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Mini-Review

Proteomics of human spermatozoa

Judit Castillo [1,*], Alberto de la Iglesia[1,3] Marina Leiva[1], Meritxell Jodar[1,2], and Rafael Oliva [1,2,*]

[1]Molecular Biology of Reproduction and Development Research Group, Departament de Biomedicina, Facultat de Medicina i Ciències de la Salut, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Fundació Clínic per a la Recerca Biomèdica, Universitat de Barcelona (UB), Barcelona, Spain
[2]Biochemistry and Molecular Genetics Service, Biomedical Diagnostic Center (CDB), Hospital Clínic de Barcelona, Barcelona, Spain
[3]Present address: Université Paris Cité, INSERM, CNRS, Institut Cochin, F-75014 Paris, France

*Correspondence address. Molecular Biology of Reproduction and Development Research Group, Departament de Biomedicina, Facultat de Medicina i Ciències de la Salut, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Fundació Clínic per a la Recerca Biomèdica, Universitat de Barcelona (UB), Casanova 143, 08036 Barcelona, Spain. E-mail: juditcastillo@ub.edu (J.C.) https://orcid.org/0000-0002-9407-2675; Molecular Biology of Reproduction and Development Research Group, Departament de Biomedicina, Facultat de Medicina i Ciències de la Salut, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Fundació Clínic per a la Recerca Biomèdica, Universitat de Barcelona (UB), Casanova 143, 08036 Barcelona, Spain. E-mail: roliva@ub.edu (R.O.) https://orcid.org/0000-0003-4876-2410

ABSTRACT

Proteomic methodologies offer a robust approach to identify and quantify thousands of proteins from semen components in both fertile donors and infertile patients. These strategies provide an unprecedented discovery potential, which many research teams are currently exploiting. However, it is essential to follow a suitable experimental design to generate robust data, including proper purification of samples, appropriate technical procedures to increase identification throughput, and data analysis following quality criteria. More than 6000 proteins have been described so far through proteomic analyses in the mature sperm cell, increasing our knowledge on processes involved in sperm function, intercommunication between spermatozoa and seminal fluid, and the transcriptional origin of the proteins. These data have been complemented with comparative studies to ascertain the potential role of the identified proteins on sperm maturation and functionality, and its impact on infertility. By comparing sperm protein profiles, many proteins involved in the acquisition of fertilizing ability have been identified. Furthermore, altered abundance of specific protein groups has been observed in a wide range of infertile phenotypes, including asthenozoospermia, oligozoospermia, and normozoospermia with unsuccessful assisted reproductive techniques outcomes, leading to the identification of potential clinically useful protein biomarkers. Finally, proteomics has been used to evaluate alterations derived from semen sample processing, which might have an impact on fertility treatments. However, the intrinsic heterogeneity and inter-individual variability of the semen samples have resulted in a relatively low overlap among proteomic reports, highlighting the relevance of combining strategies for data validation and applying strict criteria for proteomic data analysis to obtain reliable results. This mini-review provides an overview of the most critical steps to conduct robust sperm proteomic studies, the most relevant results obtained so far, and potential next steps to increase the impact of sperm proteomic data.

Keywords: sperm / proteomics / mass spectrometry / male infertility / biomarkers / semen quality

