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Differential Proteomic Analysis of Human Sperm: A Systematic Review to Identify Candidate Targets to Monitor Sperm Quality

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Purpose: The advent of proteomics provides new opportunities to investigate the molecular mechanisms underlying male infertility. The selection of relevant targets based on a single analysis is not always feasible, due to the growing number of proteomic studies with conflicting results. Thus, this study aimed to systematically review investigations comparing the sperm proteome of normozoospermic and infertile men to define a panel of proteins with the potential to be used to evaluate sperm quality.

Materials and Methods: A literature search was conducted on PubMed, Web of Science, and Scopus databases following the PRISMA guidelines. To identify proteins systematically reported, first the studies were divided by condition into four groups (asthenozoospermia, low motility, unexplained infertility, and infertility related to risk factors) and then, all studies were analysed simultaneously (poor sperm quality). To gain molecular insights regarding identified proteins, additional searches were performed within the Human Protein Atlas, Mouse Genome Informatics, UniProt, and PubMed databases.

Results: Thirty-two studies were included and divided into 4 sub-analysis groups. A total of 2752 proteins were collected, of which 38, 1, 3 and 2 were indicated as potential markers for asthenozoospermia, low motility, unexplained infertility and infertility related to risk factors, respectively, and 58 for poor sperm quality. Among the identified proteins, ACR, ACRBP, ACRV1, ACTL9, AKAP4, ATG3, CCT2, CFAP276, CFAP52, FAM209A, GGH, HPRT1, LYZL4, PRDX6, PRSS37, REEP6, ROPN1B, SPACA3, SOD1, SPEM1, SPESP1, SPINK2, TEKT5, and ZPBP were highlighted due to their roles in male reproductive tissues, association with infertility phenotypes or participation in specific biological functions in spermatozoa.

Conclusions: Sperm proteomics allows the identification of protein markers with the potential to overcome limitations in male infertility diagnosis and to understand changes in sperm function at the molecular level. This study provides a reliable list of systematically reported proteins that could be potential targets for further basic and clinical studies.

Keywords: Biological processes; Male infertility; Proteome; Proteomics; Spermatozoa

INTRODUCTION

On a global scale, infertility affects 8%–12% of reproductive-age couples. Of these, the male factor is responsible for 20-30% of the total, yet contributes to 50% of overall infertility cases [1]. A complex set of aetiologies and risk factors are known to correlate with male infertility. Nevertheless, the precise mechanisms responsible for male infertility remain unknown in 30%–50% of patients [2]. The cornerstone tool to diagnose male infertility is semen analysis, facilitating the clinical evaluation of both macro and microscopic aspects of semen samples [3,4]. These parameters can crudely distinguish fertile from infertile men according to the World Health Organization (WHO) guidelines [5]. However, the reality is that semen analysis has a relatively poor diagnostic value; this technique does not identify defects associated with the functional and molecular aspects of human sperm, and it fails to reliably predict fertilization potential and pregnancy success [6-8]. To improve infertility diagnosis, additional sperm functional tests have emerged to further evaluate key features of spermatozoa such as DNA fragmentation, assessment of reactive oxygen species and oxidative stress, membrane ion channels, acrosome reaction and mitochondrial function [4,9]. Nonetheless, these tests generally lack accuracy, often require a subjective interpretation of data, and also fail to explain underlying molecular causes of infertility [6,10].

Currently, proteomics is a powerful source of information to further understand and characterize the molecular mechanisms underlying both physiological and pathological conditions. In the reproductive field, several analyses have sought to characterize the proteome of ejaculated spermatozoa [11,12]. Indeed, spermatozoa are an ideal cell type for such studies as they can be isolated as a highly purified and relatively homogeneous material source. Additionally, since spermatozoa are considered quiescent cells presumably lacking active transcription and translation, the sperm proteome reflects the protein content of mature cells [13]. Regarding male infertility, proteomics has been applied to further understand changes in the sperm proteome and identify dysregulated pathways in certain clinical conditions [14,15]. In these proteomic studies, ejaculated spermatozoa from normozoospermic and infertile men were compared to identify differentially expressed proteins (DEPs). Through bioinformatic workflows, the DEPs are analysed to unveil altered pathways and defective biological processes allowing the molecular understanding of deregulated mechanisms [16,17]. Such bioinformatics analyses are also useful to identify potential biomarkers and therapeutic targets for subsequent investigation [17,18].

Considering the high number of available proteomic studies performed on human sperm, it is not feasible to draw robust conclusions based on single studies. The main objective of this systematic review was to compare human sperm proteomes from infertile and normozoospermic men and to identify a panel of candidate protein that could serve as potential biomarkers to evaluate male infertility. Also, using the available information related to those proteins in spermatozoa, we intended to highlight the most relevant examples.

MATERIALS AND METHODS

1. Protocol and registration

This review was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) [19,20]. The review protocol was registered on the international prospective register for systematic reviews – PROSPERO – before data extraction was completed (CRD42021257114).

2. Search strategy

An extensive search of the literature, published before 1st January 2023, was performed using the PubMed, Web of Science, and Scopus databases. Four classes of terms were defined and combined using Boolean operators to direct searchers to proteomic studies that analysed sperm samples from donors with different conditions related to male infertility. The terms included in each class are summarized in Supplement Table 1. Relevant articles referenced in the included studies were further evaluated for potential inclusion in this review.

3. Selection criteria

Among the identified studies, only those conforming to six inclusion criteria were selected: 1) publication in an indexed journal with full text in English or Portuguese; 2) freshly ejaculated human sperm samples were evaluated according to WHO guidelines [5]; 3) studies comparing a minimum of two experimental classes: (a) a group composed of men with poor-sperm quality re-

lated to significant changes in sperm parameters (concentration, motility, morphology, DNA fragmentation, ROS levels); male infertility conditions; poor reproductive outcomes; or conditions affecting fertility potential (lifestyle, systemic diseases, other relevant biological factors); (b) a control group composed of normozoospermic healthy donors; 4) spermatozoa isolated from whole semen samples using washing protocols (simple washing gradient or density washing gradient); 5) quantitative proteomic analyses; and 6) proteins were described using the gene name and/or UniProt/SwissProt ID. Review articles, metanalyses, commentaries, and proteomic analyses performed in other species were positively excluded and recorded, with descriptive exclusion criteria, in Supplement Table 2.

4. Data collection

Literature searches and study screens were independently performed by two reviewers (POC and JM). Disagreements between the judgments of the two primary reviewers were resolved by a third individual (JVS). From each included proteomic study, information related to the number of participants (n), participants' age, clinical condition evaluated, proteomic methodologies and parameters used, and the number of DEPs were collected. Additionally, DEPs were retrieved and recorded with the respective fold-change (increased or decreased). To avoid redundancy, all DEPs were mapped in the UniProt database and annotated with UniProtKB/Swiss-Prot accession number (downloaded on 5th January 2023) (Supplement Table 3).

5. Quality of evidence assessment

To evaluate the quality of the included proteomic studies, the risk of bias assessment QUADAS-2 tool was employed [21]. Reviewers' judgments were aided by available signalling questions. Each question was answered with "yes", "no" or "unclear", where "yes" indicates a low risk of bias. The risk of bias was classified as "low", "high", or "some concern". The plot and graph of the risk of bias were created through the Robvis application [22]. These evaluations were performed independently, by two reviewers, as described above.

6. Identification of protein candidates

To identify a list of potential protein candidates as sperm quality markers, two complementary approaches were used. Firstly, to identify protein candidates for specific male infertility conditions, four subgroups were defined: (i) asthenozoospermia (AZS); (ii) low sperm motility; (iii) unexplained infertility; and (iv) infertility related to risk factors. Proteins were considered candidates if they were reported in a minimum of three independent analyses. Secondly, to unveil protein candidates associated with poor sperm quality under different conditions, all included studies were cross compared. Proteins were highlighted as candidates if they were identified in at least two distinct conditions and at least four independent analyses. In both approaches, a Venn diagram analysis was performed to cross-compare increased and decreased DEPs using the JVenn tool [23]. In both analyses, proteins with inconsistent expression patterns in different studies were excluded from further evaluation.

