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The combined human sperm proteome: cellular pathways and implications for basic and clinical science

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BACKGROUND: The human sperm cell is very well suited for proteomic studies, as it is accessible, can be easily purified and is believed to be transcriptionally and translationally silent. The recent use of advanced proteomic approaches is clearly challenging the understanding of sperm biology. The aims of this review are to discuss the various human sperm proteomic studies, to create a compiled list of all the sperm proteins described to date and to re-assess the potential functional implications.

METHODS: A search of the scientific literature available in the PubMed/Medline database at 31 December 2012 was conducted for studies on human sperm proteomics. The complete list of proteins obtained was carefully analysed using different bioinformatics tools, including *Reactome*, a knowledgebase of biological pathways.

RESULTS: A total of 30 studies were identified. The proteomics studies have resulted in the identification of 6198 different proteins, an important proportion of which (around 30%) are known to be expressed in the testis. The proteins were assigned to various functional pathways, including metabolism, apoptosis, cell cycle, meiosis and membrane trafficking, among others. Unexpectedly, the sperm cell also contains a range of proteins involved in RNA metabolism and translational regulation, as well as proteins usually located in organelles believed to be absent in sperm, such as cytoplasmatic ribosomes and peroxisomes. Additionally, some proteins whose levels seem to be altered in low-quality sperm might have clinical relevance.

CONCLUSIONS: The analysis of the most complete sperm proteome available to date indicates the presence of several cellular protein pathways previously ignored in the male gamete. Confirming the activity of each of these pathways and understanding their biological significance will certainly boost the knowledge of human sperm and male fertility and infertility in the next years.

Key words: spermatozoa / proteomics / sperm function / male fertility

Introduction

Proteomics is defined as the systematic analysis of all the proteins in a cell, tissue or organism at a given moment and under a particular set of conditions (Aebersold and Mann, 2003; Domon and Aebersold, 2006; Cox and Mann, 2007). Human sperm is particularly amenable to proteomic studies, as it is responsible for delivering the paternal genetic and epigenetic information to the next generation, and is an accessible cell that can be easily purified and analysed (Oliva *et al.*, 2008, 2009). The function of this cell is determined by the presence of a very unusual proteomic composition resulting in an extremely condensed chromatin structure, a large flagellum and all the machinery needed to generate energy and perform the specialized cellular functions required for fertilization (Baccetti and Afzelius, 1976; Oliva and Dixon, 1991; Oliva, 2006; Ramalho-Santos *et al.*, 2009).

By their nature, sperm cells are intrinsically unusual in many cellular and functional features and the peculiarities of sperm should always be taken into consideration. Sperm cells are the end product of spermatogenesis, a differentiation process occurring in the testicular seminiferous tubules of any normal male after puberty. During spermiogenesis, the final post-meiotic phase of spermatogenesis, most of the cytoplasm is lost (and presumably a number of cytoplasmic proteins), and the chromatin undergoes extensive remodelling, as most of the histones are replaced by highly basic protamines (Oliva and Dixon, 1991; Oliva, 2006; Balhorn, 2007; Oliva and Castillo, 2011b). Protamines, which constitute the most abundant proteins in the sperm nucleus, enhance chromatin condensation in such a way that transcription is silenced, due to the inaccessibility of most genes to the transcription machinery (Miller *et al.*, 2010; Oliva and Castillo, 2011a). To compensate for this lack of transcription, there is prominent transcriptional activity at the beginning of spermiogenesis, controlled by testis-specific transcription factors and coactivators (Queralt and Oliva, 1995; Sassone-Corsi, 2002; Tanaka and Baba, 2005). Spermatogenesis is in fact supported by a precise regulation of gene expression, including the expression of several testis-specific proteins (Dezso *et al.*, 2008). The established paradigm is thus that no nuclear transcription activity occurs in ejaculated sperm (reviewed in Miller *et al.*, 2010; Amaral and Ramalho-Santos, 2013). Additionally, and although this has been disputed, as we will discuss, the prevalent theory is that there is no protein synthesis (at least from nuclear-encoded genes) in sperm (see Baker, 2011). Even if some protein synthesis occurs, sperm translational activity would be reduced when compared with somatic cells, simplifying proteomic studies. On the other hand, the sperm proteomics dilemma is that it may be difficult to determine if a certain protein is there to fulfil a specific sperm function, or it is simply a leftover from spermatogenesis, with no relevant role in the male gamete.

Moreover, it is well established that sperm are not able to accomplish their purpose without undergoing the maturation process called capacitation (Visconti *et al.*, 1998, 2002; De Jonge, 2005). Although the molecular characteristics associated with acquisition of the ability to fertilize the ooctye are still poorly understood, it is thought that without capacitation no acrosome reaction occurs in sperm. The acrosome reaction is the fusion of the sperm plasma membrane with the outer acrosomal membrane and the eventual release of the acrosomal contents (acrosomal exocytosis) that occurs before a sperm penetrates the zona pellucida of the oocyte *in vivo* (Buffone *et al.*, 2012; De La Vega-Beltran *et al.*, 2012). Capacitation involves the phosphorylation of various proteins, particularly of tyrosine residues, thus changing the sperm proteome. Hence, a number of sperm proteomic studies have focused on the comparison between non-capacitated and capacitated sperm, as we will discuss. Although this kind of study is certainly appealing, sperm capacitation may not occur at the same time in all sperm in an ejaculate (or may not happen at all in some sperm), which stresses another sperm characteristic: sperm samples are inevitably heterogeneous (Holt and Van Look, 2004). And even if there are techniques to fractionate samples, thereby increasing their homogeneity in terms of sperm quality, one should have in mind that any sperm proteomics analysis will have two types of variability: inter- and intra-sample.

Some decades ago no more than 100 different proteins had been described in the human sperm cell, and individual studies focused on one (or only a few) proteins (Felix, 1960; Mohri, 1968; Gilboa *et al.*, 1973). Today, with the advances of proteomic techniques, thousands of proteins have been described as being part of normal human sperm and this number is predicted to increase as additional work is conducted. The availability of a catalogue of the proteins that make up sperm cells is an important first step to understand many aspects of the physiology of the male gamete, as well as to identify altered proteins and/or protein modifications leading to male infertility. This review summarizes the methods used, current knowledge of the human sperm proteome and the altered proteins identified so far through differential proteomic analysis of sperm from infertile patients.

An essential initial step in the analysis of the human sperm proteome consists of purification of the sperm cells from seminal fluid and potentially contaminating cells (such as leukocytes and epithelial cells, the so-called round cells) co-existing in any semen sample (de Mateo *et al.*, 2013; Fig. 1). Although not always taken into account, the purity of the preparation is one of the most critical steps in the procedure, as any minor contamination could result in a false-positive identification, i.e. round cell proteins could be erroneously identified as sperm proteins (this is especially relevant given that round cells have much more cytoplasm than sperm). The purification step can be accomplished through density gradient centrifugation (usually using PureSperm or Percoll) or through swim-up (Fig. 1). Depletion of leukocytes using CD45 magnetic bead selection can also be used to further improve purity of the preparation. In addition to the depletion of potentially contaminating non-sperm cells, the stringency of this cell separation step, either through gradient centrifugation or other alternatives, should be chosen according to the study aims, as it determines the fitness of the selected sperm cells.