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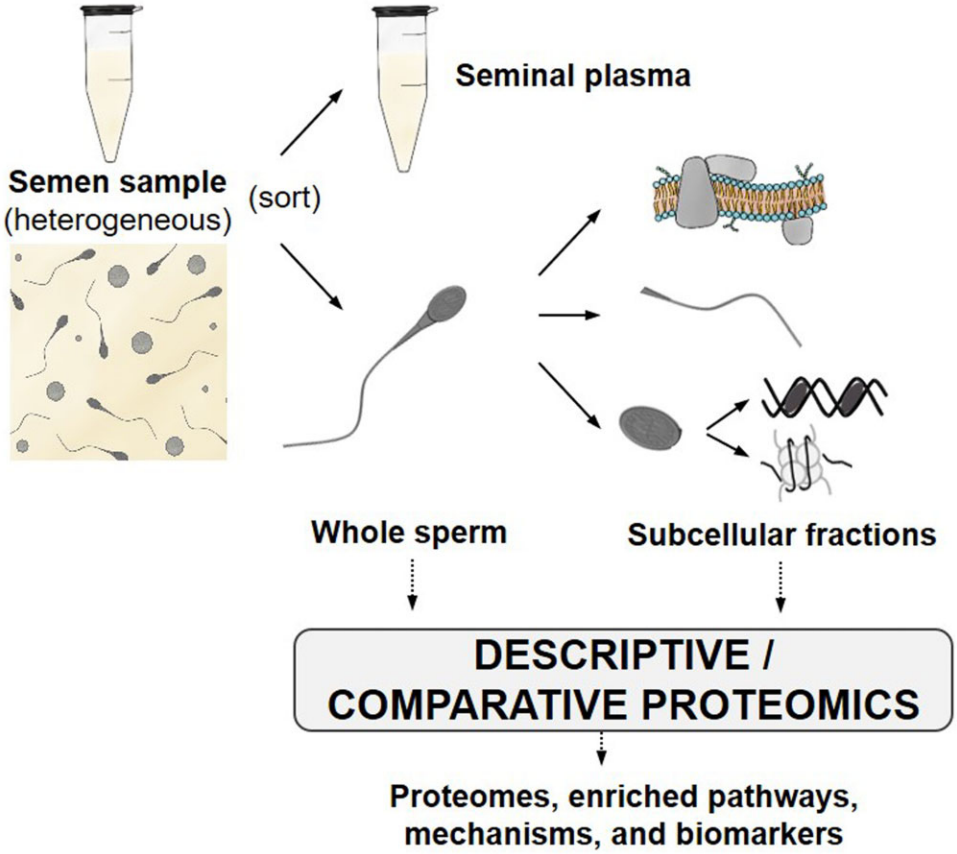
GRAPHICAL ABSTRACT



Sperm proteomics studies are based on the evaluation of either the whole cell or subcellular fractions to describe or compare protein profiles and search for enriched pathways, mechanisms and biomarkers.

Introduction

Proteins are the main effectors of cell phenotypes, and their study provides insights on molecular aspects related to function and dysfunction. Proteomics is the systematic large-scale analysis of the proteins contained in a cell, tissue, or organism and has been revealed as an extremely powerful approach for cell evaluation under normal and pathological conditions. In the field of human reproduction, a remarkable number of studies have applied proteomic strategies to evaluate sperm composition (Baker *et al.*, 2013; Amaral *et al.*, 2014a; Castillo *et al.*, 2018; Serrano *et al.*, 2022, Zhang *et al.*, 2022). A technical advantage that makes semen a suitable biological sample for proteomic studies is the relatively easy access to large numbers of male germ cells from a single individual through a non-invasive procedure, as well as the possibility to conduct cell population enrichments to overcome the intrinsic heterogeneity of the semen sample. Furthermore, the highly structural and functional compartmentalization of sperm cells enables subcellular fractionation prior to analysis, reducing the complexity of the protein extract and increasing the throughput of the procedure. The sperm-specific high degree of chromatin compaction has been related to the blockage of nuclear transcription in the ejaculated sperm, which has been also traditionally considered an advantage that simplifies the analysis and interpretation of proteomic data (Amaral *et al.*, 2014a). However, it should be considered that when evaluating subsequent biological processes required for a proper sperm function, such as capacitation, proteomic data analysis might increase in complexity. In this case, the presence of post-translational modifications biasing differences in protein abundance (Castillo *et al.*, 2019), as well as potential protein transcription through sperm mitochondrial machinery (Gur and Breitbart, 2006, 2008), must be taken into consideration.

In this mini-review, we provide concise information of the most critical steps to be considered when designing a mass spectrometry (MS)-based proteomic procedure with human sperm, from sample preparation to data analysis, to ensure reliability and robustness on the results. Furthermore, we summarize relevant data obtained from sperm proteomics descriptive and comparative studies, based on more than 80 works published from 2006 to 2022, either individually cited or as part of comprehensive reviews. This information may be complemented by additional excellent reviews on the topic (Nowicka-Bauer and Kurpisz, 2013; Amaral *et al.*, 2014a; Codina *et al.*, 2015; Griffin *et al.*, 2019; Llavanera *et al.*, 2022; Serrano *et al.*, 2022; Zhang *et al.*, 2022).