7. Detailed molecular insights of identified DEPs

Data regarding the selective expression of identified DEPs in the male reproductive tract were collected from the Human Protein Atlas (HPA) (version 22.0, downloaded on 5th January 2023) [24]. To predict any potential associations between the identified DEPs and male infertility defects and phenotypes, the Mouse Genome Informatics (MGI) database was employed (downloaded on 5th January 2023) [25]. The UniProt database provided information regarding gene ontology (downloaded on 5th January 2023). From the information collected, only terms related to sperm physiology and processes were retrieved. Finally, all DEPs were searched within the PubMed database to identify studies that report the role of these proteins in mammalian spermatozoa (until 10th January 2023).

RESULTS

1. Description of the included studies

A total of 1,133 articles were identified by a literature search of the PubMed, Web of Science, and Scopus databases and screening of references associated with such publications. After duplicate exclusion and initial screening, 423 articles were fully evaluated. A total of 32 studies [26-57] were selected for inclusion in this review (Table 1, Fig. 1). A majority of these studies used sperm samples from independent men as a control group and/or condition group (Table 1). Wu et al [52] employed samples collected from the same men

Table 1. Summary of the included proteomic studies

| No | Reference | Participants | | | Proteomic method | Number of DEPs | | Analysis group |
		Condition	Controls (#)	Cases (#)		Reported	Mapped	
1	Chhikara et al, 2023 [48]	AZS	3 normozoospermic men	12 asthenozoospermic men	Label free and LC-MS/MS	62 DEPs: ↑7 and ↓55	62 DEPs: ↑7 and ↓55	(i) AZS
2	Grande et al, 2022 [50]	Secondary hypogonadism	5 normogonadic normozoospermic men	5 hypogonadic men	TMT labelling and LC-MS/MS	43 DEPs: ↑11 and ↓32	43 DEPs: ↑11 and ↓32	Other sperm alterations
3	Liu et al, 2022 [27]	Low motility	20 normozoospermic men (high motile sperm fraction)	Same 20 normozoospermic men (low motile sperm fraction)	2DE and MALDI-TOF/TOF-MS	21 DEPs: ↑13 and ↓8	21 DEPs: ↑13 and ↓8	(ii) Low motility
4	Yang et al, 2022 [26]	AZS	7 normozoospermic men	11 asthenozoospermic men	UHPLC	1,430 DEPs: ↑701 and ↓729	1,430 DEPs: ↑701 and ↓729	(i) AZS
5	Liang et al, 2021 [38]	Severe oligoasthenoteratozoospermia	12 normozoospermic men	12 severe oligoastheno-teratozoospermic men	iTRAQ labelling and 2D-LC-MS/MS	938 DEPs: ↑226 and ↓712	938 DEPs: ↑226 and ↓712	Other sperm alterations
6	Naglot et al, 2021 [51]	Recurrent pregnancy loss	3 normozoospermic men	3 normozoospermic men whose spouse had suffered three or more miscarriages	Label free and LC-MS/MS	27 DEPs: ↑22 and ↓5	27 DEPs: ↑22 and ↓5	(iii) Unexplained infertility
7	Mohanty et al, 2020 [49]	Recurrent pregnancy loss	20 normozoospermic men	16 normozoospermic men whose spouse had suffered two miscarriages	Nano-UHPLC-MS/MS	23 DEPs: ↑8 and ↓15	23 DEPs: ↑8 and ↓15	(iii) Unexplained infertility
8	Wu et al, 2020 [52]	Hyperthermia	10 normozoospermic men	Same 10 normozoospermic men (exposed to heat)	iTRAQ labelling and 2D-LC-MS/MS	61 DEPs: ↑28 and ↓33	61 DEPs: ↑28 and ↓33	(iv) Risk factor
9	Guo et al, 2019 [53]	Globozoospermia	3 normozoospermic men	3 globozoospermic men	TMT labelling and LC-MS/MS	491 DEPs: ↑370 and ↓121	462 DEPs: ↑347 and ↓114	Other sperm alterations
10	Guo et al, 2019 [54]	AZS	5 normozoospermic men	5 asthenozoospermic men	TMT labelling and LC-MS/MS	152 DEPs: ↑84 and ↓68	152 DEPs: ↑84 and ↓68	(i) AZS
11a	Moscatelli et al, 2019 [55]	AZS	3 normozoospermic men	3 asthenozoospermic men	Label free and LC-MS/MS	86 DEPs: ↑35 and ↓51	84 DEPs: ↑34 and ↓50	(i) AZS
11b	Moscatelli et al, 2019 [55]	Severe AZS		3 severe asthenozoospermic men		88 DEPs: ↑41 and ↓47	88 DEPs: ↑41 and ↓47	(i) AZS
12	Sinha et al, 2019 [56]	AZS	5 normozoospermic men	5 asthenozoospermic men	2D-DIGE and MALDI-TOF/MS	7 DEPs: all ↓	7 DEPs: all ↓	(i) AZS
13	Xue et al, 2019 [57]	Recurrent pregnancy loss	7 fertile normozoospermic men	10 normozoospermic men whose spouse had suffered two miscarriages	iTRAQ labelling and HPLC-MS/MS	38 DEPs: ↑25 and ↓13	37 DEPs: ↑25 and ↓12	(iii) Unexplained infertility
14	Liu et al, 2018 [28]	ZP binding failure	20 normozoospermic men with IVF pregnancy	20 normozoospermic men with R-ICSI pregnancy	iTRAQ labelling and MALDI-TOF/TOF-MS	56 DEPs: ↑36 and ↓20	56 DEPs: ↑36 and ↓20	(iii) Unexplained infertility

Table 1. Continued 1

No	Reference	Condition	Participants		Proteomic method	Number of DEPs		Analysis group
			Controls (#)	Cases (#)		Reported	Mapped	
15	Nowicka-Bauer et al, 2018 [29]	AZS	10 normozoospermic men	4 asthenozoospermic men	2DE and MALDI-TOF/MS	25 DEPs: ↑18 and ↓7	23 DEPs: ↑16 and ↓7	(i) AZS
16	Wang et al, 2018 [30]	Low PR	10 normozoospermic men with highly fecundity (PR>50%)	10 normozoospermic men with low fecundity (PR=0)	2D-DIGE and MALDI-TOF/MS	25 DEPs: ↑8 and ↓17	25 DEPs: ↑8 and ↓17	(iii) Unexplained infertility
17	Saraswat et al, 2017 [31]	AZS	5 normozoospermic men	8 asthenozoospermic men	Label free and UHPLC-MS	341 DEPs: ↑167 and ↓174	341 DEPs: ↑167 and ↓174	(i) AZS
18	Liu et al, 2015 [32]	Ageing	60 healthy young men	60 old men	2DE and MALDI-TOF/MS	22 DEPs: ↑9 and ↓13	20 DEPs: ↑8 and ↓12	(iv) Risk factors
19	Liu et al, 2015 [33]	Obesity and severe AZS	3 normozoospermic men	3 obese men with/severe AZS	HPLC-MS/MS	127 DEPs: ↑22 and ↓105	125 DEPs: ↑22 and ↓103	(i) AZS; (iv) Risk factors
20a	Amaral et al, 2014 [34]	AZS	5 normozoospermic men	5 asthenozoospermic men	TMT labelling and LC-MS/MS	80 DEPs: ↑30 and ↓50	80 DEPs: ↑30 and ↓50	(i) AZS
20b	Amaral et al, 2014 [34]	Low motility	5 normozoospermic men (high motile sperm fraction)	Same 5 normozoospermic men (high motile sperm fraction)	TMT labelling and LC-MS/MS	93 DEPs: ↑47 and ↓46	93 DEPs: ↑47 and ↓46	(ii) Low motility
21	Frapsauce et al, 2014 [35]	ZP binding failure	3 normozoospermic men	3 normozoospermic men with complete ZP binding failure	2D-DIGE and MALDI-TOF/MS	12 DEPs: ↑3 and ↓9	12 DEPs: ↑3 and ↓9	(iii) Unexplained infertility
22a	Légaré et al, 2014 [36]	Idiopathic infertility	3 normozoospermic men	6 infertile men	iTRAQ labelling and LC-MS/MS	18 DEPs: ↑7 and ↓11	18 DEPS: ↑7 and ↓11	Other sperm alterations
22b	Légaré et al, 2014 [36]	IVF failure	3 normozoospermic men	4 men with a history of IVF failure	iTRAQ labelling and LC-MS/MS	33 DEPs: ↑15 and ↓18	33 DEPs: ↑15 and ↓18	(iii) Unexplained infertility
23	McReynolds et al, 2014 [43]	Blastocyst development	6 normozoospermic men and good blastocyst development	6 normozoospermic men and bad blastocyst development	1D-PAGE and LC-MS/MS	49 DEPs: ↑20 and ↓29	49 DEPs: ↑20 and ↓29	(iii) Unexplained infertility
24	Hosseinifar et al, 2013 [44]	Varicocele and oligozoospermia	20 normozoospermic men	20 oligozoospermic men with varicocele	2DE and MALDI-TOF/TOF-MS	10 DEPs: ↑1 and ↓9	9 DEPs: ↑1 and ↓8	(iv) Risk factors; Other sperm alterations
25	Intasqui et al, 2013 [45]	DF	11 normozoospermic men with low DF	6 normozoospermic men with high DF	2DE and Nano-UHPLC-ESI-MS	94 DEPs: ↑23 and ↓71	94 DEPs: ↑23 and ↓71	Other sperm alterations
26	Shen et al, 2013 [46]	AZS	30 normozoospermic men	30 asthenozoospermic men	2DE and MALDI-TOF/TOF-MS	15 DEPs: ↑4 and ↓11	15 DEPs: ↑4 and ↓11	(i) AZS
27	Zhu et al, 2013 [47]	IVF failure	3 normozoospermic men with successful IVF	3 normozoospermic men without successful IVF	TMT labelling and HPLC-MS/MS	21 DEPs: ↑5 and ↓16	21 DEPs: ↑5 and ↓16	(iii) Unexplained infertility
28	Xu et al, 2012 [42]	Null pregnancy	10 fertile normozoospermic men	10 infertile normozoospermic men	2DE and MALDI-TOF/TOF-MS	24 DEPs: ↑15 and ↓9	18 DEPs: ↑11 and ↓7	(iii) Unexplained infertility
29a	Paasch et al, 2011 [37]	DM 1	21 normozoospermic men	8 DM1 men	DIGE and MALDI-TOF/TOF-MS	8 DEPs: ↑6 and ↓2	8 DEPs: ↑6 and ↓2	(iv) Risk factors