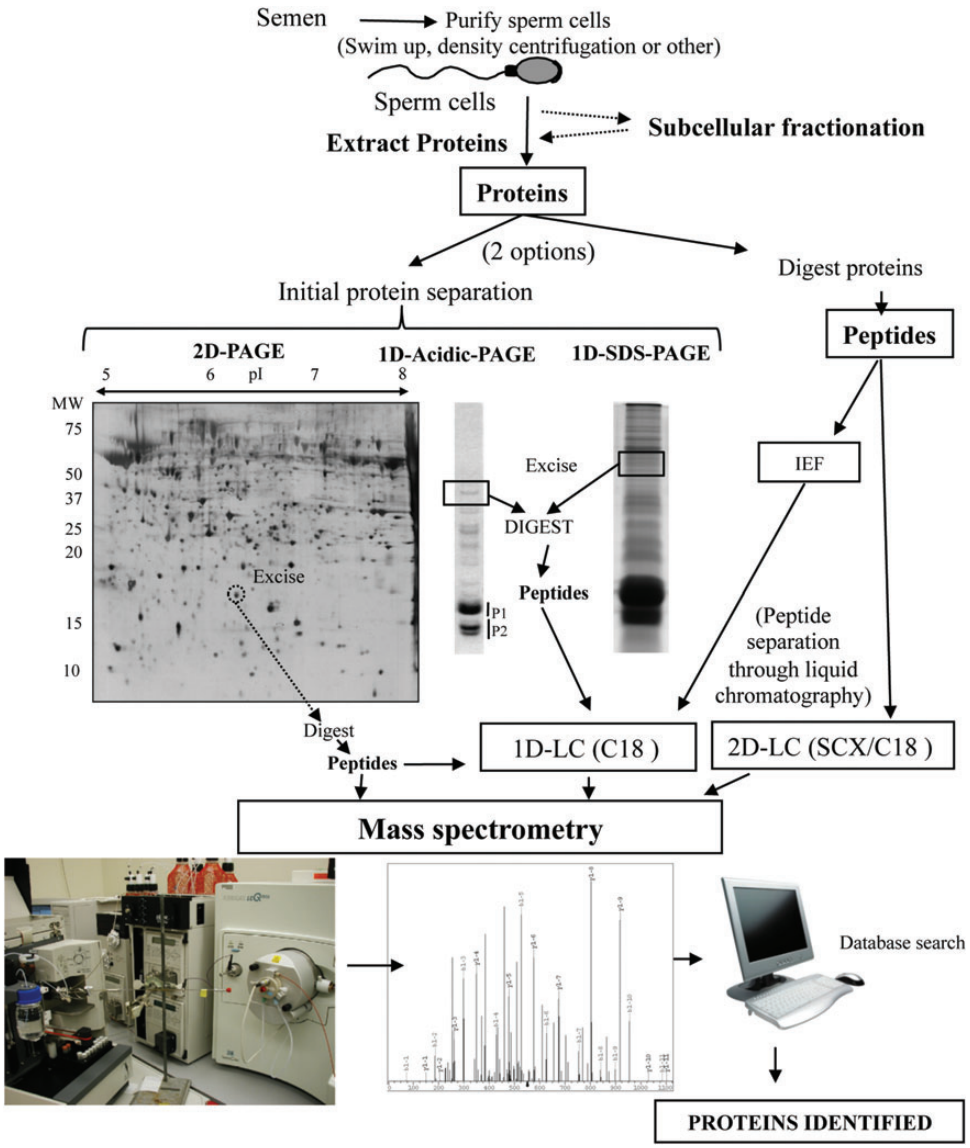

Figure 1 Human sperm proteomics strategies. The initial step in any sperm proteomics study consists on the isolation of sperm cells (from both the seminal plasma and other cells coexisting in semen). Sperm proteins are then extracted from either the whole cell or from specific subcellular compartments (in this case subcellular fractionation is performed beforehand). Once proteins are extracted, there are two technical options: (1) proteins are separated either by 1- or 2-dimensional polyacrylamide gel electrophoresis (1D-PAGE, 2D-PAGE), excised from the gels and digested (usually with trypsin) to generate peptides; 1D-acidic-PAGE may be used to resolve sperm nuclear proteins (mainly protamines: P1, P2), which cannot be resolved in a conventional sodium dodecyl sulphate (SDS)-PAGE; (2) proteins are digested without previous separation. Finally, peptides are analysed by mass spectrometry (either MALDI-MS or MS/MS) and identified by searching a protein database. In order to improve the throughput, peptides can first be separated by 1- or 2-dimensional liquid chromatography (1D-LC, 2D-LC) or by isoelectric focusing (IEF).

Another important choice when designing a proteomic study is to determine whether to target the entire cell or to perform subcellular fractionation and explore specific cell compartments (Fig. 1). An advantage of subcellular proteomics is that it may allow the detection of low abundance proteins that may well escape detection in whole cell approaches (de Mateo *et al.*, 2011a; Amaral *et al.*, 2013).

Once the sperm cells have been purified or the subcellular fractions have been obtained, solubilized proteins are digested and peptides are identified using mass spectrometry (MS), which relies on the detection of ionized molecules according to their mass-to-charge ratio (m/z; Brewis and Brennan, 2010). Essentially, there are two distinct technical options here: (i) initial protein separation followed by protein digestion and peptide identification and (ii) simple digestion of proteins to generate peptides followed by their identification (Fig. 1). Classically, proteins were initially separated using two-dimensional gel electrophoresis (2DE), an approach that has been widely used, leading to the identification of many sperm proteins (Com *et al.*, 2003; Pixton *et al.*, 2004; Chu *et al.*, 2006; Dorus *et al.*, 2006; Martínez-Heredia *et al.*, 2006, 2008; de

Mateo *et al.*, 2007). However, the throughput power of the 2DE approach is lower when compared with the option involving the separation of peptides using liquid chromatography (LC) (either of digested protein extracts or from digested proteins previously separated by one-dimensional gel electrophoresis; 1DE). For example, 2DE approaches have identified 10–200 proteins per study, whereas LC-MS/MS approaches typically resulted in the identification of around 400 to over 1000 different proteins (Table I). A disadvantage of the LC-MS/MS approach is that the potential information determined by the presence of concurrent protein post-translational modifications (PTMs) in different regions of the same protein is lost as soon as a protein is digested into peptides.

The first part of this review, which covers all the studies carried out so far using whole sperm proteomics or subcellular proteomics, provides the most complete compiled list available for the sperm proteome and analyses this set to extract some general conclusions. All the above methods can also be applied to differential proteomics involving sperm cells from infertile patients or under an experimental or physiological condition. These aspects are covered in the last section of this review. The present review extends, updates and complements other excellent reviews covering the subject of sperm proteomics in human and model species (Miller, 2006; Aitken and Baker, 2008; Barratt, 2008; Wu and Chu, 2008; Baker and Aitken, 2009; Oliva *et al.*, 2009; Brewis and Gadella, 2010; Oliva and Castillo, 2011a; Baker *et al.*, 2012; Chocu *et al.*, 2012; Dacheux *et al.*, 2012; Dorus *et al.*, 2012; Oliva, 2012; Rousseaux and Khochbin, 2012).

Methods

An exhaustive literature search was conducted using the PubMed/Medline database in order to identify human sperm proteomic articles published in English (or having at least the abstract written in English). A list of all the sperm proteins identified in 30 proteomics studies available online until the end of 2012 (the studies are shown in Table I) was compiled (Supplementary data, Table). All studies included were performed using ejaculated human sperm (human epididymal sperm and animal models were not included). To avoid redundancy, all proteins were annotated using the UniProtKB/Swiss-Prot accession number, even when these were not indicated in the original article. By merging data from the available papers, a list of 6252 different proteins, along with their literature references, was initially created. With the aim of eliminating potential false-positive identifications, we then selected the proteins from the list according to the proteomics approach and protein identification criteria used in each of the works. First of all, and seeing that matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS is somehow outdated, we considered only those proteomics studies relying on LC-MS/MS. From these, we have further excluded any study whose protein identification criteria were either not indicated, or did not meet one of the following: (i) identification of at least two peptides per protein and a false discovery rate (FDR) $< 5\%$ or (ii) an FDR $< 1\%$. By eliminating any protein that did not meet these criteria in any of studies, we created a sublist of 6198 proteins that we believe are very likely to be present in sperm.

In order to determine which biological pathways might be active in human sperm, the sublist of 6198 proteins was considered. Analyses were performed using *Reactome* (http://www.reactome.org), a free online peer-reviewed knowledgebase of biological pathways where various human biological processes are annotated by breaking them down into a series of molecular events (Croft *et al.*, 2011; Haw *et al.*, 2011; D'Eustachio, 2013). *Reactome* allows the analysis of a list of proteins by determining how they are likely to affect pathways. We performed overrepresentation analysis of the compiled list of sperm proteins. Basically, this analysis finds the *Reactome* pathways in which proteins in the list are strongly enriched. The significance of the association between the protein list and a certain pathway is expressed in two ways: a *ratio* expressing the number of proteins from the data set that map to the pathway divided by the total number of proteins constituting the pathway; and a *P-value* expressing the probability (hypergeometric test) that the association between the proteins in the data set and the pathway is explained by chance alone (P-values < 0.01 were considered significant). The eight most significant main pathways were detailed and literature searches were performed for various sperm features. Given the multitude of subjects covered, reviews are referred to at various points below. When relevant, experiments with animal models have been discussed. For instance, we searched for the existence of *knockout* (KO) mice for representative families of proteins in each pathway. To allow the detailed visualization of some of the pathways which are likely to be active in human sperm, the sperm proteome was also analysed using the *Search & Color Pathway* mapping resource of the *KEGG Pathway Database* (http://www.genome.jp/kegg/pathway.html; Kanehisa and Goto, 2000; Kanehisa *et al.*, 2012).

Lastly, we have also created a list of the proteins reported to be differentially expressed in normozoospermic and asthenozoospermic sperm. Given the low number of studies, here we have considered all the proteins reported to date, indepedent of the identification criteria used in the studies (Zhao *et al.*, 2007; Martínez-Heredia *et al.*, 2008; Chan *et al.*, 2009; Siva *et al.*, 2010; Parte *et al.*, 2012). These proteins were classified according to their main biological functions using the information available at the UniProt Knowledgebase.