Design of a sperm proteomic study

Although the development of sperm proteomic studies might be considered simple due to sample accessibility and the low complexity of the sperm in terms of active biological processes, there are critical points that should be controlled to avoid false-positive identifications (Codina *et al.*, 2015). Semen is a heterogenic fluid comprising cellular and non-cellular components accounting for 5% and 95% of the volume, respectively (Jodar *et al.*,

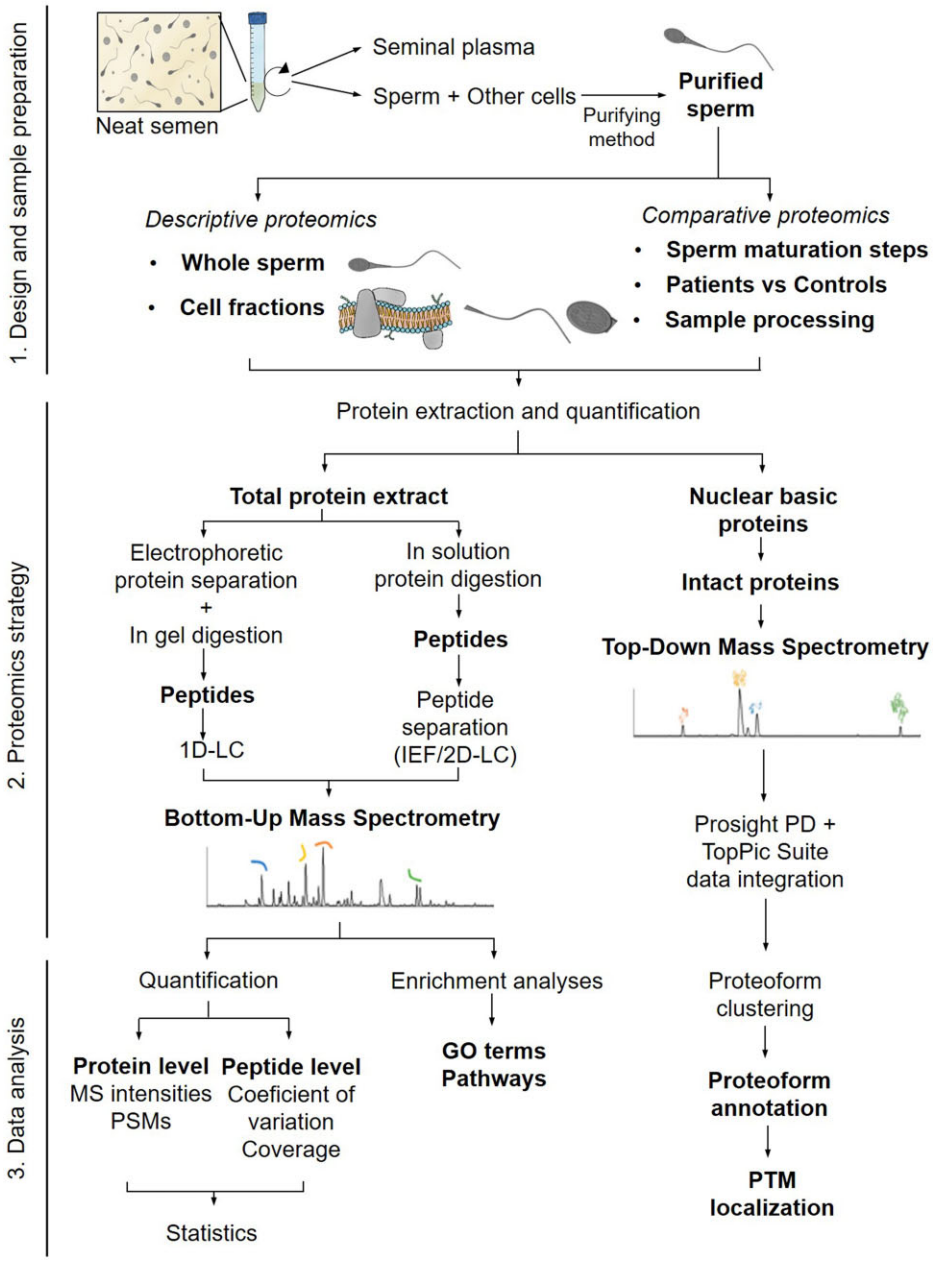


Figure 1. Critical steps in the development of proteomics studies in sperm. A sperm proteomic pipeline is divided in three main steps that should be carefully considered and well-designed. (1) Design of the study and sample preparation, which includes proper sperm isolation, purification and preparation for either descriptive proteomics (of the whole sperm or subcellular fractions such as membranes, tails and nuclei) or comparative proteomics (for the search of protein biomarkers) studies. (2) Selection of the most suitable proteomics strategy, either Bottom-Up (for the analysis of peptides) or Top-Down (for the analysis of intact proteins) mass spectrometry. When conducting Bottom-up proteomics, strategies to reduce the complexity of the protein extract, either at protein or peptide level, are recommended to increase the throughput of the technique. (3) Data analysis, the features of which will be different according to the proteomics strategy followed. For robust quantitative Bottom-Up proteomics, combined statistical analysis at both the protein and peptide level is recommended, to avoid false-positive differences. 1D-LC: one-dimensional liquid chromatography; IEF: iso-electric focusing; 2D-LC: two-dimensional liquid chromatography; GO: gene ontology; PTM: post-translational modifications.

2017). Although the cellular component mainly corresponds to spermatozoa, other cells with distinct protein and chromatin composition, such as immature spermatogenic cells and leucocytes, are also present even under normal conditions. While the presence of contaminating cells might not impact the identification of enriched biological processes associated with sperm function (Selvam *et al.*, 2019c), it would be critical in the search for useful sperm biomarkers and when focusing on sperm specific structures, such as chromatin. In those cases, false-positive identifications in non-pure sperm samples contribute to reduced reliability and robustness of the results (Amaral *et al.*, 2014a; Codina *et al.