Table 1. Continued 2

| No | Reference | Condition | Participants | | Proteomic method | Number of DEPs | | Analysis group |
			Controls (#)	Cases (#)		Reported	Mapped	
29b	Paasch et al, 2011 [37]	DM 2		7 DM2 men		10 DEPs: ↑7 and ↓3	10 DEPs: ↑7 and ↓3	(iv) Risk factors
29c	Paasch et al, 2011 [37]	Obesity		13 non-diabetic obese men		7 DEPs: ↑6 and ↓1	7 DEPs: ↑6 and ↓1	(iv) Risk factors
30	Siva et al, 2010 [39]	AZS	20 normozoospermic men	17 asthenozoospermic men	2DE and MALDI-MS/MS	8 DEPs: ↑3 and ↓5	7 DEPs: ↑3 and ↓4	(i) AZS
31a	Kriegel et al, 2009 [40]	Diabetes type 1	5 normozoospermic men	2 DM1 men	2D-DIGE and MALDI-TOF/MS	8 DEPs: ↑5 and ↓3	8 DEPs: ↑5 and ↓3	(iv) Risk factors
31b	Kriegel et al, 2009 [40]	Obesity		2 non-diabetic obese men		9 DEPs: ↑3 and ↓6	9 DEPs: ↑3 and ↓6	(iv) Risk factors
32	Martinez-Heredia et al, 2008 [41]	AZS	10 normozoospermic men	20 asthenozoospermic men	2DE and PDQuest	17 DEPs: ↑10 and ↓7	16 DEPs: ↑10 and ↓6	(i) AZS

For each study, the evaluated condition, the number of individuals included in each group, the proteomic method, the number of DEPs (reported and mapped in the UniProt database), and the analysis group are indicated.

1D: one-dimensional, 2DE: two-dimensional, AZS: asthenozoospermia, DEP: differentially expressed protein, DF: DNA fragmentation, DIGE: differential gel electrophoresis, DM1: diabetes mellitus type 1, DM2: diabetes mellitus type 2, ESI: electrospray ionization, HPLC: high-performance liquid chromatography, iTRAQ: isobaric tags for relative and absolute quantitation, IVF: *in vitro* fertilization, LC: liquid chromatography, MALDI: matrix-assisted laser desorption/ionization, MS/MS: tandem mass spectrometry, PAGE: polyacrylamide gel electrophoresis, PR: pregnancy rate, R-ICSI: rescue intracytoplasmic sperm injection, TMT: tandem mass tag, TOF: time-of-flight, UHPLC: ultra-high-performance liquid chromatography, ZP: zona pellucida, #: number of patients.
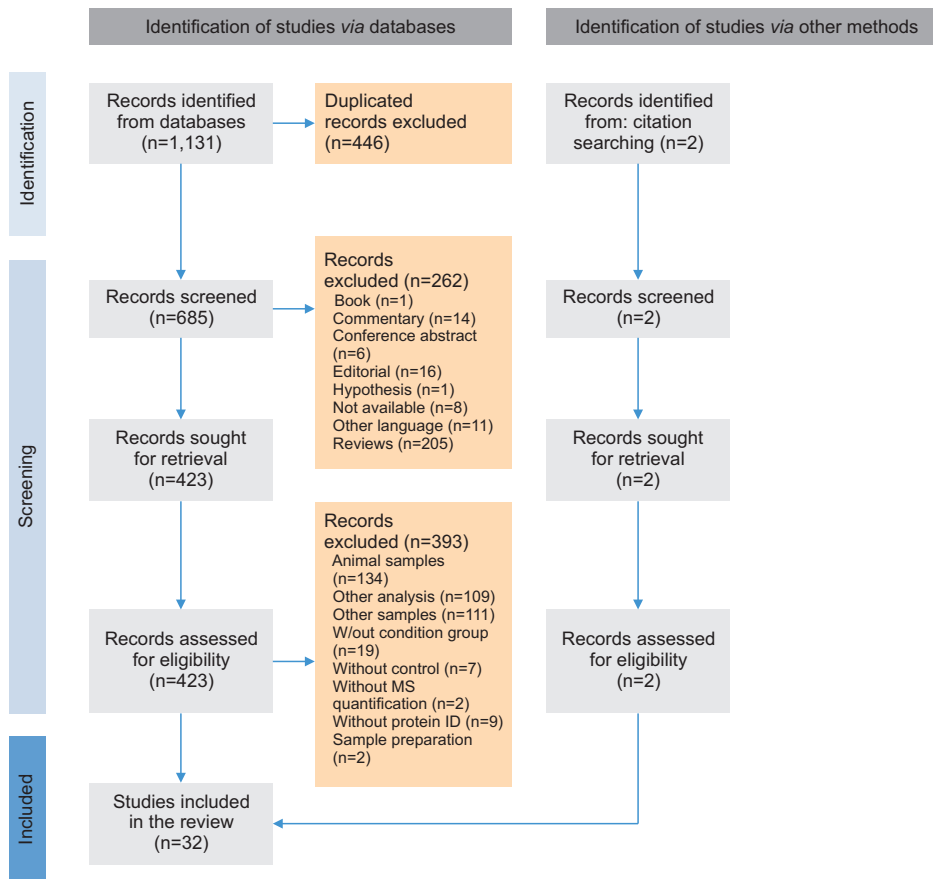
Fig. 1. Study selection. Flow diagram illustrating the selection process of proteomic studies employed in this systematic review. Systematic results search and study selection process are represented according to the PRISMA guidelines (2020). MS: mass spectrometry.

after testicular-heat exposure. Most investigations used comparison groups composed of men of similar ages (data not shown). Only Liu et al [32] used two male groups of different ages, since the major objective of this study was to determine the influence of aging on the sperm proteome. Concerning proteomic strategies, gel-based methods (one-dimensional [1D] or two-dimensional polyacrylamide gel electrophoresis [2DE or 2D-PAGE]; differential gel electrophoresis [DIGE]) and gel-free methods (tandem mass tag [TMT]; isobaric tags for relative and absolute quantitation [iTRAQ]; and free label) were the most common techniques used for protein separation, coupled with tandem mass spectrometry (MS/MS) or time-of-flight (TOF) analyses (Table 1). From the included studies, a total of 4,579 DEPs were collected, corresponding to 2,752 unique proteins according to the UniProt database (Supplement Table 3). Considering the evaluated conditions, studies were categorized into four subgroups as detailed in Table 1.

