

High-throughput sperm differential proteomics suggests that epigenetic alterations contribute to failed assisted reproduction

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STUDY QUESTION: Are there quantitative alterations in the proteome of normozoospermic sperm samples that are able to complete IVF but whose female partner does not achieve pregnancy?

SUMMARY ANSWER: Normozoospermic sperm samples with different IVF outcomes (pregnancy versus no pregnancy) differed in the levels of at least 66 proteins.

WHAT IS KNOWN ALREADY: The analysis of the proteome of sperm samples with distinct fertilization capacity using low-throughput proteomic techniques resulted in the detection of a few differential proteins. Current high-throughput mass spectrometry approaches allow the identification and quantification of a substantially higher number of proteins.

STUDY DESIGN, SIZE, DURATION: This was a case–control study including 31 men with normozoospermic sperm and their partners who underwent IVF with successful fertilization recruited between 2007 and 2008.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Normozoospermic sperm samples from 15 men whose female partners did not achieve pregnancy after IVF (no pregnancy) and 16 men from couples that did achieve pregnancy after IVF (pregnancy) were included in this study. To perform the differential proteomic experiments, 10 no pregnancy samples and 10 pregnancy samples were separately pooled and subsequently used for tandem mass tags (TMT) protein labelling, sodium dodecyl sulphate–polyacrylamide gel electrophoresis, liquid chromatography tandem mass spectrometry (LC-MS/MS) identification and peak intensity relative protein quantification. Bioinformatic analyses were performed using *UniProt Knowledgebase*, *DAVID* and *Reactome*. Individual samples ($n = 5$ no pregnancy samples; $n = 6$ pregnancy samples) and aliquots from the above TMT pools were used for western blotting.

MAIN RESULTS AND THE ROLE OF CHANCE: By using TMT labelling and LC-MS/MS, we have detected 31 proteins present at lower abundance (ratio no pregnancy/pregnancy < 0.67) and 35 at higher abundance (ratio no pregnancy/pregnancy > 1.5) in the *no pregnancy group*. Bioinformatic analyses showed that the proteins with differing abundance are involved in chromatin assembly and lipoprotein metabolism (P values < 0.05). In addition, the differential abundance of one of the proteins (SRSF protein kinase 1) was further validated by western blotting using independent samples (P value < 0.01).

LIMITATIONS, REASONS FOR CAUTION: For individual samples the amount of recovered sperm not used for IVF was low and in most of the cases insufficient for MS analysis, therefore pools of samples had to be used to this end.

WIDER IMPLICATIONS OF THE FINDINGS: Alterations in the proteins involved in chromatin assembly and metabolism may result in

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epigenetic errors during spermatogenesis, leading to inaccurate sperm epigenetic signatures, which could ultimately prevent embryonic development. These sperm proteins may thus possibly have clinical relevance.

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Key words: human sperm / differential proteomics / isotopic tandem mass tags / epigenetics / assisted reproduction

Introduction

The routine assessment of semen quality is currently based on sperm count, motility and morphology or, occasionally, on the study of sperm DNA damage (Domínguez-Fandos et al., 2007; Castillo et al., 2011; Simon et al., 2011). However, in most cases these phenotypic assessments do not provide a clue for the aetiology of male infertility. In addition, the current treatments using assisted reproduction techniques (ART), such as IVF or ICSI, are successful in only ~32% of the cases (de Mateo et al., 2009; Ferraretti et al., 2013). This situation also occurs in couples without a perceptible female factor and whose male partner is apparently normal (normozoospermic individuals). In fact, a currently unsolved issue in andrology is why some normozoospermic samples result in pregnancy after ART, while others do not. One of the reasons for the poor ART outcomes may be the existence of sperm alterations at the molecular level, which are not detected using the current routine clinic laboratory procedures (Oliva et al., 2009; Oliva and de Mateo, 2011; Oliva and Ballescà, 2012).

Changes in specific sperm protein levels are possibly involved in these potential molecular alterations and indeed several studies, using a variety of protein identification strategies, have tried to clarify this. Two kinds of studies are found in the literature: those targeted to the quantification of one or a few proteins using specific antibodies, enzymatic activity assays or direct detection (de Yebra et al., 1993; de Yebra and Oliva, 1993; Bench et al., 1998; de Yebra et al., 1998; Mengual et al., 2003; de Mateo et al., 2009; 2011b,c) and those using wider proteomic approaches relying on mass spectrometry (MS) to concurrently analyse as many sperm proteins as possible (de Mateo et al., 2007; Oliva et al., 2007, 2008; Botta et al., 2009; Liao et al., 2009; Secciani et al., 2009; Oliva and Castillo, 2011; Paasch et al., 2011; Thacker et al., 2011; Oliva, 2012). Differential proteomic studies are often carried out to compare sperm samples with defined aetiologies, such as asthenozoospermic versus normozoospermic (Zhao et al., 2007; Martínez-Heredia et al., 2008; Chan et al., 2009; Siva et al., 2010; Parte et al., 2012). On the other hand, proteomic studies aimed to investigate the correlation between sperm protein levels and ART results are scarce. The pioneer study of this kind used two-dimensional gel electrophoresis (2DE) to compare the proteome of sperm from one normozoospermic patient involved in failed IVF to that of three fertile donors, and resulted in the detection of 4 differential proteins (Pixton et al., 2004). The use of a more sensitive proteomic approach (2D fluorescence difference gel electrophoresis) to compare the proteomic mapping of sperm from 3 infertile patients (with no fertilization after IVF) to that of 3 fertile donors resulted in the identification of 12 differentially expressed proteins (Frapsauce et al., 2009). More recently, a 2DE comparison between 10 normozoospermic sperm samples associated with failed fertilization after artificial insemination and 10 sperm samples from healthy

semen donors detected 24 differentially abundant proteins (Xu et al., 2012).

Although the outcomes obtained in the aforementioned studies are relevant, it is well established that proteomic approaches based on 2DE allow the identification of a minority of the sperm proteome when compared with the detection power of current high-throughput approaches based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS; Baker et al., 2007, 2013; de Mateo et al., 2011a, 2013; Amaral et al., 2013, 2014; Wang et al., 2013). An elegant strategy to perform relative protein quantification by LC-MS/MS consists of the use of isobaric tandem mass tags (TMTs), which are reactive towards lysine residues and free amino termini of peptides (Thompson et al., 2003; Dayon and Sanchez, 2012). Despite the efficiency of this approach, that has been applied to study proteome alterations in distinct somatic cells (Dayon et al., 2010; Sinclair and Timms, 2011; Raso et al., 2012; Paulo et al., 2013; just to give a few examples), the use of the TMT technology in reproductive biology context is scarce (Zhu et al., 2013).

The aim of this study was to conduct a quantitative comparison of the proteome of sperm samples from normozoospermic individuals with different ART outcomes (i.e. *pregnancy group* versus *no pregnancy group*; but for which a similar number of morphologically normal embryos were obtained) using TMT and LC-MS/MS. We reasoned that this approach would result in the identification of sperm proteomic differences that may affect embryonic development and thus have clinical significance.

Materials and Methods

Chemicals

All reagents were supplied from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

Ethical approval

Patients undergoing IVF treatment were recruited from the Assisted Reproduction Unit of the Clinic Hospital of Barcelona, Spain. All patients signed informed consent forms and all human material was used in accordance with the appropriate ethical and Internal Review Board guidelines.

Biological material selection

A total of 31 couples with similar clinical male and female characteristics but different IVF outcomes were included in this study. The *pregnancy group* ($n = 16$) corresponds to couples who achieved pregnancy after IVF and the *no pregnancy group* ($n = 15$) to those who did not achieve pregnancy after IVF. All female partners were affected by tubal pathology, while having normal oocyte production and gestational capacity (FSH, LH and estradiol levels were normal). Routine seminal analysis was performed according to the

World Health Organization Guidelines (WHO, 2010) and all sperm samples used were normozoospermic. ICSI was not attempted in any of the couples because a substantial number of embryos were obtained using IVF and good-quality embryos were transferred in all the cases. Noteworthy, apart from the contrasting ART outcomes, no statistically significant differences were detected in any of the clinical variables in the two groups of samples (Table I, Supplementary data, Table S1).

Sperm preparation

Fresh semen samples were obtained by masturbation after 3–5 days of sexual abstinence. Samples were prepared on a density gradient using Puresperm® (Nidacon, Mölndal, Sweden), according to the manufacturer's protocol. The recovered Puresperm fraction not used for IVF was washed in Ham's F-10 nutrient mixture (Life Technologies™, Paisley, UK) and stored at -20°C in Ham's F-10 supplemented with 7% (v/v) glycerol. Only samples containing no contaminating round cells were used.

Quantitative proteomics

The general strategy used is depicted in Figure 1. Two pools of sperm samples (*pregnancy group* and *no pregnancy group*) containing 150 million sperm each (15 million from 10 samples for each group) were used. The TMT duplex™ Isotopic Label Reagent Set (Thermo Scientific, Rockford, IL, USA) was used for labelling. Basically, this approach relies on the labelling of proteins (or peptides) from distinct samples with specific isobaric tags (i.e. with the same mass) which, upon fragmentation, give rise to unique reporter ions in the MS/MS spectrum. The intensities of the reporter ions can be used to calculate relative amounts of protein between samples. The two pools of labelled proteins were mixed and resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and the bands were excised. After in-gel digestion, peptides were separated by one-dimensional liquid chromatography and analysed by MS/MS.