*, 2015). Therefore, adequate sample purification procedures are demanded as an indispensable step prior to protein extraction (Fig. 1). A recommended strategy to evaluate the impact of the presence of contaminating cells in sperm samples is measuring the proportion of non-sperm cells over total spermatozoa (Codina *et al.*, 2015). In some particular cases, such as in the study of sperm chromatin-associated proteins, additional

approaches as the expression analysis of specific leucocyte markers are also recommended to discard the somatic cell contribution to the results (Castillo et al., 2014a).

Another layer of complexity of sperm samples relies on the fact that protein lysates contain a few high abundant proteins giving rise to a higher proportion of peptides that may mask those from low abundant proteins. This fact can derive in poor identification capacity of some protein groups and loss of information from specific cell structures (Amaral et al., 2014a; Codina et al., 2015). Taking the advantage of sperm morphological compartmentalization, a relatively simple strategy to overcome this limitation consists on the study of specific sperm structures by subcellular fractionation, such as the membrane, the tail, or the nucleus (Naaby-Hansen and Herr, 2010; Nixon et al., 2011; Amaral et al., 2013; Baker et al., 2013; Castillo et al., 2014a,b) (Fig. 1). The influence of high-abundant proteins is also overcome by reducing protein extract complexity before identification by liquid chromatography followed by tandem mass spectrometry (LC-MS/MS), either at protein or peptide level (Fig. 1). Initial proteomic studies in human sperm were based on the processing of protein spots excised after two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (Martínez-Heredia et al., 2006). That strategy evolved over the years by applying different approaches for protein separation before digestion into peptides, such as mono-dimensional PAGE, or for peptide separation after digestion, such as isoelectrofocusing (Figure 1) (Amaral et al., 2014a; Baker et al., 2008; Codina et al., 2015). Nowadays, the separation of peptides by two-dimensional liquid chromatography is the strategy showing the highest throughput, which consists in an offline prefractionation of the peptide mixture by liquid chromatography and the subsequent analysis of the individual fractions by the standard LC-MS/MS (Nägele et al., 2004) (Fig. 1). These variations have remarkably increased the identification throughput from hundreds to thousands of sperm proteins (Wang et al., 2013; Codina et al., 2015) (Fig. 1).