Human sperm proteome

Human sperm proteomics is a recent field of investigation. Pioneering proteomic analyses of different human somatic cells were completed in the final years of the last century (Ostergaard *et al.*, 1997; Arnott *et al.*, 1998; to give a few examples). Likewise, the first publications with titles containing 'human sperm proteome' also date to that time, but were carried out to identify a small number of specific sperm surface proteins recognized by antisperm antibodies in the serum of infertile men (Shetty *et al.*, 1999, 2001). An in-depth proteomic analysis of human sperm came out as late as 2005 (Johnston *et al.*, 2005) but the identity of the proteins in this initial study was not reported. Since then various researchers have been using different approaches to comprehensively analyse the human sperm proteome (Table I).

Whole sperm cell proteomics

Initial analyses were carried out by 2DE and MALDI-TOF MS and resulted in the identification of around 100 proteins (Martínez-Heredia *et al.*, 2006; de Mateo *et al.*, 2007; Li *et al.*, 2007). The nature of some of these proteins was occasionally puzzling, suggesting that proteomic analyses would be efficient tools to identify novel sperm attributes. For instance, and although sperm are believed to be transcriptionally and translationally silent, it turns out that sperm contain a number of proteins involved in transcription and protein synthesis (Martínez-Heredia *et al.*, 2006; Oliva *et al.*, 2009). What the purpose of these proteins is (if any) has yet to be defined but this finding certainly inspired fruitful discussions (Amaral and Ramalho-Santos, 2013). The subsequent use of LC-MS/MS resulted in the identification of thousands of sperm proteins (Baker *et al.*, 2007; Wang *et al.*, 2013). The first of these studies relied on the identification of proteins present in soluble and insoluble fractions of sperm lysates and has shown that a great proportion of the sperm proteome

Table I Human sperm proteomics studies.

References	Number and type of samples	Sperm preparation	Protein separation method	Proteomics approach	Protein identification criteria	Number of proteins described	Main outcomes
Descriptive proteomics							
Whole sperm							
Johnston *et al.* (2005)	1 fertile	Washing	Detergent soluble and insoluble fractions; SDS-PAGE, in-gel digestion	LC-MS/MS	≥2 peptides identified per protein; Xcorr ≥2.2	0	First comprehensive identification of the human sperm proteome (but the nature of the proteins were not described)
Martínez-Heredia *et al.* (2006)	11 normozoospermic	50% Percoll fractionation	2DE, in-gel digestion	MALDI-TOF MS	MOWSE score ≥1000 (<1000 if peaks were clean and better than those corresponding to trypsin)	98	First comprehensive description of the human sperm proteome
de Mateo *et al.* (2007)	10 donors, 47 patients	50% Percoll fractionation	2DE, in-gel digestion	MALDI-TOF MS	MOWSE score ≥1000 (<1000 if peaks were clean and better than those corresponding to trypsin)	131	Correlation between sperm proteomics, DNA integrity and protamine content
Li *et al.* (2007)	12 donors	Percoll fractionation	2DE, in-gel digestion	MALDI-TOF/TOF MS	Not indicated	16	High-resolution 2-D reference map of human fertile sperm proteins
Baker *et al.* (2007)	8 normozoospermic	Percoll gradient fractionation	Detergent soluble and insoluble fractions; SDS-PAGE, in-gel digestion	LC-MS/MS	≥2 peptides identified per protein; Xcorr ≥1.5 for 1+ ≥2.0 for 2+, ≥2.5 for 3+	1053	First comprehensive description of the human sperm proteome using LC-MS. Analysis of the metabolic proteome
Wang *et al.* (2013)	32 healthy, fertile donors	60% Percoll fractionation	In-solution digestion, LC	LC-MS/MS	≥2 peptides identified per protein (672 proteins were identified based in 1 peptide only); FDR < 1%	4675	Largest description of the sperm proteome performed so far
Subcellular fractions: nuclei							
de Mateo *et al.* (2011a)	4 normozoospermic	Washing; isolation of sperm nuclei using CTAB treatment	SDS-PAGE, 1D-acid gel and 2DE, in-gel digestion	LC-MS/MS	≥2 peptides identified per protein; Xcorr ≥1.5 for 1+, ≥2.0 for 2+, ≥2.5 for 3+, ≥2.75 for 4+, 3 for >4+	403	First comprehensive description of the proteome of isolated nuclei sperm
Tails							

Kim *et al.* (2007)	Not indicated	Isolation of fibrous sheaths by a multi-step mechanical and chemical dissection procedure	SDS-PAGE, in-gel digestion	MS/MS and Edman degradation	Not indicated	9	First analysis of human sperm fibrous sheath proteome. First evidence of a role for an ADP/ATP carrier family member in glycolysis
Amaral *et al.* (2013)	4 normozoospermic	50% Percoll fractionation, CD45 MACS; isolation of sperm tail by sonication and ultracentrifugation in sucrose gradient	SDS-PAGE, in-gel digestion	LC-MS/MS	≥2 peptides identified per protein (and at least 1 unique peptide per protein); Xcorr ≥2 for 2+, ≥2.25 for 3+, ≥2.5 for 4+; FDR < 5%	1049	First comprehensive description of the human sperm tail proteome. Analysis of the metabolic proteome
Heads and tails							
Baker *et al.* (2013)	Normozoospermic	Percoll fractionation; isolation of head and tail fractions by sucessive cycles of sonication and Percoll centrifugation	SDS-PAGE, in-gel digestion	LC-MS/MS	MASCOT score >35 (plus manual inspection of every singleton); FDR < 1%	1429	First joint description of human sperm head and tail fractions (521 and 721 proteins were exclusively found in the head and the tail, respectively)
Membranes							
Naaby-Hansen *et al.* (2010)	5 normozoospermic	Percoll fractionation and/or swim-up, biotinylation	2DE, in-gel digestion	LC-MS and Edman degradation	Not indicated	13	Identification of calcium-binding proteins associated with the human sperm plasma membrane
Naaby-Hansen and Herr, (2010)	Normozoospermic	Percoll and/or swim-up, radioiodination and biotinylation	2DE, in-gel digestion	LC-MS and Edman degradation	Not indicated	7	Identification of heat shock protein chaperones that are accessible for surface labelling on human sperm
Nixon *et al.* (2011)	Healthy normozoospermic donors	Percoll fractionation, isolation of detergent-resistant membrane fractions	In-solution digestion	LC-MS/MS	≥2 peptides identified per protein; MASCOT score >35	124	Comprehensive description of the proteome of human sperm detergent-resistent membranes. Identification of proteins putatively involved in the mediation of sperm–oocyte interaction
Gu *et al.* (2011)	5 normozoospermic	Swim-up, biotinylation	SDS-PAGE, in-gel digestion	LC-MS/MS	≥2 peptides identified per protein; Xcorr ≥1.5 for 1+, ≥2 for 2+; FDR < 0.5%	1019	Description of a common pattern of cell surface proteins in human sperm and embryonic stem cells
Comparative proteomics							
Failed fertilization versus fertilization after IVF							

Continued

Table I *Continued*

References	Number and type of samples	Sperm preparation	Protein separation method	Proteomics approach	Protein identification criteria	Number of proteins described	Main outcomes
Pixton *et al.* (2004)	1 patient, 3 fertile controls	90% Percoll gradient fractionation	2DE, in-gel digestion	LC-MS/MS and MALDI-TOF MS	MASCOT software	4	First proteome comparison of different quality sperm. Identification of 4 proteins differentially expressed
Frapsauce *et al.* (2009)	3 patients, 3 fertile controls	*PureSperm* fractionation	2DE-DIGE, in-gel digestion	LC-MS/MS and MALDI-TOF MS	≥4 peptides identified per protein; MASCOT score >53	12	Identification of 12 differentially expressed proteins
Failed fertilization versus fertilization after artificial insemination							
Xu *et al.* (2012)	10 normozoospermic infertile, 10 healthy fertile	Swim-up	2DE, in-gel digestion	MALDI-TOF MS	GPS Explorer protein confidence index ≥95%	24	Identification of 24 differentially expressed proteins
Asthenozoospermic versus normozoospermic							
Zhao *et al.* (2007)	8 asthenozoospermic, 8 fertile controls	60% Percoll fractionation	2DE, in-gel digestion	MALDI-TOF MS	Not indicated	10	Identification of 10 differentially expressed proteins
Martínez-Heredia *et al.* (2008)	20 asthenozoospermic, 10 fertile controls	50% Percoll fractionation	2DE, in-gel digestion	MALDI-TOF MS	MOWSE score ≥1000 (<1000 if peaks were clean and better than those corresponding to trypsin)	17	Identification of 17 differentially expressed proteins
Chan *et al.* (2009)	20 asthenozoospermic, 20 fertile controls	50% Percoll fractionation	2DE, in-gel digestion	MALDI-TOF MS	Not indicated	12	Identification of 12 differentially phosphorylated proteins
Siva *et al.* (2010)	17 asthenozoospermic, 20 normozoospermic	70% Percoll fractionation	2DE, in-gel digestion	MALDI-MS/MS	Total ion score cut-off of 25 and % CI > 95%	75	Identification of 8 differentially expressed proteins
Parte *et al.* (2012)	4 severe asthenozoospermic, 4 normozoospermic	Washing	IMAC with a phosphoprotein enrichment kit, in-solution digestion	Nano UPLC-MS	≥1 peptide identified per protein; FDR < 5%	66	Identification of 66 differentially regulated phosphoproteins
Teratozoospermic versus normozoospermic							
Liao *et al.* (2009)	1 globozoospermic (20 ejaculates), 12 fertile donors	75% Percoll fractionation	2DE-DIGE, in-gel digestion	MALDI-TOF/TOF	Protein identifications were accepted when the observed and predicted isoelectric points and relative molecular weights were consistent; FDR < 5%	35	Identification of 35 differentially expressed proteins in round-headed sperm compared with normal sperm
Others							
Thacker *et al.* (2011)	3 oligoasthenozoospermic, 1 fertile donor	Not indicated	2DE-DIGE, in-gel digestion	MALDI-TOF/TOF MS and LC-MS	Not indicated	4	Identification of 4 differentially expressed proteins