Protein solubilization

Proteins were solubilized in Lysis Buffer (7 M urea, 2 M thiourea, 1% (w/v) CHAPS, 1% (w/v) *N*-octylglucopyranoside, 18 mM dithiothreitol (DTT) and 2.4 mM phenylmethylsulphonyl fluoride (PMSF) for 1 h at room temperature, with constant shaking, as described previously (Martinez-Heredia et al., 2006; de Mateo et al., 2011a; Amaral et al., 2013).

TMT labelling

Proteins (100 μg) from the two pooled samples (*pregnancy group* and *no pregnancy group*) were precipitated with 80% (v/v) cold acetone and separately labelled according to the manufacturer's recommendations. Briefly, proteins were incubated in 45 mM triethyl ammonium bicarbonate with 0.1% SDS, reduced in 9.5 mM tris(2-carboxyethyl)phosphine (TCEP) for 1 h at 55°C and alkylated with 17 mM iodoacetamine for 30 min, at room temperature, in the dark. TMT label reagents (0.8 mg each) were equilibrated to room temperature, dissolved in 24 μl anhydrous dimethyl sulphoxide (DMSO) and added to the reduced and alkylated proteins (the *pregnancy group* was labelled with TMT-127 and the *no pregnancy group* with TMT-126). After incubation for 1 h at room temperature, reactions were quenched by incubating for 15 min in 0.28% hydroxylamine. Labelled samples were then combined in equal amounts.

SDS-PAGE

Acetone precipitated proteins were suspended in Laemmli buffer (60 mM Tris HCl, pH 6.8, 2.2% (w/v) SDS, 5% (v/v) glycerol, 0.1 M DTT), and incubated for 10 min at 90°C . After cooling, a total of 150 μg of labelled proteins were separated by SDS-PAGE (12% acrylamide gel with a 3.9% acrylamide stacking gel). The gel was fixed with 50% methanol 10% acetic acid and stained with EZBlue™ Gel Staining Reagent for 5 min (until a protein smear was just visible).

LC-MS/MS

The entire gel lane was carefully cut into small pieces (~ 1 mm intervals) that were then processed for MS analysis as described before (Amaral et al., 2013). Essentially, the gel slices were digested with 150 ng trypsin (Promega, Madison, WI, USA) at 37°C overnight, according to the manufacturer's recommendations. Tryptic peptides were separated by means of nano liquid chromatography using a Proxeon EASY-nLC (Thermo Fisher Scientific, Waltham, MA, USA) with a flow rate of 300 nl/min, an EASY C18 trap column (5 μm , 120 \AA , 100 μm inner diameter \times 2 cm length), and an EASY C18 analytical column (3 μm , 120 \AA , 75 μm inner diameter \times 10 cm length). The following linear gradient, using Solvent B (97% acetonitrile, 0.1% formic acid) and Solvent A (3% acetonitrile, 0.1% formic acid), was employed: 5–35% buffer B (150 min); 35–100% buffer B (5 min). MS/MS analysis was performed using an LTQ Orbitrap Velos (Thermo Fisher Scientific) with a nano electrospray ion source with precursor ion selection in the

Table I Groups of samples used in the differential proteomic comparison ($n = 10 + 10$).

		Pregnancy group	No pregnancy group	P-value
Male partner	Age	35.70 \pm 4.14	35.80 \pm 2.53	0.949
	Semen volume (ml)	4.11 \pm 2.31	3.59 \pm 1.69	0.573
	Sperm concentration (million sperm/ml semen)	67.15 \pm 23.14	55.02 \pm 21.91	0.244
	Sperm motility (% progressively motile sperm)	61.36 \pm 14.36	60.68 \pm 14.06	0.916
Female partner	Age	30.30 \pm 4.35	31.00 \pm 2.26	0.659
	Body mass index (kg/m^2)	22.71 \pm 1.46	22.51 \pm 2.50	0.829
	Basal FSH levels (U/l)	5.39 \pm 1.13	5.52 \pm 0.83	0.773
	Basal LH levels (U/l)	4.67 \pm 1.89	4.97 \pm 2.00	0.734
	Basal E2 levels (U/l)	56.70 \pm 27.97	42.20 \pm 23.55	0.226
	Number of oocytes collected	14.20 \pm 2.97	13.80 \pm 3.97	0.802
Embryos	Number of embryos obtained	9.30 \pm 4.19	8.30 \pm 2.21	0.513

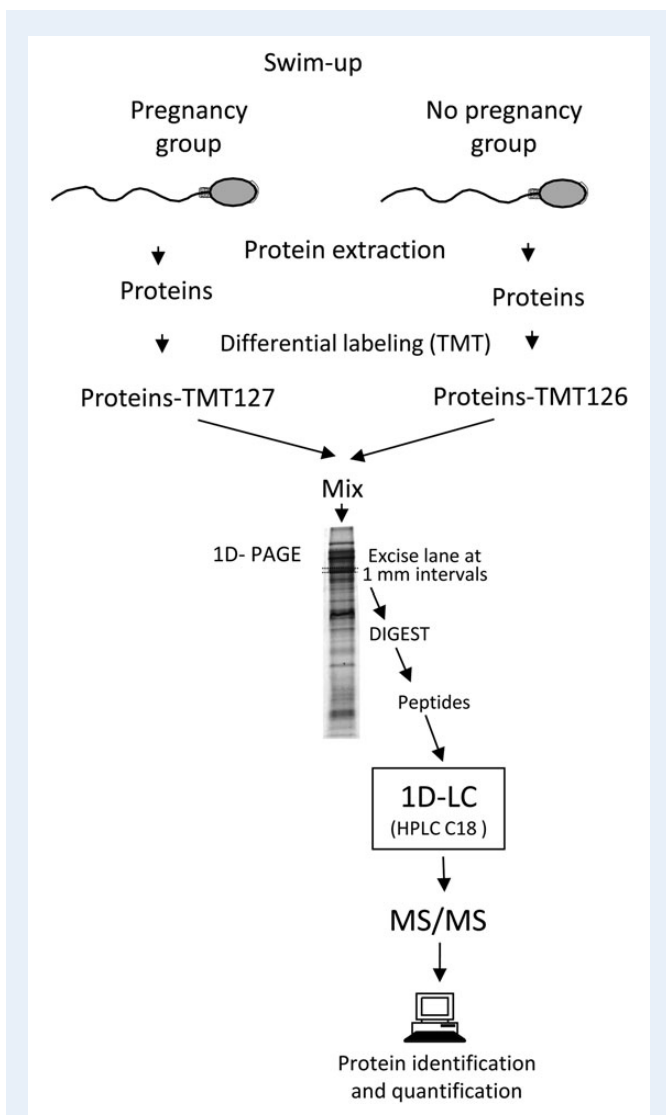


Figure 1 Overall methodological approach used to determine relative protein levels in normozoospermic samples differing in assisted reproduction outcome. A total of 20 normozoospermic samples from infertile patients undergoing IVF were included in this study: 10 samples from couples that achieved pregnancy after IVF (*pregnancy group*) and 10 samples from couples that did not achieve pregnancy after IVF (*no pregnancy group*). Proteins were extracted from all the samples individually, then pooled into *pregnancy* and *no pregnancy* groups, labelled with TMT duplex isobaric mass tags, joined and separated by monodimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis. After electrophoresis, 1 mm² bands were excised from the gel and processed for liquid chromatography mass spectrometry analysis (LC-MS/MS). Proteins were identified and their relative abundance quantified in order to detect putative proteomic alterations in *no pregnancy group* samples.

Orbitrap at 30 000 of resolution, selecting the 15 most intense precursor ions, with the collision energy of 35, in positive ion mode. MS/MS data acquisition was completed using Xcalibur 2.1 (Thermo Fisher Scientific). For identification of TMT-labelled peptides, higher energy collisional dissociation with fixed collision energy of 40% was the fragmentation method used.

Protein identification and quantification

Data were processed using Proteome Discoverer 1.4 (Thermo Fisher Scientific). For database searching, processed data were submitted to the in-house *Homo sapiens* UniProtKB/Swiss-Prot database (release 2012-04; 88 323 protein entries) using SEQUEST version 28.0 (Thermo Fisher Scientific). The following search parameters were used: two maximum miss cleavage for trypsin; TMT-labelled lysine and methionine oxidation as dynamic modifications; cysteine carbamidomethylation as fixed modification; 25 ppm precursor mass tolerance; 20 mmu fragment mass tolerance and 20 ppm TMR integration tolerance. Criteria used to accept identification included a minimum of two peptides (and at least one unique peptide) matched per protein, with a false discovery rate of 0.01.