Despite this comprehensive information on sperm composition, it is worth highlighting that protamines, the most abundant and functionally important proteins of the sperm cell (Oliva and Dixon, 1991; Oliva, 2006; Balhorn, 2007), are not identified by conventional MS strategies (de Mateo et al., 2011). This is due to the high abundance of arginine residues clusters in protamine sequences, which confers the required positive charge to tightly package DNA but precludes obtaining peptides long enough to be detectable (Oliva, 2006; Balhorn, 2007; Soler-Ventura et al., 2018; de la Iglesia et al., 2023). This particularity has promoted the adaptation of analytical techniques to identify proteins with no need of enzymatic digestion through Top-Down proteomics (Soler-Ventura et al., 2020; Arauz-Garofalo et al., 2021) (Fig. 1).

However, not only proper sample preparation is essential to obtain robust proteomic results but also accurate criteria for protein identification, quantification, data mining, and validation (Codina et al., 2015; Castillo et al., 2018) (Fig. 1). For instance, a strict selection criterion that would widely reduce the chances of false positive findings during protein quantification would be to only consider those proteins with at least one unique peptide quantified by ≥2 peptide-to-spectrum matches in all evaluated samples for further statistical analyses, and with a coefficient of variation <50% in at least 75% of the samples (Barrachina et al., 2019). Also, it is important to conduct similar statistical analyses at both protein and peptide levels, since it is possible to identify proteins with significant difference abundance although the corresponding individual peptides do not show statistical differences, and those should be discarded from the results (Castillo et al., 2019).

Descriptive proteomics helps in identifying sperm cell functions

Protein discovery in the human sperm cell

The most complete catalogue of human sperm proteins published to date compiled 6871 non-redundant proteins from 46 proteomic studies using ejaculated spermatozoa, either intact or fractionated, selected following strict quality criteria based on technical aspects (Castillo et al., 2018). Through the analysis of significant overrepresented pathways within sperm proteome, a wide number of active processes were discovered in the male gamete, including metabolism of carbohydrates, lipids, nucleotides, RNAs and proteins, as well as membrane trafficking, apoptosis, meiosis, and cell cycle (Amaral et al., 2014a). Additionally, deeper evaluation of sperm proteome through Gene Ontology (GO) annotations and comparison with phenotypic data from the Mouse Genome Annotation database revealed interesting sets of proteins with potential implication in fertilization and pre-implantation embryo development (Castillo et al., 2018).

While studies assessing whole sperm have increased our knowledge of the global molecular composition of the male gamete (Amaral et al., 2014a), evaluation of subcellular fractions have provided novel insights on sperm metabolism and contribution to fertilization and beyond. In particular, specific evaluation of human sperm tail proteome showed overrepresentation of mitochondrial fatty acid oxidation and peroxisomal metabolism pathways, highlighting lipid metabolism as an endogenous source of energy that might be more predominant than previously thought (Amaral et al., 2013). Regarding the process of fertilization, sperm membrane proteomics allowed the identification of calcium-binding proteins, heat shock proteins, and proteins with affinity for zona pellucida that may act as interactors with the oocyte (Naaby-Hansen and Herr, 2010; Nixon et al., 2011). Proteomics on isolated sperm nuclei and chromatin, instead, revealed the presence of an unexpected remarkable number of transcription factors, chromatin remodelling proteins, and chromatin-associated proteins known to undergo epigenetic functions in other cell types (de Mateo et al., 2011; Baker et al., 2013; Castillo et al., 2014a,b; Castillo et al., 2015). The proteomic study of sperm chromatin has been also complemented with the application of Top-Down MS. This proteomic variant has allowed the identification of a complex combination of unmodified and modified protamine forms, entailing a milestone in the description of the protein composition of the sperm nucleus and the search of potential alterations in patients (Soler-Ventura et al., 2020; Arauz-Garofalo et al., 2021). Altogether, these data supported potential roles for human sperm proteins transferred to the oocyte upon fertilization in the transmission of epigenetic information to the zygote.

Although much relevant functional information has been obtained by the *in silico* analysis of sperm protein catalogues, discovery proteomics is only the first step to decipher cell biology. To ascertain the potential role of sperm proteins and their impact on fertility, it is required to complement these results with proteomic strategies targeting alterations under pathological conditions, as discussed in further sections.