Paasch *et al.* (2011)	8 type-1 diabetic, 7 type-2 diabetic, 13 non-diabetic obese, 21 normozoospermic and clinically healthy	*ISolate* fractionation	2DE-DIGE, in gel digestion	MALDI-TOF/TOF MS and LC-MS/MS	MASCOT score >56; FDR < 5%	42	Identification of 12 (type-1 diabetic), 71 (type-2 diabetic) and 13 (non-diabetic obese) differentially expressed proteins. Increased amounts of eppin protein complex components in sperm from the 3 groups of patients
Functional proteomics							
Capacitated versus non-capacitated sperm							
Ficarro *et al.* (2003)	Normozoospermic healthy donors	80% Percoll fractionation, overnight incubation in capacitation medium	2DE, anti-phosphotyrosine immunoblots; IMAC in whole protein digests	LC-MS/MS	Manual validation	18	Identification of 16 proteins that undergo tyrosine phosphorylation during human sperm capacitation
Secciani *et al.* (2009)	120 normozoospermic	Washing or swim-up, 3 h incubation in capacitation medium	2DE, in-gel digestion	MALDI-TOF MS and MS/MS	≥4 peptides identified per protein; MASCOT score ≥70	58	Identification of 29 proteins differentially expressed in capacitated sperm, swim-up selected capacitated sperm and ejaculated sperm
Identification of targets for *S*-nitrosylation							
Lefièvre *et al.* (2007)	Normozoospermic donors	90% Percoll fractionation, incubation with NO donors, biotinylation	SDS-PAGE, in-gel digestion	MALDI-TOF MS/MS	MASCOT software	240	Identification of 240 *S*-nitrosylated proteins in human sperm
Identification of targets for sumoylation							
Vigodner *et al.* (2013)	Normozoospermic	*PureSperm* fractionation	Immunoprecipitation using anti-SUMO antibodies, Bis-Tris NuPAGE, in-gel digestion	LC-MS/MS	≥2 peptides identified per protein; FDR < 1%	55	Identification of 55 SUMO targets proteins in human sperm
Identification of proteins likely involved in sperm–oocyte interaction							
Redgrove *et al.* (2011)	Normozoospermic healthy donors	88% Percoll fractionation, 3 h incubation in capacitation medium, biotinylation	2D BN-PAGE, in-gel digestion	LC-MS/MS	≥2 peptides identified per protein; manual spectra validation	22	Identification of 2 sperm surface multimeric protein complexes that likely mediate sperm–zona pellucida interaction

Details of the human sperm proteomics studies performed so far are indicated. The studies are categorized as descriptive, comparative and functional. SEQUEST and MASCOT are peptide identification search engines that use a probability-based scoring algorithm to analyse MS data against sequence databases. MOWSE score and XCorr are peptide identification correlation scores reported by SEQUEST and MASCOT, respectively, and represent the degree of similarity between the experimental mass spectrum and a theoretical mass distribution. MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; LC, liquid chromatography; MS, mass spectrometry; FDR, false discovery rate; CTAB, Hexadecyltrimethylammonium bromide; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; MOWSE, molecular weight search; IMAC, immobilized metal affinity chromatography; UPLC, ultra performance liquid chromatography.

comprised enzymes involved in various metabolic pathways (Baker *et al.*, 2007). Additionally, the Baker *et al*. (2007) study described a number of sperm surface receptors, such as the prolactin receptor, which could have a role in regulating sperm function. In fact, in a follow-up study, the same group has shown that prolactin has a prosurvival effect on human sperm (Pujianto *et al.*, 2010). The largest sperm proteome described so far included 4675 proteins (Wang *et al.*, 2013). Wang *et al*. compared their protein list with published sperm transcriptome data and found little overlap, attesting the importance of sperm studies at the protein level.

Subcellular proteomics

In recent years, catalogues of proteins from isolated sperm subcellular fractions, namely sperm heads and tails have also been generated (Table I). As already explained, this kind of study is particularly relevant, not only because it suggests what the specific cellular localization of each protein is but also because it allows the identification of less abundant proteins. Subcellular proteomics analyses are especially appropriate in a compartmentalized cell such as sperm, where distinct compartments have clear and specific cellular roles.

To this extent, our group has recently characterized the proteome of isolated sperm nuclei (de Mateo *et al.*, 2011a) and tails (Amaral *et al.*, 2013) and obtained particularly interesting data. For instance, we have described various sperm chromatin-related proteins, such as histone variants, transcription factors and zinc fingers, which may contribute to epigenetic marking and/or early embryonic development (de Mateo *et al.*, 2011a; Oliva and Ballescà, 2012). On the other hand, analysis of isolated tail fractions resulted in the identification of a range of metabolic enzymes and suggested the likely activity of various metabolic pathways that have been neglected in the male gamete. Also surprising was the finding of peroxisomal proteins in sperm, a cell believed to be devoid of peroxisomes (Amaral *et al.*, 2013). Similarly, others have isolated the human sperm fibrous sheath, a unique cytoskeletal structure defining the extent of the tail principal piece, and have identified a new ADP/ATP carrier protein along with glycolytic enzymes, evidencing a probable role of this adenosine nucleotide translocase carrier in glycolysis (Kim *et al.*, 2007). In a recent study, Baker *et al*. jointly analysed and compared the proteome of isolated heads and tails from the same sperm samples and the results corroborated the idea that most of the sperm proteins have a compartmentalized expression (Baker *et al.*, 2013). For instance, most of the proteins involved in energy production are localized to the tail, while most of the proteases are localized to the head.

Various sperm membrane proteins are putatively involved in sperm–oocyte interaction and indeed proteomic analyses of isolated human sperm membrane proteins have added new insights into the plausible molecular mediation of capacitation and fertilization. Using distinct enrichment approaches, membrane calcium-binding proteins (Naaby-Hansen *et al.*, 2010), membrane heat shock proteins (HSPs; Naaby-Hansen and Herr, 2010) and membrane proteins with an affinity for the zona pellucida (Nixon *et al.*, 2011) have been identified. Also interesting was the finding of a common pattern between the cell surface protein signature of sperm and embryonic stem cells (Gu *et al.*, 2011). Worth mentioning is the proteomic characterization of isolated acrosomal matrix from epididymal mouse sperm, which resulted in the identification of 1026 proteins (Guyonnet *et al.*, 2012). The employment of a similar approach using human samples is certainly warranted.

Towards the complete human sperm proteome

In order to generate the most complete catalogue of human sperm proteins, we have put together the outcomes of all sperm proteomics studies performed so far (Table I) and have then selected proteins according to the identification criteria used in each of the studies (see the section Methods). By doing this, we report a list of 6198 different proteins (Supplementary data, Table). To identify the biological pathways likely to be active in human sperm, the combined human sperm proteome was analysed using the *Reactome* database. Overrepresentation analysis suggests that the most significant pathways in the male gamete are those involved in: (i) *metabolism*; (ii) *protein metabolism*; (iii) *membrane trafficking*; (iv) *RNA metabolism*; (v) *apoptosis*; (vi) *cell cycle*; (vii) *hemostasis* and (viii) *meiosis*. Each of these will be detailed and discussed (Fig. 2; Table II). *Developmental biology* and *extracellular matrix organization* were also detected as putative sperm pathways but with a lower probability (higher *P-values*; Table II). Moreover, and although *signal transduction* was not detected as a significant pathway, some signalling pathways seem to be active, including signalling by (i) insulin receptor; (ii) Wnt; (iii) Ras; (iv) interleukins and (v) constitutively active epidermal growth factor receptor. For space restrictions, these topics will not be discussed.