Quantitative analysis was performed using Proteome Discoverer software. The reporter ion intensities were corrected according to the isotopic purities provided by the manufacturer. The relative protein level ratios between the two groups were determined from the TMT fragment peak intensities and were calculated using the median intensities of reporter ions for each protein. The ratios were log₂ transformed to reduce variance heterogeneity (Boehm et al., 2007; Gan et al., 2007) and further normalized using the value of the median ratio of all the proteins. A 95% confidence interval was calculated (median ± 1.96 SD) and all median ratios not contained in this interval were considered up- or down-regulated (Jung et al., 2009; Karp et al., 2010; Podwojski et al., 2012). Importantly, all the proteins detected using this method had a ratio > 1.5 (more abundant proteins) or < below 0.67 (less abundant proteins). Additionally, and in order to validate the results obtained, the MS spectra of all the differential proteins detected were manually checked.

Differential proteome analysis

Proteins identified in different amounts in the two groups were classified according to subcellular localization and biological function(s) using the information available at the UniProt Knowledgebase (UniProtKB/Swiss-Prot) website (<http://www.uniprot.org>). The list of differential proteins was also analysed using the bioinformatics tool DAVID v6.7 (Database for Annotation, Visualisation and Integrated Discovery; <http://david.abcc.ncifcrf.gov/>) in order to identify overrepresented Gene Ontology (GO) *Biological Process* terms (Huang et al., 2009a,b). The significance of gene-enrichment analysis was expressed by an EASE Score, corresponding to a modified Fisher exact *P* value (*P* values < 0.05 were considered significant). Furthermore, the Reactome database (<http://www.reactome.org/>) was used to perform overrepresentation analysis, with the aim of recognizing those biological pathways likely to be affected in sperm samples from couples that did not achieve pregnancy. The significance of the association between the protein list and a certain pathway was expressed by a *P* value, representing the probability (one-tailed Fisher's exact test) that the association between the proteins in the dataset and the pathway is explained by chance alone (*P* values < 0.001 were considered significant).

Western blotting

To further confirm the proposal that there may be altered levels of proteins involved in sperm chromatin assembly with a potential epigenetic role in the male gamete and validate the MS results, we have used western blotting to compare the expression of SRSF protein kinase I (SRPK1; detected at higher levels in the *no pregnancy group*), not only in the pools of samples used for MS, but also in additional individual samples. SRPK1 expression was also analysed using 11 samples (5 and 6 from *no pregnancy* and *pregnancy groups*, respectively), using rabbit anti-human SRPK1 polyclonal antibody (0.5 µg/ml) and 35 µg total protein/sample, essentially as described before (Amaral et al., 2013). For loading controls, a mouse anti-human alpha-tubulin monoclonal antibody (clone DM1A purified mouse immunoglobulin;

1 µg/ml) was used. Detection was carried out using the Amersham Biosciences ECL Plus™ Western Blotting Analysis System (GE Healthcare, Buckinghamshire, UK) and a LAS-3000 imaging system (Fujifilm, Tokyo, Japan). Band intensities were measured using Quantity One 1D software version 4.5.0 (Bio-Rad, Hercules, CA, USA) and the results were expressed by the ratio of band intensities (SRPK1/alpha-tubulin).

Determination of protamine 1 (P1)/protamine 2 (P2) ratio

In order to monitor the protamination status on the two groups of sperm samples (and thus to assess overall sperm chromatin packaging and protection), the P1/P2 ratio was also determined. Towards this goal, the insoluble fractions of eight samples (four from each group) were processed as described previously (Castillo *et al.*, 2011). Basically, extracted protamines, along with external standards of known protamine concentrations, were run on acid-urea polyacrylamide gels (acid-urea PAGE). Band intensities were quantified using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA) and used to determine the amounts of P1 and P2 in each of the samples. Quantification calculations were based on a regression curve constructed with the protamine standards.

Statistical analyses

Statistical analyses were performed using IBM SPSS statistics software (version 21.0; SPSS Corp., Chicago, IL, USA). One-sample Kolmogorov–Smirnov test was used to check for normal distribution and the independent sample *t*-test was used to compare: (i) the male and female parameters between the two groups of couples included in the study; (ii) SRPK1 expression between the two groups of sperm samples; (iii) P1/P2 ratio between the two groups of sperm samples. A *P* value of <0.05 was considered significant.

Results

Normozoospermic sperm proteome

LC-MS/MS of the 2 groups of sperm samples (comprising 10 different samples each, loaded onto a single gel lane) resulted in the identification of 1717 proteins (Supplementary data is available at the Peptide Atlas protein repository; Dataset Identifier: PASS00459; <http://www.peptideatlas.org/PASS/PASS00459>). A GO Biological Process term analysis of all the proteins identified is shown in the Supplementary data, Table SII. Of all the proteins identified, 137 proteins turn out to be novel proteins (i.e. proteins not reported in any of the human sperm proteomic studies performed so far; Supplementary data, Fig. S1). These include proteins from different sperm subcellular parts, such as the nucleus (e.g. histone H2B type F-M; transcription factor E3; small ubiquitin-related modifier 2) or the mitochondrion [e.g. NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 3; mitochondrial import inner membrane translocase subunit Tim 17-B; propionyl-CoA carboxylase alpha chain], as well as secreted proteins, that may participate in sperm–oocyte interactions (e.g. alpha-1B-glycoprotein; alpha-2-HS-glycoprotein; beta-2-glycoprotein 1).

Sperm samples associated with failed/successful ART outcomes differ in the relative abundance of several proteins

The comparison of the proteomes of the *no pregnancy* (case) and *pregnancy* (control) groups of sperm samples using TMT-126 and TMT-127 peak intensities (Fig. 2) resulted in the detection of 66

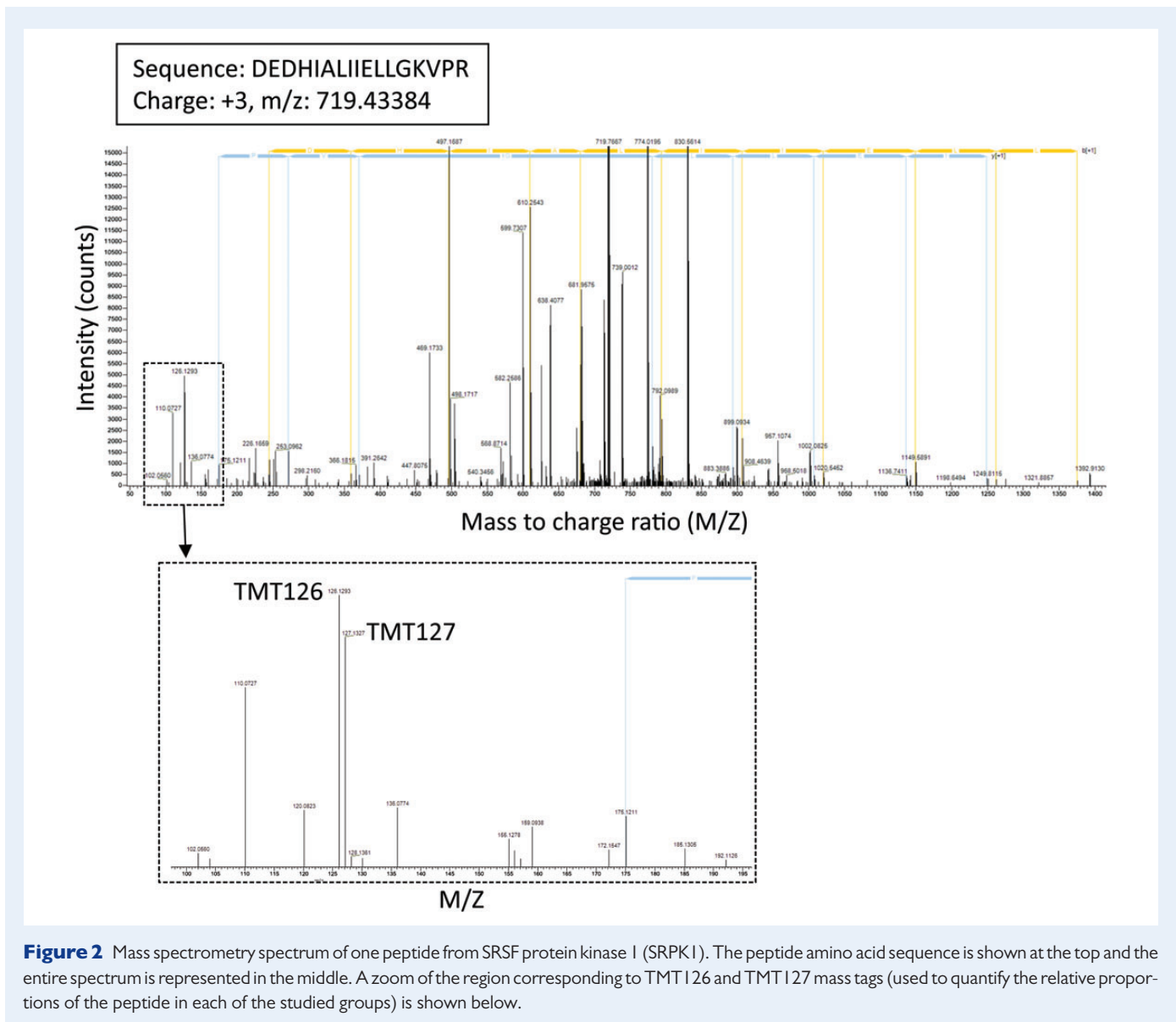
differentially expressed proteins (ratio *no pregnancy* group/*pregnancy* group < 0.67 or > 1.5; Tables II and III). Of the 66 differential proteins detected, 31 were found at lower levels in the *no pregnancy* group (ratio < 0.67; Table II), whereas 35 were found at higher levels (ratio > 1.5; Table III). Noteworthy, the groups of higher or lower abundant proteins seem to correspond to different categories. For example, a first analysis highlights that the sub-list of higher abundant proteins includes six core histones (H2A type 1-A, H2A type 1-C, H2A type 2-C, H2B type 1A, H3 and H4), while none was found in the sub-list of lower abundant proteins. On the other hand, the lower abundant proteins includes two with possible roles in early development (left-right determination factor 1 and growth/differentiation factor 15) and three proteins related to lipid metabolism (lipase member 1, lipoprotein lipase and apolipoprotein).