Transcriptional origin of sperm protein content

Data mining of descriptive proteomic reports have provided relevant information on the potential tissue origin of the proteins

and inter-communication between cells and fluids (Castillo *et al.*, 2018). Integrative analysis of the human sperm proteome with expression data from testis, epididymis, seminal vesicles, and prostate highlighted 165 human sperm proteins that may be incorporated after testicular maturation (Castillo *et al.*, 2018). These results pointed to the participation of seminal plasma in the final protein composition of the male gamete, potentially providing components that may be essential for oocyte fertilization and further developmental processes (Sullivan and Saez, 2013; Jodar *et al.*, 2017; Castillo *et al.*, 2018). In this regard, several groups have focused their recent research analysis of extracellular vesicles isolated from the seminal plasma and have demonstrated their attachment to the sperm surface (Neyroud *et al.*, 2021; Barrachina *et al.*, 2022). In fact, proteomic studies in seminal extracellular vesicles have identified proteins required for sperm motility or fertilization (Lin *et al.*, 2019). However, available proteomic data should be taken carefully since the isolating methods used so far do not ensure proper exclusion of contaminating sperm debris, demanding further technical development and validation.

Sperm proteomic changes concomitant to the acquisition of fertilizing ability

After spermatogenesis, the sperm cell requires further maturation, both in the male and female reproductive tracts, before attaining fertilizing ability. During epididymal transit, the spermatozoon acquires motility while it matures its membrane composition and stabilizes chromatin structure (Gervasi and Visconti, 2017; Skerrett-Byrne *et al.*, 2022). However, being motile and well-shaped is not enough to reach and fertilize the oocyte. Sperm capacitation is required to hyperactivate the male gamete and gain the ability to undergo the acrosome reaction, oocyte membrane penetration, and sperm–oocyte fusion in the female reproductive tract (Liu *et al.*, 2007; Stival *et al.*, 2016; Castillo *et al.*, 2018). Considering the male gamete as transcriptionally inert, proteins have been suggested as the main actors causing these sequential and regulated physiological changes, and proteomics is seen as a golden tool to increase this knowledge.

The most suitable strategy to evaluate the relevance of proteins involved in the acquisition of sperm fertilizing ability, and to identify potential novel protein candidates, is the conduction of quantitative proteomics comparing protein levels among different maturation stages. This approach is limited for the study of epididymal maturation in humans due to practical and ethical issues. However, it is easily applicable to evaluate capacitation and the acrosome reaction, which can be induced by *in vitro* incubatation of ejaculated sperm depleted of seminal plasma with specific media. In fact, a remarkable number of comparative studies have been conducted in capacitated sperm (Ficarro *et al.*, 2003; Secciani *et al.*, 2009; Wang *et al.*, 2015; Yu *et al.*, 2015; Castillo *et al.*, 2019; Hernández-Silva *et al.*, 2020; Chhikara *et al.*, 2023). The challenge of analysing these reports is to understand the mechanisms leading to distinct protein abundances in the sperm after the treatment. Translation of nuclear encoded mRNAs by the sperm mitochondrial machinery has been suggested during capacitation, which would explain the increased abundance detected for some sperm proteins in many reports studying this process (Gur and Breitbart, 2006, 2008). However, it is required to also consider the participation of mechanisms of protein modification, degradation and translocation in these changes (Ficarro *et al.*, 2003; Ickowicz *et al.*, 2012; Battistone *et al.*, 2014; Sati *et al.*, 2014; Wang *et al.*, 2015; Puga Molina *et al.*, 2017; Ritagliati *et al.*, 2018). When a particular biological process induces the elimination of post-translational modifications, it favours the identification of peptides that were not identified in the initial sample due to the change in their mass. This fact leads to biased increases in protein quantities (Castillo *et al.*, 2019).