Cellular pathways suggested by the sperm proteome

Metabolism

Unsurprisingly, *Reactome* identified *metabolism* as the most significant pathway in human sperm. Metabolism is intimately associated with the production of energy, as well as with the synthesis of essential molecules on the one hand and the elimination of toxic molecules on the other. Worth mentioning, human sperm metabolic studies are inevitably performed *in vitro* and although the formulations of culture media can be changed in order to mimic the conditions found by sperm in the reproductive tracts (epididymis, where these cells are stored until ejaculation; and female reproductive tract, that sperm must cross in order to reach the oocyte), the actual contribution of the various sperm metabolic pathways *in vivo* is difficult to establish. Knowing which pathways are putatively active (i.e. which enzymes are there) is definitely a good starting point.

Carbohydrate metabolism

It is clear that human sperm can use carbohydrates to produce ATP, although the actual contribution of each pathway potentially involved (namely glycolysis and mitochondrial respiration) is unclear. Sperm can take up exogenous hexoses using facilitative transporters (GLUTs), which are important to maintain sperm motility and fertilization ability (Purcell and Moley, 2009; Bucci *et al.*, 2011). Of note, apart from the isoforms already known to exist in human sperm, proteomics data added two members to the list: GLUTs 10 and 14. Once inside sperm, glucose is converted to glucose 6-phosphate, which cannot cross the cell membrane, and can follow distinct routes: glycolysis (Supplementary data, Fig. S1), the pentose phosphate pathway (PPP; Supplementary data, Fig. S2) and glycogen synthesis.

That glycolysis has a critical role in sperm function is suggested by an increasing body of data. Glycolysis converts glycolysable substrates into pyruvate, generating ATP and reducing equivalents. In aerobic

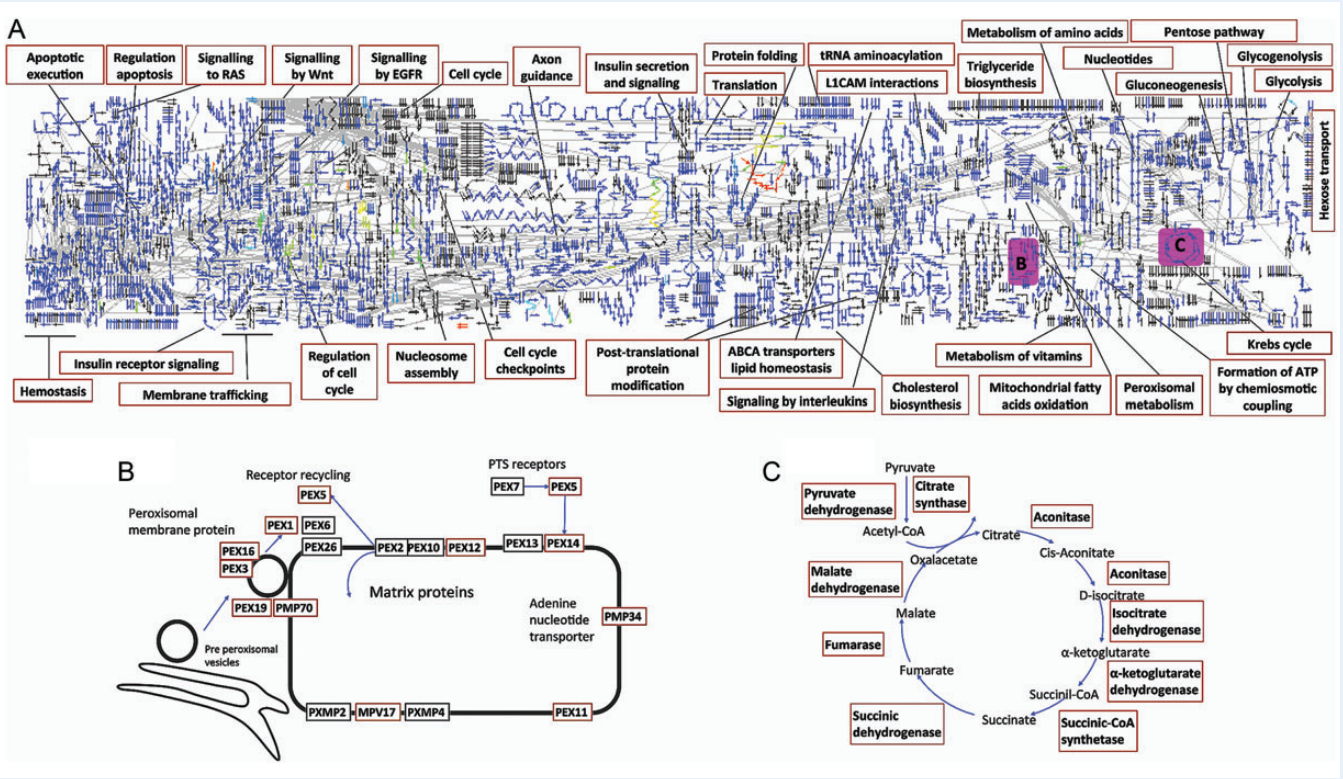

Figure 2 Reaction map showing the cellular pathways likely to be active in human sperm. The compiled list of 6198 sperm proteins was analysed using the *Reactome* database in order to find out which biological pathways are probably active in the male gamete and the result is shown in (**A**). Coloured arrows in the reaction map symbolize events or pathways detected in the human sperm cell. Grey arrows represent pathways present in a hypothetical cell but corresponding to proteins not detected in the compiled list of sperm proteins. Two of the pathways are shown in greater detail in (**B** and **C**). (B) Some of the peroxisomal proteins detected in the sperm proteome are boxed in red (adapted from the Keggs Pathways database). (C) The TCA. All the enzymes boxed in red have been detected in the sperm cell.

conditions, pyruvate is usually converted to acetyl-CoA, which enters the tricarboxylic acid cycle (TCA) and ultimately participates in mitochondrial oxidative phosphorylation (OXPHOS; which is much more ATP productive than glycolysis). In anaerobiosis, lactate fermentation occurs. In sperm, glycolytic enzymes are tethered in the fibrous sheath, whereas the mitochondrial electron transfer chain (ETC) localizes to the midpiece. Such compartmentalization led some scientists to suggest that glycolysis would be the main source of sperm ATP, even in aerobic conditions (the Warburg effect), similarly to what happens in tumour cells, or in fast axonemal transport (Zala *et al.*, 2013). This idea instigated the so-called 'sperm energy debate', which basically discusses whether the ATP needed to fuel sperm activities comes from glycolysis or OXPHOS (Ramalho-Santos *et al.*, 2009). Whatever the case may be, both pathways seem to be relevant for sperm function. Interestingly, most of the sperm glycolytic enzymatic steps are catalysed by spermatogenic cell-specific protein isoforms and knocking out of various of these enzymes in mice models resulted in impaired male fertility (Miki *et al.*, 2004; Odet *et al.*, 2008; Danshina *et al.*, 2010), undoubtedly showing the relevance of glycolysis to sperm function. Of note, the severity of the results was recently shown to depend on the mouse strain used (Odet *et al.*, 2013), obscuring any parallel between mouse models and humans. In addition to ATP production, mouse sperm glycolysis may also contribute to a pH-dependent flagellar beat frequency regulation (Mannowetz *et al.*, 2012). In humans, stimulation of glycolysis may promote sperm motility and capacitation (Rogers and Perreault, 1990; Bone *et al.*, 2001; Williams and Ford, 2001). Interestingly, human sperm seem to convert exogenous labelled pyruvate into lactate, with no trace of oxidation in the TCA, which is suggestive of the preponderance of glycolysis (Hereng *et al.*, 2011).

*Reactom*e suggests that human sperm have a functional PPP (Table II; Supplementary data, Fig. S2). Mouse sperm seem to need to produce NADPH via the PPP in order to achieve fertilization (Urner and Sakkas, 1999, 2005) and results from KO mice suggest that transaldolase is essential for sperm mitochondrial function and male fertility (Perl *et al.*, 2006). In human sperm the production of NADPH by the PPP may be needed for gluthathione peroxidase antioxidant defence (Williams and Ford, 2004) and for capacitation (Miraglia *et al.*, 2010).

When exogenous glucose is not available, glucose may be synthesized by gluconeogenesis, a pathway that may also be active in sperm. The existence of functional gluconeogenesis was demonstrated in dog sperm, and this seems to be required for the achievement of *in vitro* capacitation in the absence of glucose (Albarracin *et al.*, 2004). Furthermore, gluconeogenesis in dog sperm was proposed to be linked to glycogen metabolism. Indeed, dog sperm seems to be able to synthesize and store glycogen, as well as to break down stored glycogen (Ballester *et al.*, 2000; Palomo *et al.*, 2003). Evidence for the activity of these pathways

Table II Biological pathways likely active in human sperm.