The differential proteins belong to particular GO categories and are involved in specific cellular pathways

The categorization of the differential proteins according to the information available at the Uniprot Knowledgebase resulted in very interesting outcomes (Fig. 3). Concerning cellular localization (Fig. 3A), it is interesting to appreciate that the sub-list of lower abundant proteins comprises various membrane/secreted proteins (12 out of 31 proteins; 39%). In contrast, the majority of proteins in the sub-list of higher abundant proteins are mitochondrial (23%), membranar/secreted (17%) or nuclear (16%). Similarly, the sets of higher abundant and lower abundant proteins are associated with different cellular functions (Fig. 3B). Worth mentioning, all differential proteins involved in nucleosome assembly were detected at higher levels in the *no pregnancy* group, whereas all cellular trafficking proteins were less abundant in this group (Fig. 3B). However, this first analysis was only indicative of a trend and had no statistical significance associated.

We then questioned whether the differentially detected proteins were involved in explicit biological processes and, in such a case, if the sets of up- and down-regulated proteins were distinct. To answer these questions, we have performed GO terms enrichment analysis using DAVID. The outcomes obtained confirmed that the sets of proteins detected in lower and higher levels in the *no pregnancy* group are involved in distinctive biological processes (Table IV). To this extent, sperm from the *no pregnancy* group have higher levels of proteins involved in DNA packaging, nucleosome organization and chromatin/nucleosome assembly and lower levels of proteins with roles in intracellular transport and sexual reproduction (*P* values < 0.05). Additionally, biological processes related to apoptosis were also detected as enriched in the *no pregnancy* group.

With the aim of determining if higher or lower abundant proteins could affect specific cellular pathways and to elucidate how would the differential proteins contribute to failed ART outcomes in *no pregnancy* samples, Reactome pathways analysis was also carried out (Table V). Confirming our initial observations, analysis of the group of proteins present at lower abundance using Reactome resulted in the detection of *lipoprotein metabolism* (and specifically high-density lipoprotein (HDL)-mediated lipid transport) as a significant pathway (*P* values < 0.0001). On the other hand, and corroborating the outcomes obtained with DAVID, Reactome recognized *meiosis* and *chromosome maintenance* as pathways in which the higher abundance proteins might be involved (*P* values < 0.001).



The protamine 1 (P1)/protamine 2 (P2) ratio is not significantly different in the two groups of samples

The results indicate that in the group of normozoospermic samples associated with failed ART outcomes the P1/P2 ratio is 1.71 ± 0.37 (mean \pm SD) and in the pregnancy group the P1/P2 ratio is 1.57 ± 0.28 (mean \pm SD). No statistically significant differences were detected between the two groups ($P = 0.573$).

The levels of proteins with a potential sperm epigenetic function may be altered in normozoospermic samples associated with failed ART outcomes

The outcomes of the three analyses performed (manual protein functional categorization according to the information available at the Uniprot Knowledgebase; DAVID GO Biological Process terms

enrichment and *Reactome* pathways analysis) are all in agreement with the idea that normozoospermic samples associated with failed ART outcomes may have altered levels of proteins involved in sperm chromatin assembly (which may have an epigenetic role in the male gamete).

The results obtained confirmed that the level of SRPK I was higher in the pooled *no pregnancy* samples compared with the pooled *pregnancy* samples (Fig. 4A) and this was further corroborated by the analysis of additional independent sperm samples (Fig. 4B). Indeed, there was a statistically significant difference in the relative amount of SRPK I in the two sets of samples (P value < 0.01).

Discussion

Unravelling the putative causes of the increasing number of clinical cases of idiopathic male infertility is certainly one of the biggest challenges in modern andrology. Although reaching such a goal may be very difficult due to the multitude of possible reasons and the fact that infertility is,

Table II Lower abundant proteins in normozoospermic samples that did not achieve pregnancy (n = 31).

UniprotKB/ Swiss-Prot accession number	Protein name	Ratio: no pregnancy/ pregnancy
B0QY90	Eukaryotic translation initiation factor 3 subunit I	0.25
P31947	Isoform 2 of I4-3-3 protein sigma	0.38
Q02383	Semenogelin-2	0.41
B4DI70	cDNA FLJ53509, highly similar to Galectin-3-binding protein	0.43
Q6XZB0	Lipase member I	0.43
P04279-2	Isoform 2 of Semenogelin-1	0.44
P02768	Serum albumin	0.44
B3KRV7	Lipoprotein lipase	0.45
P54886-2	Isoform short of Delta-1-pyrroline-5-carboxylate synthase	0.48
Q99988	Growth/differentiation factor 15	0.49
Q6BCY4-2	Isoform 2 of NADH-cytochrome b5 reductase 2	0.49
O43592	Exportin-T	0.49
O75610	Left-right determination factor 1	0.50
P0C7M6	IQ domain-containing protein F3	0.50
B7Z1J9	Parkinson disease 7 domain-containing protein 1	0.51
P05109	Protein S100-A8	0.51
Q8N427	Thioredoxin domain-containing protein 3	0.52
Q96RL7-4	Isoform 4 of vacuolar protein sorting-associated protein 13A	0.52
Q5H943	Kita-kyushu lung cancer antigen 1	0.53
P02649	Apolipoprotein E	0.54
P35237	Serpin B6	0.54
F5GX33	Calcium-binding mitochondrial carrier protein Aralar2	0.55
Q92599-3	Isoform 3 of Septin-8	0.56
P0CG04	Ig lambda-1 chain C regions	0.56
O95336	6-phosphogluconolactonase	0.56
O14980	Exportin-1	0.56
B4DY72	Heat shock protein 105 kDa	0.57
P51148	Ras-related protein Rab-5C	0.57
O15084	Serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit A	0.57
Q96KX2	F-actin-capping protein subunit alpha-3	0.59
B4DPM9	Serine hydroxymethyltransferase	0.60

Table III Higher abundant proteins in normozoospermic samples that did not achieve pregnancy (n = 35).

UniprotKB/ Swiss-Prot accession number	Protein name	Ratio: no pregnancy/ pregnancy
Q53HC9	Protein TSSCI	2.44
F8VV32	Lysozyme C	2.37
Q9H095-2	Isoform 2 of IQ domain-containing protein G	2.37
Q0P670	Uncharacterized protein C17orf74	2.29
Q8N5K1	CDGSH iron-sulphur domain-containing protein 2	2.28
P05164-2	Isoform H14 of myeloperoxidase	2.04
P04083	Annexin A1	1.98
E9PIC0	Succinate dehydrogenase [ubiquinone] cytochrome b small subunit, mitochondrial	1.94
P24752	Acetyl-CoA acetyltransferase, mitochondrial	1.94
Q8IZ16	Uncharacterized protein C7orf61	1.93
Q5SRN2	Uncharacterized protein C6orf10	1.92
Q96A08	Histone H2B type 1-A	1.91
P62805	Histone H4	1.87
O43837-2	Isoform A of isocitrate dehydrogenase [NAD] subunit beta, mitochondrial	1.86
Q5TEC6	Histone cluster 2, H3, pseudogene 2	1.86
B7Z1I2	Aspartate aminotransferase	1.86
P10644	cAMP-dependent protein kinase type I-alpha regulatory subunit	1.80
P16152	Carbonyl reductase [NADPH] I	1.80
Q96SB4	SRSF protein kinase I	1.79
E7EPB3	60S ribosomal protein L14	1.79
Q5TGZ0	Mitochondrial inner membrane organizing system protein 1	1.77
P13646-3	Isoform 3 of Keratin, type I cytoskeletal 13	1.76
E5RJX2	40S ribosomal protein S20	1.74
Q96IX5	Up-regulated during skeletal muscle growth protein 5	1.73
Q16777	Histone H2A type 2-C	1.70

Continued

by definition, a couple's (rather than an individual's) problem, modern omics technologies are expected to contribute to the elucidation of the molecular mechanisms that are at the origin of this disorder. In this

study we have used TMT protein labelling and MS to identify potential quantitative proteomic alterations in otherwise normal sperm samples, which may have contributed to embryonic development failure after

Table III Continued

UniprotKB/ Swiss-Prot accession number	Protein name	Ratio: no pregnancy/ pregnancy
O43464-3	Isoform 3 of serine protease HTRA2, mitochondrial	1.68
P49721	Proteasome subunit beta type-2	1.65
P14174	Macrophage migration inhibitory factor	1.63
Q93077	Histone H2A type 1-C	1.60
O14841	5-oxoprolinase	1.58
Q96QV6	Histone H2A type 1-A	1.58
Q5T5M1	Aquaporin 7	1.56
O14949	Cytochrome <i>b-c1</i> complex subunit 8	1.56
F8WAS3	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5	1.56
O76024	Wolfamin	1.54

successful IVF. While other sperm (and oocyte) molecular alterations cannot be ruled out, our data add further support to the increasing body of evidence suggesting that a tight regulation of sperm protein levels might be needed for normal embryonic development. Very interestingly, several of the differential proteins detected using our approach point to a range of cellular alterations that would result in inaccurate sperm epigenetic signatures.