The role of post-translational modifications in the acquisition of sperm fertilizing capability has motivated the application of proteomic pipelines targeting specific modified peptides. Label-free quantitative phosphoproteomics has allowed the specific identification of tyrosine-phosphorylated proteins, and highlighted insulin growth factor 1 receptor (IGF1R) tyrosine kinase as being required to regulate sperm capacitation in humans (Wang *et al.*, 2015). Similarly, lysine acetylated proteins involved in sperm energy and carbohydrate metabolism, as well as associated with zona pellucida receptor complex, have been identified in the acetylproteome of human capacitated sperm, stressing the impact of this modification in the process of fertilization (Sun *et al.*, 2014). The crucial role of glycosylation on cell–cell interactions and modulation of the sperm surface, which are essential for sperm capacitation, the acrosome reaction and sperm–oocyte interaction, has also motivated the conduction of glycoproteomics (Xin *et al.*, 2022). Of note, a set of seminal plasma glycosylated proteins was revealed to interact with the gametes to maintain sperm structure and stability, inhibit premature sperm capacitation, and facilitate sperm survival in the female reproductive tract (Saraswat *et al.*, 2016; Sun *et al.*, 2020).

Additional proteomic-based approaches arose to improve protein identification. This is the case of Multiple-Reaction Monitoring (MRM) MS, which provides higher specificity and sensitivity and is optimal to identify selected targeted proteins within complex mixtures. A recent report applied this strategy to study the abundance of the HSP70 chaperone protein family members along capacitation, showing no significant differences in any of the detected isoforms (Grassi *et al.*, 2022). These results contrast with the suggested role of HSPA2 in human sperm motility, capacitation and relocation of surface proteins needed for sperm interaction with the zona pellucida (Redgrove *et al.*, 2012), and may support the importance of protein modifying mechanisms in its function.

Proteomic studies have emerged as a valuable source of information about proteins and protein mechanisms involved in the acquisition of human sperm fertilizing ability. However, when comparing the different studies, both intra- and inter-proteomic report variabilities are detected. This is mainly due to the artificial induction of sperm maturation stages, which might not accurately mimic the natural trigger of the process. Also, the complete sperm population present in the semen sample is generally used for assessment, when in the female tract only a small proportion of the spermatozoa will be able to reach the oocyte. It is thus important to follow strict criteria for data analyses, as well as to combine data from different studies applying different proteomic approaches, to increase the chances of obtaining a comprehensive picture of the post-testicular sperm maturation protein events and translate these results to the evaluation of male infertility.

Proteomic alterations in infertile patients and potential clinical implications

Asthenozoospermia is the most well studied phenotype from a proteomic perspective, most likely due to the availability of large amounts of material suitable for analysis (Zhao *et al.*, 2007; Martínez-Heredia *et al.*, 2008; Amaral *et al.*, 2014a,b;

Hashemitabar et al., 2015; Liu et al., 2015; Cao et al., 2018; Guo et al., 2019; Sinha et al., 2019; Yang et al., 2022). Concordant results among studies have shown alterations on proteins related to energy production, including glycolysis, gluconeogenesis, oxidative phosphorylation and fatty acid oxidation, in addition to sperm tail structure and cytoskeleton (Amaral et al., 2014a,b; Liu et al., 2015; Asghari et al., 2017; Saraswat et al., 2017; Cao et al., 2018; Moscatelli et al., 2019; Yang et al., 2022). Consistently, similar differences were also detected among sperm fractions differing in motility within the same normozoospermic samples (Amaral et al., 2014b). Of note, the seminal plasma proteome has also revealed protein differences associated with low sperm motility (Saraswat et al., 2017; Barrachina et al., 2019; Lin et al., 2019; Murdica et al., 2019; Wu et al., 2019), reinforcing the intercommunication and protein transfer from seminal plasma to sperm, either free or encapsulated in extracellular vesicles (Jodar et al., 2017; Barrachina et al., 2022).

In contrast, proteomic studies on oligozoospermic men are scarce, likely because of the limiting amounts of material and the difficulties for proper sample purification (Légaré et al., 2014; Giacomini et al., 2015; Liang et al., 2021). Despite that, many efforts have been dedicated to identify protein alterations in conditions leading to oligozoospermia, such as secondary hypogonadism, to identify altered abundances of what proteins related to spermatogenesis impairment and to epididymis and prostate epithelial cell secretory function (Grande et al., 2019).