2. Quality appraisal

The bias risk of 30 of the included studies was evalu-

ated as "low", whilst two studies [38,47] were appraised with "some concerns" (Supplement Fig. 1). As determined by the QUADAS-2 tool, the studies included in this systematic review were classified as good quality.

3. Candidate protein markers for AZS

AZS is one of the most common causes of male infertility (~20% of cases) and is characterized by a reduction (total motility <40%) or total lack of motility [5]. Among the focused conditions, the sperm proteome related to AZS was the most often analysed (n=12) (Table 1). One of the included studies presented two independent analyses performed in samples from asthenozoospermic and severe asthenozoospermic (total motility <12%) patients [55]. Another investigation included samples from obese men with severe AZS (total motility <20%) [33]. The study conducted by Chhikara et al [48] contains several sub-analyses, but only DEPs identified among ejaculated samples of normozoospermic and asthenozoospermic men were considered. A total of 1,998 unique DEPs were identified in AZS sperm, of which 878 were exclusively increased and

992 were exclusively decreased (Fig. 2A). As potential protein candidates, 12 increased and 26 decreased proteins were identified (Table 2). Of these 38 proteins, ACRBP, TEKT5, ACR, ACRV1, CCIN, IZUMO4, AKAP4, SPACA3, GARIN3, ACTRT2, LYZL4, and ZPBP have enriched expression in testis, REEP6 is enhanced in testis, SPINK2 is enriched in epididymis, and ACP3 is a prostate-enriched protein (Table 2). Furthermore, ACP3, ACRBP, CA2, REEP6, ACR, CD46, SPINK2, AKAP4 and ZPBP were associated with fertility defects in mice (Table 2). Although not mentioned in the MGI database, two proteins (PRDX6 and SOD1) were also associated with male infertility phenotypes

in mice. *Prdx6* null mice presented spermatozoa with abnormal chromatin structure, low motility, impaired capacitation, decreased sperm/oocyte interaction, and fertility competence (reviewed by O'Flaherty [58]). *Sod1* null mice had altered spermatogenesis, presenting spermatozoa with low motility, impaired capacitation, inability to penetrate ZP, and reduced fertilization potential [59-62].

4. Candidate protein markers for low sperm motility

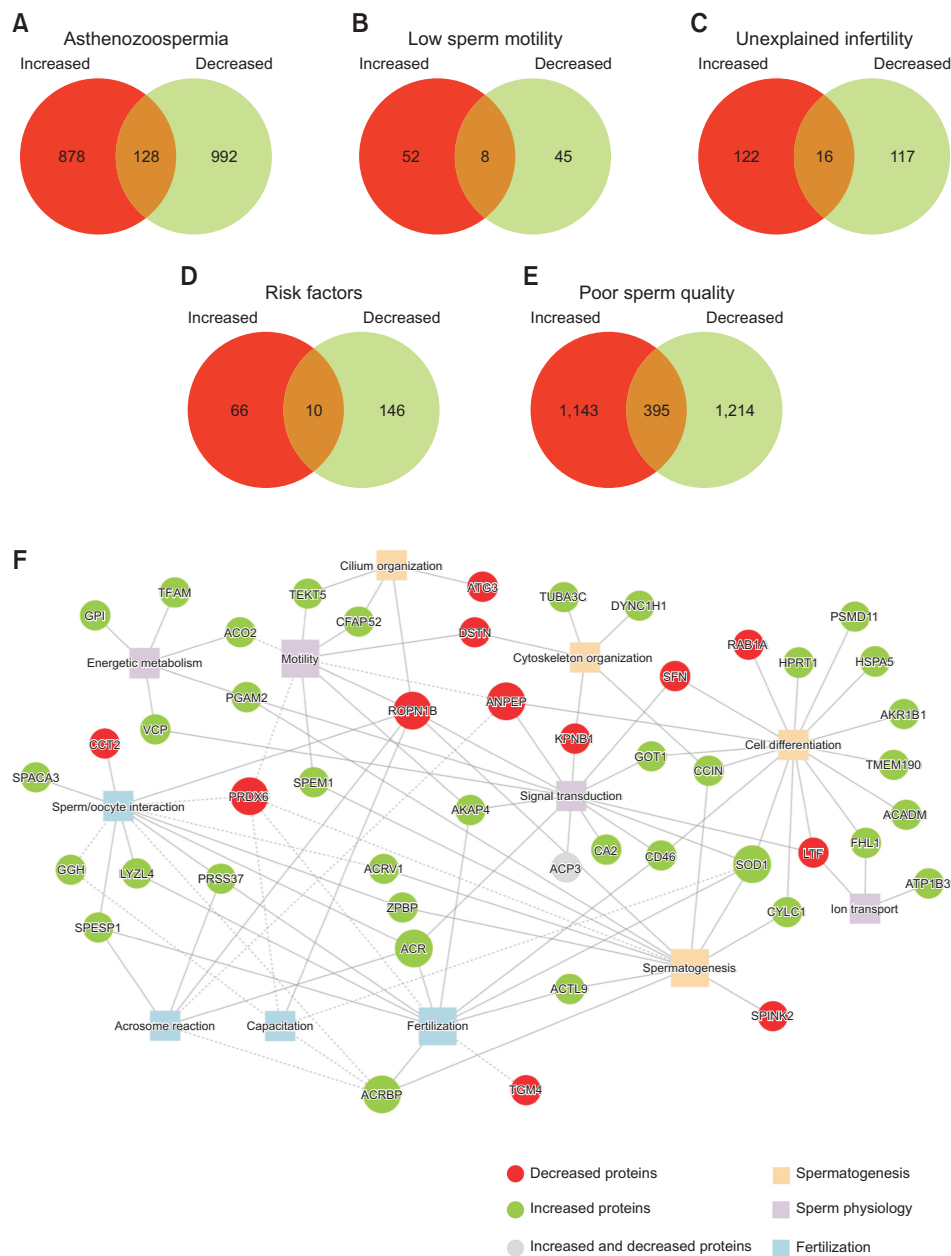Regarding semen analysis, motility is one of the major predictors of sperm function [5]. After spermato-

Fig. 2. Differentially expressed proteins (DEPs) in male infertility-related conditions and overall poor-sperm quality. Number of DEPs (increased and decreased) in (A) asthenozoospermia, (B) low sperm motility, (C) unexplained infertility, (D) infertility related to risk factors, (E) poor sperm quality. (F) DEPs related to the retrieved biological processes in the UniProt database (solid line) and PubMed search (dash line). Larger circles represent proteins associated with a higher number of biological processes. Larger squares represent biological processes with a higher number of associated proteins.