In fact, a number of the differential proteins detected are localized to the chromatin. Of note, 6 out of the 14 histones (43%) identified in this study (Table III) were detected at significantly higher levels in the *no pregnancy group*. Additionally, analysis of the sub-set of more abundant proteins indicates enrichment in *nucleosome assembly* process (DAVID and Reactome). The increased levels of histones in samples that did not result in pregnancy may indicate a deficient nucleohistone to nucleoprotamine transition during spermiogenesis, which could result in abnormal sperm imprinting and/or in reduced paternal chromatin protection (Govin et al., 2004; Torregrosa et al., 2006; de Mateo et al., 2011c; Oliva and Castillo, 2011; Oliva and Ballescà, 2012). However, the relative amounts of protamines (P1/P2 ratio) in the *no pregnancy group* seemed to be normal and comparable to the *pregnancy group*. Thus, and because it is well-established sperm DNA damage is associated with alterations in protamination (Aoki et al., 2005; de Mateo et al., 2007; Castillo et al., 2011), these outcomes support the idea that no substantial nuclear DNA damage is present in these samples. On the other hand, SRPK1 levels were higher in the *no pregnancy group*.

SRPK1 specifically phosphorylates serine residues within arginine/serine-rich substrate regions and shuttles between the nucleus and the cytoplasm, is highly expressed in the testis and, among other functions, may contribute to sperm chromatin condensation (for review see Gianakourous et al., 2011). Indeed, one of the testis-specific substrates of SRPK1 is PI (Papoutsopoulou et al., 1999). Importantly, protamine phosphorylation might be critical for the correct binding of protamine

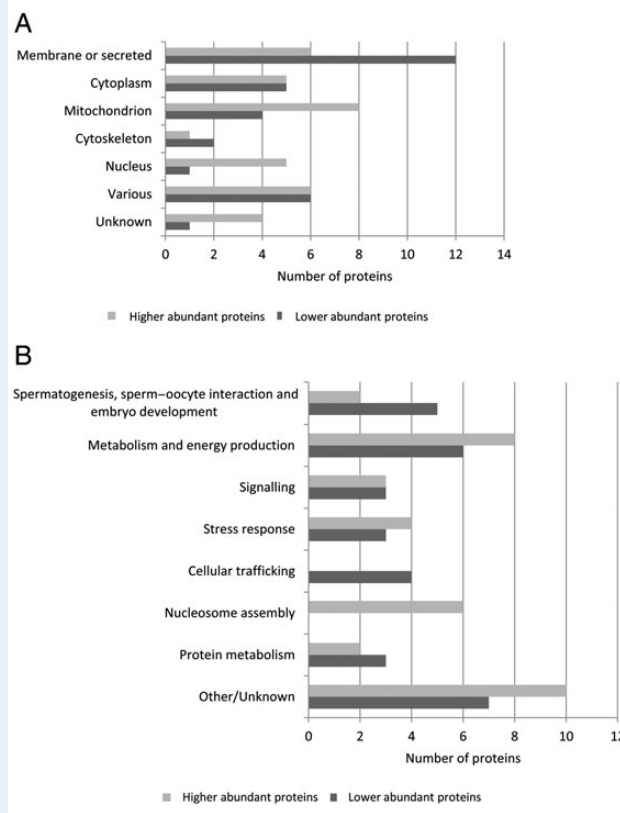


Figure 3 Cellular localizations and functions of the differential proteins detected. The protein cellular localizations (**A**) and functions (**B**) were determined according to the information available at the UniProtKB/Swiss-Prot website. Proteins were grouped as those that were present at higher abundance and those there were present at lower abundance in *no pregnancy group* compared with *pregnancy group*.

to the sperm DNA (Oliva and Mezquita, 1986; Oliva and Dixon, 1991; Oliva, 2006; Jodar and Oliva, 2014). Therefore, a deregulation of protamine phosphorylation could result in an abnormal distribution of the genes in the histone and protamine sperm chromatin regions (believed to have an epigenetic role; Arpanahi et al., 2009; Hammoud et al., 2009, 2011; Jodar et al., 2011; Oliva and Ballescà, 2012; Paradowska et al., 2012; Castillo et al., 2013) and potentially interfere with embryo development, without affecting sperm fertilization ability.

Furthermore, any putative epigenetic modification occurring during spermatogenesis could result in metabolic adjustments and conversely, metabolic shifts may also induce chromatin alterations. Indeed, a growing body of data is showing that there is a crosstalk between a cell's metabolome and its epigenome (for reviews see Gut and Verdin, 2013; Hino et al., 2013; Martinez-Pastor et al., 2013). For instance, the levels of HDL cholesterol in patients presenting with hypercholesterolemia were correlated with epigenome-wide changes (Guay et al., 2012a,b; Kim et al., 2012). To this extent, our data suggest that sperm samples that did not result in pregnancy after IVF have reduced levels of proteins with functions in lipid/lipoprotein metabolism: lipase member I (LIPI), apolipoprotein E (APOE) and lipoprotein lipase (LPL). LPL is a member of the triglyceride lipase family known to be expressed in human testis (Nielsen et al., 2010) and LIPI is a testis-specific

Table IV GO Biological Process terms enrichment of proteins differentially expressed in the *no pregnancy* group.

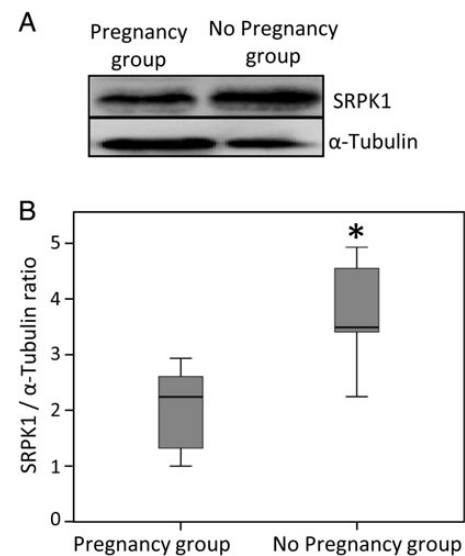
GO biological process term	GO identification	P-value
Lower abundant proteins		
Regulation of cellular component organization	GO: 0051128	1.9×10^{-2}
Sexual reproduction	GO: 0019953	1.9×10^{-2}
Cellular localization	GO: 0051641	2.7×10^{-2}
Regulation of biological quality	GO: 0065008	3.3×10^{-2}
Macromolecule localization	GO: 0033036	4.3×10^{-2}
Regulation of cellular component size	GO: 0032535	4.6×10^{-2}
Intracellular transport	GO: 0046907	4.9×10^{-2}
Higher abundant proteins		
Nucleosome assembly	GO: 0006334	3.4×10^{-6}
Chromatin assembly	GO: 0031497	3.9×10^{-6}
Protein-DNA complex assembly	GO: 0065004	4.6×10^{-6}
Nucleosome organization	GO: 0034728	5.0×10^{-6}
DNA packaging	GO: 0006323	1.3×10^{-5}
Macromolecular complex subunit organization	GO: 0043933	1.7×10^{-4}
Macromolecular complex assembly	GO: 0065003	1.2×10^{-3}
Chromosome organization	GO: 0051276	2.9×10^{-3}
Cellular component assembly	GO: 0022607	4.2×10^{-3}
Cellular component biogenesis	GO: 0044085	7.0×10^{-3}
Negative regulation of apoptosis	GO: 0043066	9.6×10^{-3}
Regulation of apoptosis	GO: 0042981	1.7×10^{-2}
Organelle organization	GO: 0006996	2.2×10^{-2}
Cellular component organization	GO: 0016043	2.8×10^{-2}
Oxidation reduction	GO: 0055114	4.5×10^{-2}

phospholipase (Hiramatsu *et al.*, 2003; Wen *et al.*, 2003). APOE has a key role in the transport and catabolism of lipoprotein particles and interestingly, male heterozygous knockout mice for a related member of the apolipoprotein family (APOB) were infertile, although their sperm were apparently normal (a phenotype resembling our *no pregnancy* individuals; Huang *et al.*, 1995). Moreover, a recent study suggests an association between APOA1 up-regulation and inhibition of a family of bromodomain epigenetic readers (Chung *et al.*, 2011), thus linking epigenetic alterations with the expression of an APO protein.