An intriguing group of patients studied from a proteomic perspective is that formed by normozoospermic men with no known genetic and hormonal alterations, and whose partner is unable to become pregnant despite no female factor being reported (de Mateo et al., 2007; Selvam et al., 2019b). Studies on these males with unexplained infertility have evaluated ART outcome, including fertilization rate (Torra-Massana et al., 2021), embryo quality (Jodar et al., 2020), and pregnancy achievement (Azpiazu et al., 2014; McReynolds et al., 2014; Xue et al., 2019; Rivera-Egea et al., 2021), and much valuable data has been obtained. For instance, alterations in the abundance of proteins involved in epigenetic marking of the paternal chromatin were observed when no pregnancy was achieved after IVF (Azpiazu et al., 2014). Additionally, several components related to sperm telomere function correlated with poor embryo quality (Jodar et al., 2020), and dysregulation of mitochondrial proteins was found in patients experiencing fertilization failure (Torra-Massana et al., 2021). Recurrent pregnancy loss has been also studied by sperm proteomics, showing alterations in proteins involved in a wide set of functions, including energy production, oxidative stress, and the maintenance of cytoskeleton, acrosome, and chromatin (Mohanty et al., 2020; Xue et al., 2019; Naglot et al., 2021; Fernandez-Encinas et al., 2022).

Additional groups of patients studied by proteomics analyses have been those affected by oxidative stress and associated clinical conditions, such as varicocele, smoking, obesity, and male accessory gland infection and inflammation (Agarwal et al., 2019; Cannarella et al., 2020; Fernandez-Encinas et al., 2022; Ribeiro et al., 2022). Of note, important complementary information has been provided by the differential proteomic studies using animal models (Kim et al., 2013; Griffin et al., 2019; Skerrett-Byrne et al., 2022). Furthermore, sperm protein alterations have been reported in testicular cancer patients (Selvam et al., 2019a; Dias et al., 2020; Martins et al., 2020), consistent with a potential paracrine deregulation of testicular homeostasis (Barrachina et al., 2022).

The application of proteomics has also revealed the impact of sperm processing on the protein composition of the male gamete. For instance, cryopreservation procedures seem to affect a wide range of pathways involved in energy production, oxidative stress, and membrane functionality, including acrosomal integrity, mitochondrial membrane potential, capacitation, and the acrosome reaction (Bogle et al., 2017; Fu et al., 2019; Li et al., 2019; Corda et al., 2022; Hezavehei et al., 2022). The effect of the different sperm selection methods has been also studied as a way to improve ART, although further research in this topic is clearly needed (Giacomini et al., 2015; Torabi et al., 2017).

Concluding remarks

The study of the human mature sperm using proteomic strategies has resulted in valuable data allowing us to unravel many processes involved in sperm function and dysfunction, with an impact on oocyte fertilization and male infertility. However, to ensure a robust interpretation of the results, it is necessary to know and understand all those characteristics that make the semen a unique model to study. When working with sperm, identification of proteins does not necessarily imply functionality, but could represent leftovers of past processes taking place during spermatogenesis. In addition, it is important to consider the intrinsic presence of inter- and intra-individual variability in the studies, due to semen heterogeneity, with multiple populations of sperm differing in quality and ability to acquire fertilizing potential.

Furthermore, experiments focused on the discovery of sperm proteomics biomarkers have shown, so far, a relatively low concordance between different laboratories, hampering the validation of protein candidates, and the translational potential to be applied in the routine activity of the reproductive clinics. This limitation might be related to imprecise sample preparation or non-robust data analysis pipelines. Current newly developed strategies for proteomics data analysis highlight the importance of strict inclusion criteria of differentially abundant proteins and the relevance of combined data analysis at both protein and peptide levels (Barrachina et al., 2019; Castillo et al., 2019; Torra-Massana et al., 2021). When there is no consistency among the relative abundance of several peptides belonging to the same protein, it is important to understand why they show different behaviours. In some cases, this difference is explained by proteolytic events or presence of genetic variants, isoforms or post-translational modifications on that specific peptide. Thus, accurate sample preparation, a robust proteomic pipeline design and appropriate proteomic data analysis are the backbone for the proper development of reliable semen proteomics studies.

Data availability

Data sharing is not applicable to this article as no new data were created or analysed.

Authors' roles

J.C., A.d.l.I., M.L., M.J., and R.O. participated in the manuscript design, drafting and editing. All authors critically reviewed and approved the final version of the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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