Table 2. Candidate protein markers for male infertility-related conditions

UniProtKB	Gene name	Protein name	#	Expression level	Reference	Tissue expression	Male fertility defects	Biological process
(i) Asthenozoospermia								
P15309	ACP3	Prostatic acid phosphatase	4	↑	[26,31,34,54]	Prostate (enriched)	Abnormal prostate gland	Signal transduction
P60981	DSTN	Destrin	3	↑	[34,55]	-	-	Cytoskeleton organization; Motility
O00231	PSMD11	26S proteasome non-ATPase regulatory subunit 11	3	↑	[26,55]	-	-	Cell differentiation
P15144	ANPEP	Aminopeptidase N	3	↑	[26,31,55]	-	-	Cell differentiation; Signal transduction
P26640	VARS1	Valine-tRNA ligase	3	↑	[26,31,54]	-	-	-
P30041	PRDX6	Peroxiredoxin-6	3	↑	[26,31,34]	-	-	Cell differentiation
P62820	RAB1A	Ras-related protein Rab-1A	3	↑	[26,31,54]	-	-	Cell differentiation
P78371	CCT2	T-complex protein 1 subunit beta	3	↑	[26,29,31]	-	-	Sperm/oocyte interaction
Q14974	KPNB1	Importin subunit beta 1	3	↑	[26,31,33]	-	-	Cytoskeleton organization; Signal transduction
Q96FW1	OTUB1	Ubiquitin thioesterase OTUB1	3	↑	[26,55]	-	-	-
Q9HOI9	TKTL2	Transketolase-like protein 2	3	↑	[26,31,54]	-	-	-
Q9HB40	SCPEP1	Retinoid-inducible serine carboxypeptidase	3	↑	[26,54,55]	-	-	-
P17174	GOT1	Aspartate aminotransferase, cytoplasmic	4	↓	[26,54,55]	-	-	Cell differentiation; Signal transduction
Q8NEB7	ACRBP	Acrosin-binding protein	4	↓	[26,31,48,54]	Testis (enriched)	Abnormal acrosome and nucleus, decreased sperm motility	Fertilization; Spermatogenesis
Q92820	GGH	Gamma-glutamyl hydrolase	4	↓	[26,54,55]	-	-	-
Q96M29	TEKT5	Tektin-5	4	↓	[26,31,33,34]	Testis (enriched)	-	Cilium organization; Motility
Q99798	ACO2	Aconitate hydratase, mitochondrial	4	↓	[26,34,55]	-	-	Energy metabolism
P00441	SOD1	Superoxide dismutase [Cu-Zn]	3	↓	[26,48,54]	-	-	Fertilization; Cell differentiation; Spermatogenesis; Signal transduction
P00918	CA2	Carbonic anhydrase 2	3	↓	[26,48,54]	-	Reduced male fertility; dilated efferent testis ductules and rete testis	Signal transduction
Q96HR9	REEP6	Receptor expression-enhancing protein 6	3	↓	[31,55]	Testis (enhanced)	OZS, TZS, AZS, impaired acrosome reaction	-
P06744	GPI	Glucose-6-phosphate isomerase	3	↓	[26,34,55]	-	-	Energy metabolism

Table 2. Continued 1

UniProtKB	Gene name	Protein name	#	Expression level	Reference	Tissue expression	Male fertility defects	Biological process
P10323	ACR	Acrosin	3	↓	[26,54,55]	Testis enriched	Delayed fertilization	Acrosome reaction; Fertilization; Signal transduction; Sperm/oocyte interaction
P15121	AKR1B1	Aldo-keto reductase family 1 member B1	3	↓	[26,33,54]	-	-	Cell differentiation
P15529	CD46	Membrane cofactor protein	3	↓	[26,55]	-	Abnormal fertilization	Cell differentiation; Fertilization; Signal transduction
P20155	SPINK2	Serine protease inhibitor Kazal-type 2	3	↓	[26,31,48]	Epididymis (enriched)	Azoospermia	Spermatogenesis
P26436	ACRV1	Acrosomal protein SP-10	3	↓	[26,31,54]	Testis (enriched)	-	Spermatogenesis
Q13642	FHL1	Four and a half LIM domains protein 1	3	↓	[26,31,34]	-	-	Cell differentiation; Ion transport
Q13939	CCIN	Calicin	3	↓	[26,31,33]	Testis (enriched)	-	Cell differentiation; Cytoskeleton organization; Spermatogenesis
Q1ZYL8	IZUMO4	Izumo sperm-egg fusion protein 4	3	↓	[26,48,54]	Testis (enriched)	-	-
Q5JQC9	AKAP4	A-kinase anchor protein 4	3	↓	[26,29,31]	Testis (enriched)	Impaired sperm motility	Fertilization; Motility; Signal transduction
Q5JRX3	PITRM1	Presequence protease, mitochondrial	3	↓	[26,33,55]	-	-	-
Q8IXA5	SPACA3	Sperm acrosome membrane-associated protein 3	3	↓	[26,31,54]	Testis (enriched)	-	Sperm/oocyte interaction
Q8IYQ7	THNSL1	Threonine synthase-like 1	3	↓	[26,55]	-	-	-
Q8TC56	GARIN3	Golgi-associated RAB2 interactor protein 3	3	↓	[26,31,48]	Testis (enriched)	-	-
Q8TDY3	ACTRT2	Actin-related protein T2	3	↓	[26,33,48]	Testis (enriched)	-	-
Q96KX0	LYZL4	Lysozyme-like protein 4	3	↓	[26,34,54]	Testis (enriched)	Normal fertility	Fertilization; Sperm/oocyte interaction
Q9BS86	ZPBP	Zona pellucida-binding protein 1	3	↓	[26,48,54]	Testis (enriched)	Abnormal sperm morphology	Sperm/oocyte interaction; Spermatogenesis
Q9H3G5	CPVL	Probable serine carboxypeptidase CPVL	3	↓	[26,54,55]	-	-	-
(ii) Low motility								
P11021	HSPA5	Endoplasmic reticulum chaperone BiP	2	↑	[27,34]	-	-	Cell differentiation
(iii) Unexplained infertility								
Q5JQC9	AKAP4	A-kinase anchor protein 4	4	↓	[30,35,42,57]	Testis (enriched)	Impaired sperm motility	Fertilization; Motility; Signal transduction
P02788	LTF	Lactotransferrin	3	↓	[43,49,51]	-	-	Cell differentiation; Ion transport; Signal transduction
P0DPH7	TUBA3C	Tubulin alpha-3C chain	3	↓	[28,30,35]	-	-	Cytoskeleton organzitation

Table 2. Continued 1

UniProtKB	Gene name	Protein name	#	Expression level	Reference	Tissue expression	Male fertility defects	Biological process
(iv) Infertility related to risk factors								
P02788	LTF	Lactotransferrin	3	↑	[37]	-	-	Cell differentiation; Ion transport; Signal transduction
Q6UWU2	GLB1L	Beta-galactosidase-1-like protein	3	↓	[37]	Testis (enhanced)	-	-

Highlighted protein candidates in (i) asthenozoospermia, (ii) low sperm motility, (iii) unexplained infertility, and (iv) infertility related to risk factors. For each protein, this table indicates selective expression in male reproductive tissues, the male infertility defects/phenotypes observed in knock-out mice, and the relevant biological processes (retrieved from UniProt database).
AZS: asthenozoospermia, OZS: oligozoospermia, TZS: teratozoospermia, #: number of counts.

genesis and epididymal maturation, a fraction of spermatozoa still presents low motility, or even immotility, providing a useful biological sample to determine the molecular mechanisms responsible for sperm (im)motility. Two studies analysed the proteome of the low motility sperm fraction of normozoospermic men (Table 1). A total of 105 unique proteins were identified, of which 52 were increased and 45 were decreased (Fig. 2B). Only one protein (HSPA5) was described in both studies as decreased in sperm with low motility (Table 2).

5. Candidate protein markers for unexplained male infertility and poor reproductive outcomes

In unexplained male infertility, men present normal semen analysis and physical and endocrine abnormalities related to infertility were excluded. Yet, men with these conditions cannot establish a successful pregnancy [6,63]. In these cases, the female infertility factor has been excluded a *priori* either by clinical evaluation or oocyte morphological analysis. The ten included studies in this category were related to recurrent pregnancy loss (n=3), ZP binding failure (n=2), low pregnancy rate (n=1), null pregnancy (n=1), *in vitro* fertilization (IVF) failure (n=2), and inappropriate blastocyst development (n=1) (Table 1). In these proteomic analyses both comparison groups were composed of normozoospermic men. From the included studies, 255 unique DEPs were identified, 122 being exclusively increased and 117 exclusively decreased (Fig. 2C). Three decreased DEPs (AKAP4, LTF, and TUBA3C) were systematically reported in these studies (Table 2). Although not mentioned in the HPA, TUBA3C is a testis-specific α-tubulin isoform, amenable to acetylation, present in human sperm [64].

6. Candidate protein markers for infertility associated with risk factors

A multitude of causes and risk factors have been associated with the increasing male infertility incidence; these include genetic defects (diabetes mellitus [DM] type 1), acquired factors (for example varicocele, obesity, and DM type 2), and biological factors (ageing) [2]. Although those causes and risk factors are biologically distinct, their presence is correlated with a significant decrease in sperm quality [65]. In this section, six studies were included to unveil common DEPs in infertility associated with risk factors: hyperthermia (n=1), DM

type 1 (n=2), DM type 2 (n=1), obesity (n=3), varicocele (n=1), and ageing (n=1) (Table 1). A total of 222 unique proteins were identified, of which 66 were increased, 146 were decreased and 10 were both increased and decreased (Fig. 2D). Two proteins (LTF and GLB1L) were positively identified as potential markers (Table 2). GLB1L expression is enhanced in the testis (Table 2).