On a slightly different note, it was also interesting to find out that our sub-list of proteins present at lower abundance also includes proteins that have been implicated in spermatogenesis/sperm function. The examples are: (i) the testis-specific F-actin-capping protein, which is predominantly localized to the sperm neck region and seems to play an important role in sperm architecture and male fertility (Miyagawa *et al.*, 2002; Geyer *et al.*, 2009); (ii) the 14-3-3 protein sigma (SFN), an adapter protein implicated in the regulation of several signalling pathways

Table V Reactome pathway analysis results of the lower abundant and higher abundant proteins in the *no pregnancy* group as compared with the *pregnancy* group.

Pathway	Pathway identifier	P-value
Lower abundant proteins		
Lipoprotein metabolism	REACT_6823	5.6×10^{-4}
HDL-mediated lipid transport	REACT_13621	1.7×10^{-4}
Higher abundant proteins		
Meiosis	REACT_111183	1.1×10^{-5}
Meiotic recombination	REACT_27271	1.6×10^{-6}
Meiotic synapsis	REACT_75792	2.7×10^{-6}
Chromosome Maintenance	REACT_22172	2.9×10^{-5}
Nucleosome assembly	REACT_22344	1.0×10^{-6}
Telomere maintenance	REACT_7970	2.5×10^{-6}

**Figure 4** Western blot quantification of SRPK1 in sperm samples of infertile patients differing in assisted reproduction outcome. (A) Expression of SRPK1 in *pregnancy* ($n = 10$) and *no pregnancy* ($n = 10$) pooled samples (the same used for LC-MS/MS). (B) Box plot corresponding to SRPK1 expression (normalized to alpha-tubulin) in independent samples (*pregnancy* group: $n = 6$; *no pregnancy* group: $n = 5$). SRPK1/alpha-tubulin was expressed in arbitrary units. Asterisk denotes a statistically significant difference between the two groups (P value < 0.01).

in which epimutations in the corresponding gene have been reported in association with poor semen parameters (Houshdaran *et al.*, 2007); and family members of this protein have distinct binding partners in the seminiferous epithelium (Sun *et al.*, 2009) and (iii) exportin I, known to mediate the nuclear export of both RNAs and proteins bearing a leucine-rich nuclear export signal, that is involved in nucleocytoplasmic transport during spermatogenesis (Whiley *et al.*, 2012). In accordance,

both intracellular transport and sexual reproduction were GO biological process terms found to be enriched in the sub-set of less abundant proteins.

Ultimately, and although the role of paternal proteins in early embryonic development (with a few exceptions) is uncertain, it is tempting to speculate that the detected lower levels of certain sperm proteins, such as the left-right determination factor 1 (LEFTY1) and growth/differentiation factor 1 (GDF15) may prevent embryonic development. LEFTY1 is a member of the transforming growth factor (TGF) beta superfamily of proteins that may be involved in the left-right asymmetry determination of organ systems during development and seems to play important roles in decidualization and embryo implantation and to participate in stem cell pluripotency and cell fate decisions (Raya and Izpisua Belmonte, 2006; Tabibzadeh and Hemmati-Brivanlou, 2006; Tabibzadeh, 2011). Our results also raise a potential involvement of this protein in sperm cell dysfunction. Similarly, GDF15, which also belongs to the TGF beta superfamily, seems to be able to promote cell proliferation and differentiation during mouse prostate development and has been described as an abundant cytokine in human seminal plasma (Böttner et al., 1999; Noorali et al., 2007; Soucek et al., 2010). Then again, several members of the TGF beta superfamily have been indicated as key mediators of testis development and spermatogenesis (Loveland et al., 2007; Fan et al., 2012).

To sum-up, sperm proteomic epigenetic alterations may be the origin of the stalling of embryo development after successful IVF in at least some idiopathic infertile couples. Our data suggest that proteomic alterations occurring during spermiogenesis may alter sperm epigenetic signatures, ultimately resulting in abnormal embryo development. These proteomic alterations seem to be subtle, not affecting major features of sperm quality, but putatively influencing sperm genetic chromatin distribution and imprinting. Ultimately, some of the differential proteins described in this study may be effective sperm biomarkers and the assessment of their expression in sperm (using, for example, a sperm-specific protein array) could ultimately be used to help anticipate ART results.

Supplementary data

Supplementary data are available at <http://humrep.oxfordjournals.org/>.

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Authors' roles

R.A. and J.M.E.: participation in study design, execution, analysis and critical discussion. A.A., J.C. and R.O.: participation in study design, execution, analysis, manuscript drafting and critical discussion. M.G., J.L.B. and J.B.: execution and critical discussion.

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Conflict of interest

The authors have no conflict of interest to declare.

References

- Amaral A, Castillo J, Estanyol JM, Ballescà JL, Ramalho-Santos J, Oliva R. Human sperm tail proteome suggests new endogenous metabolic pathways. *Mol Cell Proteomics* 2013; **12**:330–342.
- Amaral A, Castillo J, Ramalho-Santos J, Oliva R. The combined human sperm proteome: cellular pathways and implications for basic and clinical science. *Hum Reprod Update* 2014; **20**:40–62.
- Aoki VW, Moskovtsev SI, Willis J, Liu L, Mullen JB, Carrell DT. DNA integrity is compromised in protamine-deficient human sperm. *J Androl* 2005; **26**:741–748.
- Arpanahi A, Brinkworth M, Iles D, Krawetz SA, Paradowska A, Platts AE, Saida M, Steger K, Tedder P, Miller D. Endonuclease-sensitive regions of human spermatozoal chromatin are highly enriched in promoter and CTCF binding sequences. *Genome Res* 2009; **19**:1338–1349.
- Baker MA, Reeves G, Hetherington L, Müller J, Baur I, Aitken RJ. Identification of gene products present in Triton X-100 soluble and insoluble fractions of human spermatozoa lysates using LC-MS/MS analysis. *Proteomics Clin Appl* 2007; **1**:524–532.
- Baker MA, Naumovski N, Hetherington L, Weinberg A, Velkov T, Aitken RJ. Head and flagella subcompartmental proteomic analysis of human spermatozoa. *Proteomics* 2013; **13**:61–74.
- Bench G, Corzett MH, de Yebra L, Oliva R, Balhorn R. Protein and DNA contents in sperm from an infertile human male possessing protamine defects that vary over time. *Mol Reprod Dev* 1998; **50**:345–353.
- Boehm AM, Pütz S, Altenhöfer D, Sickmann A, Falk M. Precise protein quantification based on peptide quantification using iTRAQ. *BMC Bioinformatics* 2007; **8**:214.
- Botta T, Blescia S, Martínez-Heredia J, Lafuente R, Brassesco M, Ballescà JL, Oliva R. Identification of proteomic differences in oligozoospermic sperm samples. *Rev Int Androl* 2009; **7**:4–9.
- Böttner M, Suter-Crazzolara C, Schober A, Unsicker K. Expression of a novel member of the TGF-beta superfamily, growth/differentiation factor-15/macrophage-inhibiting cytokine-1 (GDF-15/MIC-1) in adult rat tissues. *Cell Tissue Res* 1999; **297**:103–110.
- Castillo J, Simon L, de Mateo S, Lewis S, Oliva R. Protamine/DNA ratios and DNA damage in native and density gradient centrifuged sperm from infertile patients. *J Androl* 2011; **32**:324–332.
- Castillo J, Amaral A, Oliva R. Sperm nuclear proteome and its epigenetic potential. *Andrology* 2013. doi: 10.1016/j.rbmo.2011.08.010 [Epub ahead of print].
- Chan C-C, Shui H-A, Wu C-H, Wang C-Y, Sun G-H, Chen H-M, Wu G-J. Motility and protein phosphorylation in healthy and asthenozoospermic sperm. *J Proteome Res* 2009; **8**:5382–5386.
- Chung C, Coste H, White JH, Mirguet O, Wilde J, Gosmini RL, Delves C, Magny SM, Woodward R, Hughes SA et al. Discovery and characterization of small molecule inhibitors of the BET family bromodomains. *J Med Chem* 2011; **54**:3827–3838.
- Dayon L, Sanchez JC. Relative protein quantification by MS/MS using the Tandem Mass Tag Technology. *Methods Mol Biol* 2012; **893**: 115–127 [Internet].

