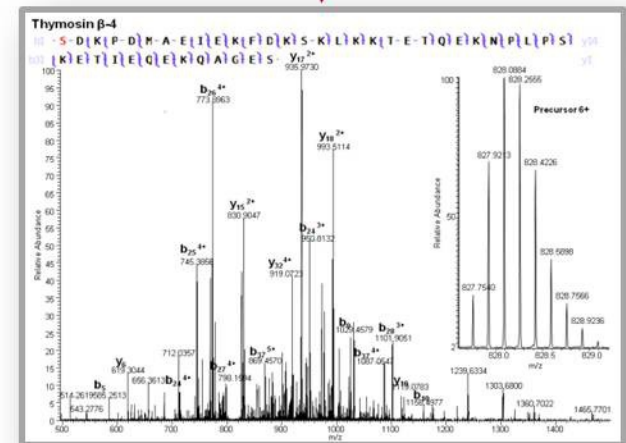
Internal calibration, Baseline subtraction, Smooth, Spectra alignment, Peak detection, Normalization

Statistical analyses for marker characterization

ANOVA, PCA, hierarchical clustering...

Top Down MS

Fragmentation of intact endogenous peptides/proteins



Sequence and PTM identifying pepti/proteoforms of interest