7. Candidate protein markers of poor sperm quality

From the 2,752 unique proteins identified in all the studies included in this review (Supplement Table 3), 1,143 and 1,214 proteins were exclusively increased and decreased, respectively (Fig. 2E). Amongst these, 11 increased and 47 decreased DEPs were highlighted as potential protein markers of poor sperm quality (Table 3). Twenty-four proteins (ACO2, ACRBP, ACRV1, ACTRT2, ANPEP, CA2, CCIN, CD46, FHL1, GARIN3, GGH, GLB1L, GOT1, IZUMO4, KPNB1, LYZL4, OTUB1, PITRM1, RAB1A, SPACA3, SPINK2, TEKT5, THNSL1, and ZPBP) were previously identified in the subgroup analyses (Table 2, 3). Among the 58 highlighted proteins, 22 (H2AC1, ACRBP, ZPBP, CCIN, GARIN3, ACRV1, TEKT5, FAM205A, LYZL4, ACTRT2, PRSS37, CYLC1, IZUMO4, FAM209A, ACTL9, FNDC8, TMEM190, GARIN4, SPESP1, LYZL1, SPACA3, and SPEM1) are testis-enriched, two (GLB1L and CFAP276) are testis-enhanced, one is enriched epididymis (SPINK2), and one (TGM4) is prostate-enriched (Table 3). Fifteen proteins (TGM4, DYNC1H1, ACRBP, ZPBP, CD46, PRSS37, CA2, FAM209A, ACTL9, HPRT1, SPINK2, CFAP276, SPESP1, SPEM1, and ROPN1B) were associated with defects in male fertility such as abnormal sperm morphology, impaired/reduced fertilization, and abnormal male reproductive tissues morphology (Table 3).

8. Biological processes associated with the identified DEPs

A total of 50 different biological processes were retrieved from the UniProt database associated with the previously identified protein candidates (Supplement Table 4). Among these, 13 were highlighted as being relevant for sperm physiology: ion transport, signal transduction, energy metabolism (including glycolysis and aerobic respiration), spermatogenesis, cell differentiation, cytoskeleton organization, cilium organization, motility, capacitation, sperm/oocyte interaction, acro-

Table 3. Potential protein markers for poor sperm quality

UniProtKB	Gene name	Protein name	#	Conditions (references)	Tissue expression	Male fertility defects	Biological process
Increased proteins							
P31947	SFN	14-3-3 protein sigma	5	AZS [26,31]; LM [34]; RPL [51,57]	-	-	Cell differentiation; Signal transduction
P15144	ANPEP	Aminopeptidase N	4	AZS [26,31,55]; Idiopathic Infertility [36]	-	-	Cell differentiation; Signal transduction
P49221	TGM4	Protein-glutamine gamma-glutamyltransferase 4	4	AZS [31]; DF [45]; IVF failure [36]; RPL [57]	Prostate (enriched)	Impaired copulatory plug formation, reduced fertilization	-
P52597	HNRNPF	Heterogeneous nuclear ribonucleoprotein F	4	AZS [26,54]; Hyperthermia [52]; Severe OAT [38]	-	-	-
P56192	MARS1	Methionine--tRNA ligase, cytoplasmic	4	AZS [26,54]; GZS [53]; LM [34]	-	-	-
P62820	RAB1A	Ras-related protein Rab-1A	4	AZS [26,31,54]; Severe OAT [38]	-	-	Cell differentiation
Q14204	DYNC1H1	Cytoplasmic dynein 1 heavy chain 1	4	AZS [26,55]; GZS [54]; RPL [51]	-	Male infertility	Cytoskeleton organization
Q14974	KPNB1	Importin subunit beta 1	4	AZS [26,31]; GZS [53]; AZS and obesity [33]	-	-	Cytoskeleton organization; Signal transduction
Q96FW1	OTUB1	Ubiquitin thioesterase OTUB1	4	AZS [26,55]; GZS [53]	-	-	-

Table 3. Continued 1

UniProtKB	Gene name	Protein name	#	Conditions (references)	Tissue expression	Male fertility defects	Biological process
Q9NT62	ATG3	Ubiquitin-like-conjugating enzyme ATG3	4	AZS [26,54]; GZS [53]; Severe OAT [38]	-	Cilium organization	-
Decreased proteins							
Q96QV6	H2AC1	Histone H2A type 1-A	4	GZS [54]; AZS [31,48]; ZP binding failure [28]	Testis (enriched)	-	-
Q8NEB7	ACRBP	Acrosin-binding protein	8	AZS [26,31,54]; RPL [57]; GZS [53]; ZP binding failure [28]; Severe OAT [38]	Testis (enriched)	Abnormal acrosome and nucleus, and decreased sperm motility	Fertilization; Spermatogenesis
Q92820	GGH	Gamma-glutamyl hydrolase	8	AZS [26,54,55]; GZS [53]; LM [34]; DM-1 and 2 [37]	-	-	-
Q9BS86	ZPBP	Zona pellucida-binding protein 1	8	Hyperthermia [52]; AZS [26,48,54]; GZS [53]; ZP binding failure [28]; Idiopathic infertility [36]; Severe OAT [38]	Testis (enriched)	Abnormal sperm morphology	Sperm/oocyte interaction; Spermatogenesis
Q13939	CCIN	Calicin	7	AZS [26,31,54]; GZS [53]; AZS and obesity [33]; DF [45]; Hyperthermia [52]; Severe OAT [38]	Testis (enriched)	-	Cell differentiation; Cytoskeleton organization; Spermatogenesis
Q6UWU2	GLB1L	Beta-galactosidase-1-like protein	7	AZS [26,54] GZS [53]; DM-1 and 2, obesity [37]; Severe OAT [38]	Testis (enhanced)	-	-
Q8TC56	GARIN3	Golgi-associated RAB2 interactor protein 3	7	GZS [53]; RPL [57]; Hyperthermia [52]; AZS [26,31,48] Severe OAT [38]	Testis (enriched)	-	-
P26436	ACRV1	Acrosomal protein SP-10	6	AZS [26,31,54]; GZS [53]; IVF failure [47]; DF [45]	Testis (enriched)	-	Spermatogenesis
Q96M29	TEKT5	Tektin-5	6	AZS [26,31,34]; AZS and obesity [33]; Blastocyst development [43]; Hyperthermia [52]	Testis (enriched)	-	Cilium organization; Motility
P15529	CD46	Membrane cofactor protein	6	Hyperthermia [52]; GZS [53]; AZS [26,55]; Severe OAT [38]	-	Abnormal fertilization	Cell differentiation; Fertilization; Signal transduction
P17174	GOT1	Aspartate aminotransferase, cytoplasmic	6	LM [34]; AZS [26,54,55]; Severe OAT [38]	-	-	Cell differentiation; Fertilization; Signal transduction
Q6ZU69	FAM205A	Protein FAM205A	6	GZS [53]; AZS [26,54]; DF [45]; RPL [57]; Severe OAT [38]	Testis (enriched)	-	-
Q96KX0	LYZL4	Lysozyme-like protein 4	6	Blastocyst development [43]; GZS [53]; AZS [26,34,54]; Severe OAT [38]	Testis (enriched)	-	Fertilization; Sperm/oocyte interaction
Q8TDY3	ACTRT2	Actin-related protein T2	6	AZS [26,48]; GZS [53]; AZS and obesity [33]; Hyperthermia [52];	Testis (enriched)	-	-
A4D1T9	PRSS37	Probable inactive serine protease 37	5	AZS [26,54]; GZS [53]; Severe OAT [38]; Secondary hypogonadism [50]	Testis (enriched)	Impaired ZP binding and migration from the uterus to oviduct	Acrosome reaction; Fertilization; Sperm/oocyte interaction