- Dayon L, Turck N, Kienle S, Schulz-Knappe P, Hochstrasser DF, Scherl A, Sanchez JC. Isobaric tagging-based selection and quantitation of cerebrospinal fluid tryptic peptides with reporter calibration curves. *Anal Chem* 2010;**82**:848–858.
- de Mateo S, Martínez-Heredia J, Estanyol JM, Domínguez-Fandos D, Vidal-Taboada JM, Ballescà JL, Oliva R. Marked correlations in protein expression identified by proteomic analysis of human spermatozoa. *Proteomics* 2007;**7**:4264–4277.
- de Mateo S, Gázquez C, Guimerà M, Balasch J, Meistrich ML, Ballescà JL, Oliva R. Protamine 2 precursors (Pre-P2), protamine 1 to protamine 2 ratio (P1/P2), and assisted reproduction outcome. *Fertil Steril* 2009;**91**:715–722.
- de Mateo S, Castillo J, Estanyol JM, Ballescà JL, Oliva R. Proteomic characterization of the human sperm nucleus. *Proteomics* 2011a;**11**:2714–2726.
- de Mateo S, Ramos L, de Boer P, Meistrich M, Oliva R. Protamine 2 precursors and processing. *Protein Pept Lett* 2011b;**18**:778–785.
- de Mateo S, Ramos L, van der Vlag J, de Boer P, Oliva R. Improvement in chromatin maturity of human spermatozoa selected through density gradient centrifugation. *Int J Androl* 2011c;**34**:256–267.
- de Mateo S, Estanyol JM, Oliva R. Methods for the analysis of the sperm proteome. *Methods Mol Biol* 2013;**927**:411–422.
- de Yebra L, Oliva R. Rapid analysis of sperm nuclear proteins. *Anal Biochem* 1993;**209**:201–203.
- de Yebra L, Ballescà JL, Vanrell JA, Bassas L, Oliva R. Complete selective absence of protamine P2 in humans. *J Biol Chem* 1993;**268**:10553–10557.
- de Yebra L, Ballescà JL, Vanrell JA, Corzett M, Balhorn R, Oliva R. Detection of P2 precursors in the sperm cells of infertile patients who have reduced P2 levels. *Fertil Steril* 1998;**69**:755–759.
- Domínguez-Fandos D, Camejo MI, Ballescà JL, Oliva R. Human sperm DNA fragmentation: correlation of TUNEL results as assessed by flow cytometry and optical microscopy. *Cytometry A* 2007;**71**:1011–1018.
- Fan YS, Hu YJ, Yang WX. TGF- β superfamily: how does it regulate testis development. *Mol Biol Rep* 2012;**39**:4727–4741.
- Ferraretti AP, Goossens V, Kupka M, Bhattacharya S, de Mouzon J, Castilla JA, Erb K, Korsak V, Nyboe Andersen A. Assisted reproductive technology in Europe, 2009: results generated from European registers by ESHRE. *Hum Reprod* 2013;**28**:2318–2331.
- Frapsauce C, Pionneau C, Bouley J, de Larouzière V, Berthaut I, Ravel C, Antoine J-M, Soubrier F, Mandelbaum J. Unexpected in vitro fertilization failure in patients with normal sperm: a proteomic analysis. *Gynecol Obstet Fertil* 2009;**37**:796–802.
- Gan CS, Chong PK, Pham TK, Wright PC. Technical, experimental, and biological variations in isobaric tags for relative and absolute quantitation (iTRAQ). *J Proteome Res* 2007;**6**:821–827.
- Geyer CB, Inselman AL, Sunman JA, Bornstein S, Handel MA, Eddy EM. A missense mutation in the Capza3 gene and disruption of F-actin organization in spermatids of repro32 infertile male mice. *Dev Biol* 2009;**330**:142–152.
- Giannakouros T, Nikolakaki E, Mylonis I, Georgatsou E. Serine-arginine protein kinases: a small protein kinase family with a large cellular presence. *FEBS J* 2011;**278**:570–586.
- Govin J, Caron C, Lestrat C, Rousseaux S, Khochbin S. The role of histones in chromatin remodelling during mammalian spermiogenesis. *Eur J Biochem* 2004;**271**:3459–3469.
- Guay SP, Brisson D, Munger J, Lamarche B, Gaudet D, Bouchard L. ABCA1 gene promoter DNA methylation is associated with HDL particle profile and coronary artery disease in familial hypercholesterolemia. *Epigenetics* 2012a;**7**:464–472.
- Guay S-P, Voisin G, Brisson D, Munger J, Lamarche B, Gaudet D, Bouchard L. Epigenome-wide analysis in familial hypercholesterolemia identified new loci associated with high-density lipoprotein cholesterol concentration. *Epigenomics* 2012b;**4**:623–639.
- Gut P, Verdin E. The nexus of chromatin regulation and intermediary metabolism. *Nature* 2013;**502**:489–498.
- Hammoud SS, Nix DA, Zhang H, Purwar J, Carrell DT, Cairns BR. Distinctive chromatin in human sperm packages genes for embryo development. *Nature* 2009;**460**:473–478.
- Hammoud SS, Nix DA, Hammoud AO, Gibson M, Cairns BR, Carrell DT. Genome-wide analysis identifies changes in histone retention and epigenetic modifications at developmental and imprinted gene loci in the sperm of infertile men. *Hum Reprod* 2011;**26**:2558–2569.
- Hino S, Nagaoka K, Nakao M. Metabolism-epigenome crosstalk in physiology and diseases. *J Hum Genet* 2013;**58**:410–415.
- Hiramatsu T, Sonoda H, Takanezawa Y, Morikawa R, Ishida M, Kasahara K, Sanai Y, Taguchi R, Aoki J, Arai H. Biochemical and molecular characterization of two phosphatidic acid-selective phospholipase A1 s, mPA-PLA1 alpha and mPA-PLA1 beta. *J Biol Chem* 2003;**278**:49438–49447.
- Houshdaran S, Cortessis VK, Siegmund K, Yang A, Laird PW, Sokol RZ. Widespread epigenetic abnormalities suggest a broad DNA methylation erasure defect in abnormal human sperm. *PLoS One* 2007;**2**:e1289.
- Huang LS, Voyiaziakis E, Markenson DF, Sokol KA, Hayek T, Breslow JL. Apo-B gene knockout in mice results in embryonic lethality in homozygotes and neural-tube defects, male-infertility, and reduced HDL cholesterol ester and Apo-A-I transport rates in heterozygotes. *J Clin Invest* 1995;**96**:2152–2161 [Internet].
- Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* 2009a;**37**:1–13.
- Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 2009b;**4**:44–57.
- Jodar M, Oliva R. Protamine alterations in human spermatozoa. *Adv Exp Med Biol* 2014;**791**:83–102.
- Jodar M, Oriola J, Mestre G, Castillo J, Giwercman A, Vidal-Taboada JM, Ballescà JL, Oliva R. Polymorphisms, haplotypes and mutations in the protamine 1 and 2 genes. *Int J Androl* 2011;**34**:470–485.
- Jung K, Poschmann G, Podwojski K, Eisenacher M, Kohl M, Pfeiffer K, Meyer HE, Stühler K, Stephan C. Adjusted Confidence Intervals for the Expression Change of Proteins Observed in 2-Dimensional Difference Gel Electrophoresis. *J Proteomics Bioinform* 2009;**2**:78–87.
- Karp NA, Huber W, Sadowski PG, Charles PD, Hester SV, Lilley KS. Addressing accuracy and precision issues in iTRAQ quantitation. *Mol Cell Proteomics* 2010;**9**:1885–1897.
- Kim YR, Kim CS, Naqvi A, Kumar A, Kumar S, Hoffman TA, Irani K. Epigenetic upregulation of p66shc mediates low-density lipoprotein cholesterol-induced endothelial cell dysfunction. *Am J Physiol Heart Circ Physiol* 2012;**303**:H189–H196.
- Liao T-T, Xiang Z, Zhu W-B, Fan L-Q. Proteome analysis of round-headed and normal spermatozoa by 2-D fluorescence difference gel electrophoresis and mass spectrometry. *Asian J Androl* 2009;**11**:683–693.
- Loveland KL, Dias V, Meachem S, Rajpert-De Meyts E. The transforming growth factor-beta superfamily in early spermatogenesis: potential relevance to testicular dysgenesis. *Int J Androl* 2007;**30**:377–384.
- Martínez-Heredia J, Estanyol JM, Ballescà JL, Oliva R. Proteomic identification of human sperm proteins. *Proteomics* 2006;**6**:4356–4369.
- Martínez-Heredia J, de Mateo S, Vidal-Taboada JM, Ballescà JL, Oliva R. Identification of proteomic differences in asthenozoospermic sperm samples. *Hum Reprod* 2008;**23**:783–791.
- Martínez-Pastor B, Cosentino C, Mostoslavsky R. A tale of metabolites: the cross-talk between chromatin and energy metabolism. *Cancer Discov* 2013;**3**:497–501.