Metabolism (REACT_111217; $P = 6.5 \times 10^{-50}$; ratio = 0.54)	
Metabolism of carbohydrates ($P = 6.4 \times 10^{-17}$)	Hexose transport ($P = 1.6 \times 10^{-7}$) Glycolysis ($P = 2.2 \times 10^{-8}$) Gluconeogenesis ($P = 7.4 \times 10^{-9}$) Glycogenolysis ($P = 2.2 \times 10^{-4}$) Pentose phosphate pathway ($P = 8.5 \times 10^{-4}$)
Metabolism of lipids and lipoproteins ($P = 1.1 \times 10^{-3}$)	Triglyceride biosynthesis ($P = 2.1 \times 10^{-3}$) Mitochondrial fatty acid beta-oxidation ($P = 4.6 \times 10^{-3}$) Peroxisomal lipid metabolism ($P = 2.3 \times 10^{-3}$) Cholesterol biosynthesis ($P = 8.5 \times 10^{-3}$)
The citric acid cycle and respiratory electron transport ($P = 1.4 \times 10^{-30}$)	Pyruvate metabolism ($P = 8.5 \times 10^{-5}$) Citric acid cycle ($P = 4.4 \times 10^{-9}$) Respiratory electron transport ($P = 8.4 \times 10^{-18}$)
Metabolism of nucleotides ($P = 4.8 \times 10^{-8}$)	Purine metabolism ($P = 2.7 \times 10^{-5}$) Synthesis and interconversion of nucleotide di- and triphosphates ($P = 2.3 \times 10^{-5}$)
Metabolism of vitamins and cofactors (2.3×10^{-4})	Metabolism of water-soluble vitamins and cofactors ($P = 4.2 \times 10^{-4}$)
Metabolism of amino acids and derivatives (1.8×10^{-14})	Amino acid synthesis and interconversion ($P = 8.7 \times 10^{-3}$) Branched-chain amino acid catabolism ($P = 1.5 \times 10^{-5}$) Lysine catabolism ($P = 3.1 \times 10^{-4}$) Regulation of ornithine decarboxylase ($P = 4.3 \times 10^{-11}$) Sulfur amino acid metabolism ($P = 3.1 \times 10^{-4}$)
Metabolism of proteins (REACT_17015; $P = 1.1 \times 10^{-38}$; ratio = 0.66)	
Translation ($P = 5.0 \times 10^{-23}$)	Eukaryotic translation initiation ($P = 3.8 \times 10^{-20}$) SRP-dependent cotranslational protein targeting to membrane ($P = 3.8 \times 10^{-14}$) Eukaryotic translation elongation ($P = 6.8 \times 10^{-15}$) Eukaryotic translation termination ($P = 6.5 \times 10^{-14}$) 3′-UTR-mediated translational regulation ($P = 3.1 \times 10^{-18}$)
Protein folding ($P = 2.0 \times 10^{-10}$)	Chaperonin-mediated protein folding ($P = 9.7 \times 10^{-9}$) Post-chaperonin tubulin folding pathway ($P = 3.4 \times 10^{-8}$)
Post-translational protein modification ($P = 2.2 \times 10^{-6}$)	Asparagine N-linked glycosylation ($P = 9.4 \times 10^{-8}$)
Mitochondrial protein import ($P = 7.5 \times 10^{-8}$)	TOMM40 complex transports proteins across the outer mitochondrial membrane ($P = 4.4 \times 10^{-9}$) SAM50 complex inserts proteins into mitochondrial outer membrane ($P = 6.4 \times 10^{-3}$) MPP cleaves presequence of matrix precursors ($P = 8.7 \times 10^{-3}$)
Membrane trafficking (REACT_11123; $P = 6.5 \times 10^{-20}$; ratio = 0.69)	COPII (coat protein 2) mediated vesicle transport ($P = 1.9 \times 10^{-3}$) COPI mediated transport ($P = 4.1 \times 10^{-5}$) Clathrin-derived vesicle budding ($P = 5.4 \times 10^{-10}$) Translocation of GLUT4 to the plasma membrane ($P = 1.6 \times 10^{-15}$)
Metabolism of RNA (REACT_21257; $P = 7.6 \times 10^{-20}$; ratio = 0.65)	Metabolism of mRNA ($P = 2.7 \times 10^{-22)}$ Nonsense-mediated decay ($P = 6.4 \times 10^{-15}$) Regulation of mRNA stability by proteins that bind AU-rich elements ($P = 4.1 \times 10^{-9}$)
Apoptosis (REACT_578; $P = 1.2 \times 10^{-14}$; ratio = 0.68)	
Apoptotic intrinsic pathway ($P = 1.1 \times 10^{-4}$)	
Apoptotic execution phase ($P = 6.4 \times 10^{-5}$)	Apoptotic cleavage of cellular proteins ($P = 7.1 \times 10^{-4}$)
Regulation of apoptosis ($P = 2.0 \times 10^{-10}$)	Regulation of activated PAK-2p34 by proteasome-mediated degradation ($P = 1.2 \times 10^{-12}$)
Cell cycle (REACT_115566; $P = 2.6 \times 10^{-10}$; ratio = 0.50)	
Mitotic cell cycle ($P = 1.9 \times 10^{-8}$)	Regulation of DNA replication ($P = 5.5 \times 10^{-7}$) Mitotic G2-G2/M phases ($P = 2.8 \times 10^{-8}$) M Phase ($P = 7.9 \times 10^{-13}$) M/G1 transition ($P = 2.9 \times 10^{-6}$) Regulation of mitotic cell cycle ($P = 4.8 \times 10^{-6}$)
Chromosome maintenance (2.1×10^{-2})	Nucleosome assembly ($P = 2.2 \times 10^{-6}$) Meiotic synapsis ($P = 3.5 \times 10^{-5}$)
Hemostasis (REACT_604; $P = 3.3 \times 10^{-6}$; ratio = 0.46)	Platelet activation, signaling and aggregation ($P = 1.1 \times 10^{-4}$) Factors involved in megakaryocyte development and platelet production ($P = 3.2 \times 10^{-3}$)
Meiosis (REACT_111183; $P = 1.7 \times 10^{-4}$; ratio = 0.57)	Meiotic Recombination ($P = 6.4 \times 10^{-5}$) Meiotic Synapsis ($P = 3.5 \times 10^{-5}$)

Extracellular matrix organization (REACT_118770; $P = 1.7 \times 10^{-3}$; ratio = 0.48)	
Collagen formation ($P = 2.6 \times 10^{-6}$)	Collagen biosynthesis and modifying enzymes ($P = 3.2 \times 10^{-5}$) Assembly of collagen fibrils and other multimeric structures ($P = 3.0 \times 10^{-4}$)
Degradation of the extracellular matrix ($P = 4.9 \times 10^{-3}$)	Degradation of collagen ($P = 2.3 \times 10^{-3}$)
Developmental biology (REACT_111045; $P = 4.4 \times 10^{-3}$; ratio = 0.43)	
Axon guidance ($P = 2.9 \times 10^{-10}$)	NCAM signaling for neurite out-growth ($P = 7.9 \times 10^{-4}$) L1CAM interactions ($P = 4.3 \times 10^{-12}$)
Others	Signalling by Wnt ($P = 4.8 \times 10^{-17}$) Signalling by insulin receptor ($P = 1.1 \times 10^{-4}$) Signalling to Ras ($P = 4.5 \times 10^{-3}$) Signalling by constitutively active EGFR ($P = 4.6 \times 10^{-3}$) Signalling by interleukins ($P = 4.1 \times 10^{-3}$) Amyloids ($P = 2.2 \times 10^{-12}$)

Data were analysed using *Reactome*. *P* represents the probability that the association between the proteins in the sperm proteome data set and the pathway is explained by chance alone (*P* values < 0.01 were considered significant). *Ratio* stands for the number of proteins from the sperm proteome that map to the pathway divided by the total number of proteins constituting the pathway. For the main pathways, *Reactome* identifiers are also indicated.

in human sperm is required. Both gluconeogenesis and glycogenolysis may contribute to the long-term maintenance of human sperm motility in a medium without substrates (Amaral *et al.*, 2011).

Lipid metabolism

Several proteins involved in lipid metabolism seem to be relevant for spermatogenesis and sperm function. A well-studied example is hormone-sensitive lipase (HSL), a multifunctional enzyme that catalyses triacylglycerol hydrolysis and for which a spermatid- and sperm-specific isoform exists (Holst *et al.*, 1996). HSL-deficient mice were infertile due to spermiogenesis abnormalities ultimately resulting in severe oligoasthenoteratozoospermia (Osuga *et al.*, 2000; Chung *et al.*, 2001; Hermo *et al.*, 2008), and the fertility of these mice could be restored by the expression of either the mouse or human HSL testicular isoform (Vallet-Erdtmann *et al.*, 2004; Wang *et al.*, 2004). Likewise, targeted disruption of group VIA phospholipase A2 resulted in impaired sperm motility and reduced male fertility, and chemical inhibition of the enzyme in wild types decreased the ability of sperm to swim (Bao *et al.*, 2004). Male KO mice for fatty-acid desaturase 2 (FADS2) were also infertile because of disrupted spermatogenesis, resulting in low sperm counts, impaired motility and abnormal morphology, as well as acrosome biogenesis failure (Stoffel *et al.*, 2008; Stroud *et al.*, 2009; Roqueta-Rivera *et al.*, 2011).