Table 3. Continued 2

UniProtKB	Gene name	Protein name	#	Conditions (references)	Tissue expression	Male fertility defects	Biological process
P00918	CA2	Carbonic anhydrase 2	5	AZS [26,48,54]; Idiopathic Infertility [36]; Severe OAT [38]	-	Reduced male fertility, dilated rete testis and efferent ductulus	Signal transduction
P15259	PGAM2	Phosphoglycerate mutase 2	5	IVF failure [36]; AZS [26,54]; DF [45]; Severe OAT [38]	-	-	Energetic metabolism; Signal transduction; Spermatogenesis
P35663	CYLC1	Cylicin-1	5	AZS [26]; GZS [53]; AZS and obesity [33]; Hyperthermia [52]; Severe OAT [38]	Testis (enriched)	-	Cell differentiation; Spermatogenesis
P54709	ATP1B3	Sodium/potassium-transporting ATPase subunit beta-3	5	Hyperthermia [52]; AZS [26,55]; IVF failure [47]; Severe OAT [38]	-	-	Ion transport
P55072	VCP	Transitional endoplasmic reticulum ATPase	5	LM [34]; DF [45]; IVF failure [36]; ZP binding failure [35]; Severe OAT [38]	-	-	Energetic metabolism; Signal transduction
Q13642	FHL1	Four and a half LIM domains protein 1	5	AZS [31,34][26]; ZP binding failure [28]; Severe OAT [38]	-	-	Cell differentiation; Ion transport
Q1ZYL8	IZUMO4	Izumo sperm-egg fusion protein 4	5	GZS [53]; ZP binding failure [28]; AZS [26,48,54]	Testis (enriched)	-	-
Q5JX71	FAM209A	Protein FAM209A	5	DF [45]; Blastocyst development [43]; Severe OAT [38]; AZS [26,48]	Testis (enriched)	AZS, GZS, and abnormal acrosome morphology	-
Q8TC94	ACTL9	Actin-like protein 9	5	AZS [26]; LM [34]; GZS [53]; Ageing [32]; Hyperthermia [52]	Testis (enriched)	Abnormal sperm morphology, impaired fertilization	Fertilization; Spermatogenesis
Q8TC99	FNDC8	Fibronectin type III domain-containing protein 8	5	GZS [53]; Hypertermia [52]; Severe OAT [38]; AZS [26,48]	Testis (enriched)	-	-
Q8WZ59	TMEM190	Transmembrane protein 190	5	GZS [53]; Hyperthermia [52]; DF [45]; AZS [31]; Severe OAT [38]	Testis (enriched)	-	Cell differentiation
Q8IYT1	GARIN4	Golgi-associated RAB2 interactor protein 4	5	GZS [53]; Hypertermia [52]; Severe OAT [38]; AZS [26,48]	Testis (enriched)	-	-
Q96QE4	LRRC37B	Leucine-rich repeat-containing protein 37B	5	GZS [53]; AZS [26,31]; Hyperthermia [52]; Severe OAT [38]	-	-	-
Q99798	ACO2	Aconitate hydratase, mitochondrial	5	AZS [26,34,55]; Severe OAT [38]	-	-	Energy metabolism
P00492	HPRT1	Hypoxanthine-guanine phosphoribosyltransferase	4	Blastocyst development [43]; LM [34]; AZS [55]; Severe OAT [38]	-	Testicular atrophy	Cell differentiation
P04066	FUCA1	Tissue alpha-L-fucosidase	4	AZS [26,54]; GZS [53]; Severe OAT [38]	-	-	-
P11310	ACADM	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	4	LM [34]; AZS [26,55]; Severe OAT [38]	-	-	Cell differentiation
P20155	SPINK2	Serine protease inhibitor Kazal-type 2	4	DF [45]; AZS [26,31,48]	Epididymis (enriched)	Azoospermia	Spermatogenesis

Table 3. Continued 3

UniProtKB	Gene name	Protein name	#	Conditions (references)	Tissue expression	Male fertility defects	Biological process
P23786	CPT2	Carnitine O-palmitoyltransferase 2, mitochondrial	4	ZP binding failure [35]; AZS [26,55]; Severe OAT [38]	-	-	-
P28838	LAP3	Cytosol aminopeptidase	4	DF [45]; IVF failure [36]; Severe OAT [38]; AZS [26]	-	-	-
P56730	PRSS12	Neurotrypsin	4	AZS [26,54]; GZS [53]; Severe OAT [38]	-	-	-
Q00059	TFAM	Transcription factor A, mitochondrial	4	GZS [53]; Severe OAT [38]; IVF failure [36]; AZS [26]	-	-	Energy metabolism
Q5JRX3	PITRM1	Presequence protease, mitochondrial	4	Obesity and AZS [33]; Severe OAT [38]; AZS [26,55]	-	-	-
Q5T5A4	CFAP276	Protein CFAP276	4	AZS and obesity [33]; LM [34]; Blastocyst development [43]; Severe OAT [38]	Testis enhanced	Impaired embryo implantation	-
Q6UW49	SPESP1	Sperm equatorial segment protein 1	4	Hyperthermia [52]; GZS [53]; AZS [54]; DF [45]	Testis enriched	Decreased fertilization frequency and delayed fertilization	Acrosome reaction; Fertilization; Sperm/oocyte interaction
Q6UWQ5	LYZL1	Lysozyme-like protein 1	4	Obesity [40]; GZS [53]; AZS [26,34]	Testis enriched	-	-
Q8IXA5	SPACA3	Sperm acrosome membrane-associated protein 3	4	GZS [53]; AZS [26,31,54]	Testis enriched	-	Sperm/oocyte interaction
Q8IYQ7	THNSL1	Threonine synthase-like 1	4	AZS [26,55]; Severe OAT [38]	-	-	-
Q8N1V2	CFAP52	Cilia- and flagella-associated protein 52	4	AZS [26,34]; LM [34]; Severe OAT [38]	-	-	Cilium organization; Motility
Q8N4L4	SPEM1	Spermatid maturation protein 1	4	GZS [53]; ZP binding failure [28]; Severe OAT [38]; AZS [26]	Testis (enriched)	AZS, TZS, and impaired spermiogenesis	Spermatogenesis; Acrosome reaction; Fertilization; Sperm/oocyte interaction; Motility
Q9BZX4	ROPN1B	Ropporin-1B	4	Severe OAT [38]; AZS [26,31]; Secondary Hypogonadism [50]	Testis (enriched)	AZS, reduced male fertility	Acrosome reaction; Motility; Sperm/oocyte interaction; Spermatogenesis; Capacitation; Cilium organization; Signal transduction
Q9NR28	DIABLO	Diablo IAP-binding mitochondrial protein	4	AZS [26,34]; Blastocyst development [43]; Severe OAT [38]	-	-	-
Q9UBX1	CTSF	Cathepsin F	4	AZS [48,54]; GZS [53]; Severe OAT [38]	-	-	-

After cross-comparison of the studies included in this review, relevant protein candidates were identified. For each protein is indicated selective expression in male reproductive tissues, the male infertility defects/phenotypes observed in knock-out (KO) mice, and the relevant biological processes (retrieved from UniProt database).
AZS: asthenozoospermia, DF: DNA fragmentation, DM: diabetes mellitus, GZS: globozoospermia, IVF: in vitro fertilization, LM: low motility, OAT: oligoasthenoteratozoospermia, RPL: recurrent pregnancy loss, TZS: teratozoospermia, ZP: zona pellucida, #: number of counts.

some reaction, and fertilization (Fig. 2F, squares). From the 76 highlighted DEPs, 44 are associated with those processes (Tables 2, 3, Fig. 2F).

Additionally, the search performed on PubMed allowed the identification of additional associations between ACRV1, ACRBP, CD46, TGM4, ANPEP, GGH, ACO2, PRDX6, and SOD1 and sperm-related processes. ACRV1 is involved in sperm-oocyte interaction [66-68]. Studies performed in mice and boar spermatozoa showed ACRBP involvement in the acrosome reaction, capacitation, and sperm/oocyte interaction [69,70]. TGM4 is involved in the formation of copulatory plugs [71,72]. ANPEP activity is associated with both sperm motility and acrosome reaction in mice and humans [73-76]. A recent study reported that GGH forms a complex with T-complex protein 1 subunit beta (CCT2) and glutathione S-transferase Mu 3 protein (GSTM-3), both involved in sperm capacitation and sperm/oocyte interaction [77], suggesting the involvement of GGH in these processes. Low ACO2 levels correlate with the reduced motility of human sperm [78]. CD46 blockaded with antibodies decreased sperm/oocyte interactions [79,80]. PRDX6 activity is associated with spermatogenesis, motility, capacitation, and fertility competence [58,81,82]. Several studies have reported that SOD1 provides protection against oxidative stress during epididymal maturation and capacitation of mammalian sperm [83-85]. Moreover, increased levels of SOD1 were associated with a high fertility rate in cattle [86].