- Mengual L, Balleca JL, Ascaso C, Oliva R. Marked differences in protamine content and P1/P2 ratios in sperm cells from Percoll fractions between patients and controls. *J Androl* 2003;**24**:438–447.
- Miyagawa Y, Tanaka H, Iguchi N, Kitamura K, Nakamura Y, Takahashi T, Matsumiya K, Okuyama A, Nishimune Y. Molecular cloning and characterization of the human orthologue of male germ cell-specific actin capping protein alpha3 (cpalpha3). *Mol Hum Reprod* 2002;**8**:531–539.
- Nielsen JE, Lindegaard ML, Friis-Hansen L, Almstrup K, Leffers H, Nielsen LB, Rajpert-De Meyts E. Lipoprotein lipase and endothelial lipase in human testis and in germ cell neoplasms. *Int J Androl* 2010;**33**:e207–e215.
- Noorali S, Kurita T, Woolcock B, de Alarga TR, Lo M, Paralkar V, Hoodless P, Vielkind J. Dynamics of expression of growth differentiation factor 15 in normal and PIN development in the mouse. *Differentiation* 2007;**75**:325–336.
- Oliva R. Protamines and male infertility. *Hum Reprod Update* 2006;**12**:417–435.
- Oliva R. SBIrM: focus on proteomics and reproduction. Preface. *Syst Biol Reprod Med* 2012;**58**:177–178.
- Oliva R, Balleca JL. Altered histone retention and epigenetic modifications in the sperm of infertile men. *Asian J Androl* 2012;**14**:239–240.
- Oliva R, Castillo J. Proteomics and the genetics of sperm chromatin condensation. *Asian J Androl* 2011;**13**:24–30.
- Oliva R, de Mateo S. Medical implications of sperm nuclear quality. In: Rousseaux S, Khochbin S (eds). *Epigenetics and Human Reproduction, Epigenetics and Human Health*. Heidelberg, Germany: Springer-Verlag Berlin, 2011.
- Oliva R, Dixon GH. Vertebrate protamine genes and the histone-to-protamine replacement reaction. *Prog Nucleic Acid Res Mol Biol* 1991;**40**:25–94.
- Oliva R, Mezquita C. Marked differences in the ability of distinct protamines to disassemble nucleosomal core particles 'in vitro'. *Biochemistry* 1986;**25**:6508–6511.
- Oliva R, Martínez-Heredia J, de Mateo S, Gázquez C, Oriola J, Estanyol JM, Guimerà M, Balasch J, Balleca JL. Proteomics of human spermatozoa, protamine content and assisted reproduction outcome. *Soc Reprod Fertil Suppl* 2007;**65**:527–530.
- Oliva R, Martínez-Heredia J, Estanyol JM. Proteomics in the study of the sperm cell composition, differentiation and function. *Syst Biol Reprod Med* 2008;**54**:23–36.
- Oliva R, de Mateo S, Estanyol JM. Sperm cell proteomics. *Proteomics* 2009;**9**:1004–1017.
- Paasch U, Heidenreich F, Pursche T, Kuhlisch E, Kettner K, Grunewald S, Kratzsch J, Dittmar G, Glander H-J, Hoflack B et al. Identification of increased amounts of eppin protein complex components in sperm cells of diabetic and obese individuals by difference gel electrophoresis. *Mol Cell Proteomics* 2011;**10**:M110.007187.
- Papoutsopoulou S, Nikolakaki E, Chalepakis G, Krufft V, Chevallier P, Giannakourou T. SR protein-specific kinase I is highly expressed in testis and phosphorylates protamine I. *Nucleic Acids Res* 1999;**27**:2972–2980.
- Paradowska AS, Miller D, Spiess AN, Vieweg M, Cerna M, Dvorakova-Hortova K, Bartkuhn M, Schuppe HC, Weidner W, Steger K. Genome wide identification of promoter binding sites for H4K12ac in human sperm and its relevance for early embryonic development. *Epigenetics* 2012;**7**:1057–1070.
- Parte PP, Rao P, Redij S, Lobo V, D'Souza SJ, Gajbhiye R, Kulkarni V. Sperm phosphoproteome profiling by ultra performance liquid chromatography followed by data independent analysis (LC-MS(E)) reveals altered proteomic signatures in asthenozoospermia. *J Proteomics* 2012;**75**:5861–5871.
- Paulo JA, Kadiyala V, Banks PA, Conwell DL, Steen H. Mass spectrometry-based quantitative proteomic profiling of human pancreatic and hepatic stellate cell lines. *Genomics Proteomics Bioinformatics* 2013;**11**:105–113.
- Pixton KL, Deeks ED, Flesch FM, Moseley FLC, Björndahl L, Ashton PR, Barratt CLR, Brewis IA. Sperm proteome mapping of a patient who experienced failed fertilization at IVF reveals altered expression of at least 20 proteins compared with fertile donors: case report. *Hum Reprod* 2004;**19**:1438–1447.
- Podwojski K, Stephan C, Eisenacher M. Important issues in planning a proteomics experiment: statistical considerations of quantitative proteomic data. In: Marcus K (ed.) *Quantitative Methods in Proteomics. Methods in Molecular Biology*. Bochum, Germany: Humana Press, 2012:3–21.
- Raso C, Cosentino C, Gaspari M, Malara N, Han X, McClatchy D, Park SK, Renne M, Vadalà N, Prati U et al. Characterization of breast cancer interstitial fluids by TmT labeling, LTQ-Orbitrap Velos mass spectrometry, and pathway analysis. *J Proteome Res* 2012;**11**:3199–3210.
- Raya A, Izpisua Belmonte JC. Left-right asymmetry in the vertebrate embryo: from early information to higher-level integration. *Nat Rev Genet* 2006;**7**:283–293.
- Secciani F, Bianchi L, Ermini L, Cianti R, Armini A, La Sala GB, Focarelli R, Bini L, Rosati F. Protein profile of capacitated versus ejaculated human sperm. *J Proteome Res* 2009;**8**:3377–3389.
- Simon L, Castillo J, Oliva R, Lewis SE. Relationships between human sperm protamines, DNA damage and assisted reproduction outcomes. *Reprod Biomed Online* 2011;**23**:724–734.
- Sinclair J, Timms JF. Quantitative profiling of serum samples using TMT protein labelling, fractionation and LC-MS/MS. *Methods* 2011;**54**:361–369.
- Siva AB, Kameshwari DB, Singh V, Pavani K, Sundaram CS, Rangaraj N, Deenadayal M, Shivaji S. Proteomics-based study on asthenozoospermia: differential expression of proteasome alpha complex. *Mol Hum Reprod* 2010;**16**:452–462.
- Soucek K, Slabáková E, Ovesná P, Malenová A, Kozubík A, Hampl A. Growth/differentiation factor-15 is an abundant cytokine in human seminal plasma. *Hum Reprod* 2010;**25**:2962–2971.
- Sun S, Wong EWP, Li MWM, Lee WM, Cheng CY. 14–3–3 and its binding partners are regulators of protein-protein interactions during spermatogenesis. *J Endocrinol* 2009;**202**:327–336.
- Tabibzadeh S. Isolation, characterization, and function of EBAF/LEFTY B: role in infertility. *Ann N Y Acad Sci* 2011;**1221**:98–102.
- Tabibzadeh S, Hemmati-Brivanlou A. Lefty at the crossroads of 'stemness' and differentiative events. *Stem Cells* 2006;**24**:1998–2006.
- Thacker S, Yadav SP, Sharma RK, Kashou A, Willard B, Zhang D, Agarwal A. Evaluation of sperm proteins in infertile men: a proteomic approach. *Fertil Steril* 2011;**95**:2745–2748.
- Thompson A, Schäfer J, Kuhn K, Kienle S, Schwarz J, Schmidt G, Neumann T, Johnstone R, Mohammed AK, Hamon C. Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. *Anal Chem* 2003;**75**:1895–1904.
- Torregrosa N, Domínguez-Fandos D, Camejo MI, Shirley CR, Meistrich ML, Balleca JL, Oliva R. Protamine 2 precursors, protamine 1/protamine 2 ratio, DNA integrity and other sperm parameters in infertile patients. *Hum Reprod* 2006;**21**:2084–2089.
- Wang G, Guo Y, Zhou T, Shi X, Yu J, Yang Y, Wu Y, Wang J, Liu M, Chen X et al. In-depth proteomic analysis of the human sperm reveals complex protein compositions. *J Proteomics* 2013;**79**:114–122 [Internet].
- Wen X-Y, Hegele RA, Wang J, Wang DY, Cheung J, Wilson M, Yahyapour M, Bai Y, Zhuang L, Skaug J et al. Identification of a novel lipase gene mutated in lpd mice with hypertriglyceridemia and associated with dyslipidemia in humans. *Hum Mol Genet* 2003;**12**:1131–1143.
- Whiley PAF, Miyamoto Y, McLachlan RI, Jans DA, Loveland KL. Changing subcellular localization of nuclear transport factors during human spermatogenesis. *Int J Androl* 2012;**35**:158–169.
- World Health Organization. *WHO Laboratory Manual for the Examination and Processing of Human Semen*. 5th edn. Geneva, Switzerland: World Health Organization, 2010.
- Xu W, Hu H, Wang Z, Chen X, Yang F, Zhu Z, Fang P, Dai J, Wang L, Shi H et al. Proteomic characteristics of spermatozoa in normozoospermic patients with infertility. *J Proteomics* 2012;**75**:5426–5436.

- Zhao C, Huo R, Wang F-Q, Lin M, Zhou Z-M, Sha J-H. Identification of several proteins involved in regulation of sperm motility by proteomic analysis. *Fertil Steril* 2007;**87**:436–438.
- Zhu Y, Wu Y, Jin K, Lu H, Liu F, Guo Y, Yan F, Shi W, Liu Y, Cao X *et al.* Differential proteomic profiling in human spermatozoa that did or did not result in pregnancy via IVF and AID. *Proteomics Clin Appl* 2013;**7**:750–758.