After analysing the proteome of isolated human sperm tail fractions, we have recently proposed that, at least in the absence of glycolysable substrates, the male gamete may be able to produce ATP using fatty acids (Amaral *et al.*, 2013). Analysis of the compiled sperm proteome adds further support to the notion that sperm have the enzymatic machinery to perform beta-oxidation of different length fatty acids (Supplementary data, Fig. S3). Importantly, we have shown that inhibition of beta-oxidation with etomoxir impairs sperm motility in a time- and dose-dependent manner. Etomoxir inhibits carnitine palmitoyl-transferase I, an enzyme that was suggested to be relevant for rat sperm development (Adams *et al.*, 1998). As we have also suggested (Amaral *et al.*, 2013), human sperm may be able to catabolize very long chain fatty acids (VLCFA) using peroxisomal enzymes. In fact, the compiled sperm proteome includes several peroxisomal proteins, including catalase, the typical peroxisomal marker (Supplementary data, Fig. S4). Whether or not ejaculated sperm possess peroxisomes is uncertain, the alternative being the existence of peroxisomal proteins in sperm mitochondria. At any rate, peroxisomal metabolic pathways might be essential for normal spermatogenesis, as both patients with peroxisomal deficiencies and mice models with altered levels of specific peroxisomal proteins are infertile (Powers, 1985; Huyghe *et al.*, 2006; Kaczmarek *et al.*, 2011). After the chain is shortened by peroxisomal enzymes, VLCFAs could either be further oxidized by mitochondrial enzymes, or serve as substrates for the biosynthesis of cholesterol, a pathway also detected by *Reactome*. This might be particularly important and should be studied further given the acknowledged role of cholesterol homeostasis in sperm function (Cross, 1998; Saez *et al.*, 2011).

TCA and mitochondrial oxidative phosphorylation

Whether or not OXPHOS is one of the sources of ATP for fuelling sperm actions, an increasing body of data has shown that mitochondrial activity is intimately related to sperm function. For instance, and just to give a few examples, both the enzymatic activity of ETC complexes and the expression of ETC proteins were related to human sperm quality (Ruiz-Pesini *et al.*, 1998, 2000; Amaral *et al.*, 2007). The accumulation of multiple mitochondrial DNA (mtDNA) rearrangements was associated with loss of sperm function, and low-quality sperm seem to possess altered mtDNA levels (St John *et al.*, 2007). Furthermore, sperm motility and fertilization ability were correlated with mitochondrial membrane potential (MMP), a parameter frequently used to monitor mitochondrial functionality (Kasai *et al.*, 2002; Marchetti *et al.*, 2002, 2004; Amaral and Ramalho-Santos, 2010). Perhaps even more illustrative, the subpopulations of sperm with high MMP seem to have better fertilization potential (Gallon *et al.*, 2006; Sousa *et al.*, 2011).

Studies of the relevance of the TCA cycle in mammalian sperm function are scarce but given the association between this cycle and the activity of the ETC, one can infer that an accurate function of the TCA cycle in sperm may also be needed (Supplementary data, Fig. S5). Although analysis of rat testicular germ cells suggested that the activity of the TCA cycle may be more relevant at the spermatid stage (Bajpai *et al.*, 1998), ejaculated boar sperm seem to be capable of metabolizing exogenous citrate and

lactate through the TCA cycle (Medrano et al., 2006). Interestingly, different subunits of the pyruvate dehydrogenase complex seem to be implicated in hamster sperm capacitation and acrosome reaction (Mitra and Shivaji, 2004; Mitra et al., 2005; Kumar et al., 2008).

Nucleotide metabolism

The role of nucleotides in providing energy for flagellar movement is clearly established. An array of sperm kinases can catalyse the synthesis (and interconversion) of di- and tri-phosphate nucleotides. Some of these kinases, namely adenylate kinases and nucleoside diphosphate kinases (together with creatine kinases, carbonic anhydrases and the glycolytic system, all of which are found in the sperm proteome), may be particularly relevant by participating in a phosphotransfer network, co-ordinating a rapid flow of ATP between production and consumption cellular processes (Dzeja and Terzic, 2003; Munier et al., 2003; Noma, 2005). Noteworthy, proteomics data also confirm that human sperm contain inorganic pyrophosphatases 1 and 2, as well as progressive ankylosis protein homolog (ANKH), which is consistent with the recent identification of the inorganic pyrophosphate pathway as a significant component of mammalian sperm physiology (Yi et al., 2012).

Protein metabolism

The sperm proteome seems to be enriched in proteins related to protein metabolism (Table II; which includes all the pathways implicated in protein translation, folding, post-translation modification and protein degradation).

Protein translation and folding

Whether mature sperm are able to perform nuclear protein synthesis has been debated in the literature. Independent observations suggested that the incorporation of labelled amino acids into polypeptides by sperm was inhibited by chloramphenicol but was not affected by cycloheximide (Bragg and Handel, 1979; Ahmed et al., 1984), which led to the accepted idea that mammalian sperm are able to translate mitochondrial but not nuclear encoded proteins. Oddly enough, *de novo* protein synthesis of nuclear encoded proteins was suggested to take place during mammalian sperm capacitation in mitochondrial-type ribosomes (Gur and Breitbart, 2006). The idea of translation activity concomitant with sperm capacitation was apparently confirmed in rodent and pig sperm using proteomics approaches (Choi et al., 2008; Zhao et al., 2009). However, one should be cautious when interpreting these data, at least until the existence of functional ribosomes in sperm is confirmed, and the clarification of a reliable mechanism enabling mitoribosomes to translate nuclear-encoded transcripts is deciphered. For instance, mitochondria use a genetic code that is different from the 'universal' nuclear one (Barrell et al., 1979). Additionally, and as pointed out recently (Baker, 2011), it is difficult to understand how sperm would handle the specific proteins that Gur and Breitbart suggested were synthesized '*de novo*' in mitochondrial ribosomes during capacitation (Gur and Breitbart, 2006). How would proteins such as A-kinase-anchoring protein 4 (AKAP4; a structural and insoluble protein) be transported from the mitochondria to the flagellum? The current paradigm is thus that mature mammalian sperm are able to translate mitochondrial-encoded proteins but not nuclear-encoded ones. In any event, at least at the protein level, it seems that mature sperm contain the basic machinery to synthesize proteins, including various eukaryotic translation initiation factors and elongation factors, as well as peptide chain release factors involved in translation termination. Additionally, the sperm proteome contains numerous cytoplasmic and mitochondrial ribosomal proteins. Analysis of sperm cytoplasmic rRNA indicated that sperm possess 18S rRNA (small subunit) but not 28S rRNA (large subunit), which suggests an impaired cytoplasmic ribosome assembly (Cappallo-Obermann et al., 2011). In accordance, 18S rRNA seems to be mainly associated with a monosomal ribosome fraction indicating that sperm cytoplasmic ribosomes may be inactive, while mitochondrial 12S rRNA was associated with both monosomes and polysomes (Gur and Breitbart, 2006). Using proteomics approaches, different ribosomal protein subunits were found both in isolated sperm nucleus and tail fractions (Supplementary data, Fig. S6; de Mateo et al., 2011a; Amaral et al., 2013; Baker et al., 2013). Future studies should be performed in order to determine the integrity, accurate assembly and functional plausibility of sperm ribosome subunits. The correct folding of newly synthesized proteins is assisted by a battery of chaperone proteins, including HSPs and chaperonins (Frydman, 2001), several of which belong to the sperm proteome. Interestingly, various HSPs might be essential to spermatogenesis and/or sperm fertilization ability, as suggested by experiments using mice models (Eddy, 1999; Ikawa et al., 2001; Held et al., 2011). A functional role for some HSPs and chaperonins in human sperm has also been shown. For instance, immature sperm seems to have low levels of HSPA2 (homologue of the mouse HSP70-2; Huszar et al., 2000). More recently, the proteomic profile of human sperm from a patient with a defect in sperm–zona pellucida binding was compared with that of a fertile donor, and the results supported an association between reduced expression of HSPA2 and the sperm defect (Redgrove et al., 2012). The authors further suggested that HSPA2 co-ordinates the formation of a multimeric oocyte receptor complex involved in sperm–oocyte interaction (Redgrove et al., 2013).

Post-translational protein modifications

Numerous PTMs increase protein complexity and modulate protein functions. N-linked glycosylation may be the most common PTM (Khoury et al., 2011) and in fact the human sperm proteome includes various glycosyltransferases potentially involved in the covalent attachment of an oligosaccharide onto asparagine residues. It is well established that the sperm surface possesses various N-glycans (Pang et al., 2007) and that some of these are likely involved in fertilization. Indeed, sperm–oocyte binding is believed to rely on the recognition of zona pellucida glycoproteins by glycosylated proteins on the sperm surface. It seems that a sialyl-Lewis(x) sequence is the major carbohydrate ligand for sperm–oocyte binding (Pang et al., 2011) but the exact sperm surface receptor involved in this interaction is unknown. Sperm glycoproteomics studies will certainly help decipher this cellular recognition event.