Taken together, the retrieved biological processes can be grouped into three main categories related to the spermatozoon life journey: 1) spermatogenesis (cell differentiation, spermatogenesis, cilium organization, and cytoskeleton organization) (Fig. 2F, orange squares), 2) sperm physiology (signal transduction, ion transport, energy metabolism, motility, and capacitation) (Fig. 2F, lilac squares), and 3) fertilization (sperm/oocyte interaction, acrosome reaction and fertilization) (Fig. 2F, blue squares). The most common processes associated with the identified DEPs were cell differentiation (n=16), signal transduction (n=14), sperm/oocyte interaction (n=12), spermatogenesis (n=12), fertilization (n=11), and motility (n=9) (highlighted in Fig. 2F with bigger squares). Among the DEPs, ROPN1B (n=7), PRDX6 (n=5) ACRBP (n=5), SOD1 (n=5), ANPEP (n=4), and ACR (n=4) were DEPs associated with most processes (highlighted in Fig. 2F with bigger circles).

DISCUSSION

Male infertility conditions have a plethora of underlying causes resulting in the dysregulation of various biochemical pathways and cellular processes. These altered molecular mechanisms are reflected in qualitative and quantitative differences in the sperm proteome [12]. Although there are some literature reviews focused on sperm proteomic studies, there is a general lack of analyses to indicate which DEPs are systematically reported in male infertility conditions. We believe this present study is to be the first systematic review to critically analyse proteomic data from the spermatozoa of infertile men. As a consequence of this analysis, it was possible to identify a set of relevant DEPs which are likely to prove viable targets for further basic and clinical research to unveil male infertility biomarkers.

One of the inherent challenges of this systematic analysis was the effective combination of proteomic studies to identify candidate proteins without losing relevant targets. As indicated by this work, a profitable strategy was to combine two approaches: 1) group the studies by infertility condition; and 2) concurrently analyse all data. Thus, it was possible to establish panels of protein candidates for specific conditions (such as AZS, low motility, unexplained infertility, and infertility related to risk factors) and for overall poor sperm quality. Evidence that this strategy was successful is provided by the identification of the following proteins: ACP3, PSMD11, VARS1, PRDX6, CCT2, SOD1, REEP6, GPI, ACR, AKAP4, CPVL, HSPA5, LTF, and TUBA3C. These proteins were highlighted in subgroup analyses but would be excluded for overall poor sperm quality because they presented inconsistent expression among the different infertility conditions.

For the highlighted proteins, it was important to understand their molecular role(s) in spermatozoa, which could be helpful to string the candidates' list. Currently, biomarkers research is focused on the discovery of tissue-specific candidates which facilitate a greater understanding of specific molecular mechanisms underlying clinical conditions [87]. From the 72 identified proteins (Table 2, 3), 28 have a selective expression in the testis and 3 in other male reproductive tissues. TGM4, ACP3, and LTF are components of the seminal fluid and bind to the sperm surface through membrane proteins [88,89]. In physiological conditions, these proteins are involved in sperm processes such as motility,

capacitation, and fertilization [88,89]. However, in male infertility, it is not fully understood whether these proteins are abnormally expressed or whether the spermatozoa present molecular changes on their surface to differentially bind them. Additional proteomic studies to analyse, in parallel, altered proteins in spermatozoa and seminal fluid could clarify this point. Among the testis-selective expressed proteins, ACRBP, SPESP1, ACTL9, PRSS37, HPRT1, CFAP276, SPESP1, FAM209A, SPEM1, ACR, REEP6, AKAP4, ZPBP, and ROPN1B were associated with male infertility phenotypes in KO mice models. Considering their expression and impact upon mouse fertility, they must represent strong candidates for further investigation. Conversely, 49 identified proteins were associated with the 13 relevant processes for spermatogenesis [90,91], sperm survival, maintenance, maturation, and fertilization [92-95], suggesting that these processes may be dysregulated in patients with poor sperm quality. Two mitochondrial proteins (ACO2 and TFAM) were associated with energetic metabolism. Besides the contribution of mitochondria role to energy production, mitochondrial integrity loss has been associated with increased ROS production, oxidative stress, and apoptosis-like events in spermatozoa [96]. Thus, ACO2 and TFAM may also be indicative of mitochondrial dysfunction in poor sperm quality. ACO2, ACRBP, ACR, ACRV1, ACTL9, AKAP4, ANPEP, CCT2, CFAP52, DSTN, GGH, LYZL4, PRDX6, PRSS37, ROPN1B, SOD1, SPACA3, SPEM1, SPESP1, TEKT5, and ZPBP were associated with sperm-specific processes (motility, capacitation, sperm/oocyte interaction, acrosome reaction, and fertilization). These proteins are also strong candidates for further research as potential biomarkers.

In the included studies, some limitations were identified and can be taken into consideration for further study design. Firstly, the inconsistencies observed in the expression levels of some proteins under the same conditions may result from the proteomic methods employed. Though gel-based methods remain viable in proteomic analyses, they present certain limitations compared to gel-free methods which are more sensitive and accurate in protein quantification [97]. Since proteomics is a constantly evolving field, the emergence of new software and instrumentation may be useful for protein identification and quantification in male infertility. A pertinent example is the study performed by Yang et al [26]; these authors used a 4D proteomic strategy to study AZS allowing the identification of a higher number of DEPs (n=1,430) compared to similar studies [31,34,54,55]. Secondly, there is a limited number (or even a lack) of proteomic studies focused on the analysis of the sperm proteome in some male infertility conditions such as idiopathic infertility, globozoospermia, and ageing. Another factor that may be overlooked in proteomics analyses is participant heterogeneity. Indeed, confounders (such as age match, ethnic group, lifestyle, and region of residence) are poorly addressed in sperm proteomic analysis. Detailed participant questionnaires may help to establish more homogeneous comparison groups and so improve the identification of reliable protein candidates. In unexplained male infertility analyses, the female infertility factor was excluded by clinical evaluation or by oocyte morphological selection. Nevertheless, the molecular dysfunctions associated with the female factor were not considered and may also contribute to poor reproductive outcomes. Final relevant comment is the limited data regarding the role of the identified DEPs in mammalian spermatozoa. Most of these are exhaustively investigated in somatic cells but remain poorly characterized in this highly differentiated cell type. Consequently, the identified proteins in this analysis are obvious candidates to be further explored in future investigations.

CONCLUSIONS

Proteomics, a valuable tool to study the molecular mechanisms of male infertility and poor sperm quality, can provide large datasets of DEPs. These datasets further reflect the complexity of male infertility since the same clinical condition may originate from the dysregulation of different molecular pathways. The present review identified, for the first time, DEPs systematically reported in proteomic studies focused on male infertility. Herein, we provide a list of 76 potential protein candidates to assess sperm quality in specific conditions or which are more generally related to poor sperm quality. Some of these proteins (ACR, ACRBP, ACRV1, ACTL9, AKAP4, ATG3, CCT2, CFAP276, CFAP52, FAM209A, GGH, HPRT1, LYZL4, PRDX6, PRSS37, REEP6, ROPN1B, SPACA3, SOD1, SPEM1, SPESP1, SPINK2, TEKT5, and ZPBP) have a selective expression in male tissues or have been associated with male infertility phenotypes or biological processes

relevant to sperm function. These DEPs are obvious targets for further basic and clinical research and may be validated for screening purposes, potentiating an improved clinical diagnosis of the origins of male infertility.

Conflict of Interest

The authors have nothing to disclose.

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Author Contribution

Conceptualization: POC, JVS, MF. Data curation: POC, JVS. Formal analysis: POC. Funding Acquisition: MF, JVS. Investigation: POC, JM. Methodology: POC, JVS. Project administration: MF. Supervision: PFO, MF. Writing – original draft: POC. Writing – review & editing: POC, JH, MF, JVS.

Supplementary Materials

Supplementary materials can be found *via* https://doi.org/10.5534/wjmh.220262.

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