Besides N-linked glycosylation, there are other types of PTMs known to mediate sperm function but those are not included in the *Reactome* database as independent pathways, and were thus not traceable using this tool. Particularly relevant are protein phosphorylation and *S*-nytrosilation, nuclear protein acetylation and methylation, as well as ubiquitination events, which will be discussed below.

One of the hallmarks of sperm capacitation is the massive phosphorylation of a number of proteins. Although studying this sperm maturation event at the molecular level has been challenging, capacitation is believed to be regulated by a signalling cascade triggered by the stimulation of a soluble adenylyl cyclase that, via cyclic AMP, activates protein kinase A (PKA) resulting in the phosphorylation of various proteins (Bailey,

2010). The identities of the protein substrates and of the tyrosine kinases/phosphatases that modulate protein phosphorylation are however not completely settled and proteomic approaches have been used to identify the capacitation-associated tyrosine phosphorylated substrates. In addition, the compiled sperm proteome contains a group of protein tyrosine kinases that may be involved in capacitation. A growing body of evidence suggests that several kinases (and phosphatases) may be involved in multiple signalling events associated with spermatogenesis, epididymal maturation and sperm function (Li *et al.*, 2009; Lie *et al.*, 2009; Ijiri *et al.*, 2012). Thus, that the sperm proteome contains dozens of kinases and phosphatases is not surprising. Although less well studied, sperm function seems to be also modulated by the nytrosylation of proteins at cysteine residues. Indeed, nitric oxide (NO) enhances sperm motility and capacitation, and human sperm contains various *S*-nitrosylation targets (Lefievre *et al.*, 2007). The sperm *S*-nytrosoproteome seems to regulate the mobilization of calcium stores and enhance flagellar beating (Machado-Oliveira *et al.*, 2008).

Although most of the sperm DNA is packaged with protamines, some regions retain histones (and their tail modifications), and recent data suggest that the nucleosome retention is not random, but rather constitutes epigenetic information, allowing the activation of specific paternal genes in the early embryo (Arpanahi *et al.*, 2009; Hammoud *et al.*, 2009; Oliva and de Mateo, 2011). Various histone modifications are known to be relevant to sperm and in fact aberrant sperm histone acetylation and/or methylation have been associated with male infertility (de Mateo *et al.*, 2011c; Hammoud *et al.*, 2011; Carrell, 2012; Oliva and Ballescà, 2012). Proteomics data show that human sperm contains various enzymes implicated in the dynamic establishment and regulation of histone modifications. The activity of these enzymes might be particularly relevant during spermatogenesis (Ooi and Henikoff, 2007), ultimately determining mature sperm histone modifications and likely constituting paternal epigenomic marks.

Another PTM controlling sperm function is ubiquitination and this can be discussed at different levels. First of all, a substantial body of work is showing that the ubiquitin-proteasome pathway (UPP) is implicated in sperm function. The UPP is an ATP-dependent mechanism that mediates protein turnover by targeting ubiquitinated proteins for degradation by the 26S proteasome (Supplementary data, Figs S7 and S8). Many proteins and organelles are degraded by this pathway during spermatogenesis (Hou and Yang, 2013), and its relevance is typified by the phenotype of mice models lacking different components of the UPP, which were shown to have a block in spermatogenesis and male infertility, to varying extents (Roest *et al.*, 1996; Rodriguez and Stewart, 2007). In addition to the usual intracellular UPP, studies on sperm proteasome have indicated that fertilization is somehow controlled by a gamete-associated extracellular UPP, as proteasome-mediated proteolytic events participate in mammalian sperm–zona pellucida penetration (Zimmerman and Sutovsky, 2009; Sutovsky, 2011). Thus, it was not surprising to find various proteasomal subunits in the sperm proteome. In addition, a ubiquitin-dependent mechanism for the detection and elimination of defective mammalian sperm during their passage through the epididymis has been suggested (Sutovsky *et al.*, 2001; Baska *et al.*, 2008). Finally, ubiquitin may also tag mammalian sperm mitochondria for destruction in the zygote, contributing to the generally exclusive maternal inheritance of the mitochondrial genome (Sutovsky *et al.*, 1999; Sutovsky and Schatten, 2000). The mitochondrial protein prohibitin may be one of the sperm ubiquitinated substrates (Thompson *et al.*, 2003). A comprehensive identification of the sperm ubiquitinome would certainly result in the identification of additional substrates. The challenge would then be to determine in which of the aforementioned sperm facets (or others) each ubiquitinated protein is involved.

Membrane trafficking

Membrane trafficking is a crucial aspect of eukaryotic cell biology, allowing for the delivery of different molecules to distinct locations using membrane-enclosed vesicles (Supplementary data, Fig. S9). It includes two complementary pathways, the secretory pathway and the endocytic pathway (Lippincott-Schwartz *et al.*, 2000). In somatic cells the first involves protein synthesis in the endoplasmic reticulum (ER), transport and modification through the Golgi apparatus and delivery to internal organelles, the plasma membrane or secretory vesicles. The second encompasses the uptake of exogenous molecules (via clathrin-coated endocytosis, caveolae or macropinocytosis), with the formation of plasma membrane-derived intracellular vesicles, and their subsequent delivery to other locations for functional purposes, processing, receptor recycling (in endosomes) or digestion (in lysosomes). Although there are several specific processes within each pathway, they all share common components.

That so many different components of membrane trafficking pathways have been identified in human sperm (coat proteins, motors, adaptors, cytoskeletal links, targeting and fusion proteins) raises the possibility that the male gamete may be more dynamic than previously anticipated. However, as stated previously, it is always possible that these proteins are only present as remnants of mammalian spermiogenesis. At any rate their presence certainly seems to highlight (albeit in an indirect fashion) the importance of membrane trafficking in sperm formation. There are three main points to be made. The first relates to events that are known to be important for sperm formation, the second to processes that take place in mature gametes. Finally, one should note the presence of proteins involved in particular aspects that have yet to be clarified, and which may be interesting targets for future studies. The role of membrane trafficking in spermiogenesis is well known, especially in terms of the synthesis of acrosomal proteins in the ER and the subsequent coalescing of Golgi-derived vesicles carrying these proteins, thus producing the acrosome (Moreno *et al.*, 2000b; Ramalho-Santos and Moreno, 2001; Ramalho-Santos *et al.*, 2001, 2002). In essence, the acrosome is a large secretory vesicle formed by the fusion of smaller vesicles, the ultimate product of the secretory pathway in spermiogenesis. Thus, it does not seem surprising that many elements of this pathway are detected in the mature gamete, although whether they still have a functional role at this stage may be questioned. For example, recycling of components back to the ER or Golgi seems futile, as these organelles are being dismantled and shed by the elongating spermatid, resulting in misplaced proteins. Additionally, it is particularly interesting that *Reactome* identifies lysosome vesicle biogenesis as a likely pathway in human sperm, given the parallels that have been made between these organelles, classifying the acrosome as a sort of modified secretory lysosome, which might also receive contributions from the endocytic pathway in its biogenesis and maturation (Moreno *et al.*, 2000a; Moreno, 2003; Moreno and Alvarado, 2006).

There are, however, elements of membrane trafficking that have clearly established roles in sperm function, notably at the last stage of

the classical secretory pathway, exocytosis. In sperm this process is represented by the acrosome reaction, and proteins involved include, for example, the small GTPases rab3A and rab27 (Bustos et al., 2012). It is notable that many of the rab family-interacting proteins (regulators, activators) detected in the human sperm proteome are specific for rab3A, stressing the importance of this particular GTPase in sperm. The sperm proteome is also enriched in proteins that mediate membrane fusion and thus promote the release of acrosomal contents. These include members of the SNARE family and associated proteins, such as syntaxin 1 and 2, VAMPs, SNAP 23 (but curiously not SNAP 25), MUNC18, plus the priming ATPase NSF and its SNAP regulators, as well as synaptotagmins, which function as calcium sensors during regulated exocytosis (Ramalho-Santos et al., 2000; Tomes et al., 2002, 2005; Sousa et al., 2006; Rodriguez et al., 2012; Tsai et al., 2012).

It is also interesting to note the presence of components of the exocyst in mature human sperm. This complex is involved in vesicle docking during exocytosis and has not been studied in mammalian sperm. Interestingly, one report cites components of this complex as having a role in sperm polarization in *Drosophila* (Fabian et al., 2010). Given that membrane trafficking in general, and the exocyst in particular, is known to contribute towards the establishment and maintenance of compartments in polarized cells (Rodriguez-Fraticelli et al., 2011), a re-visitation of its role in sperm formation at this level may be warranted.

Of course the final fusion event the sperm is involved in is sperm–oocyte fusion and proteins thought to be involved in this process are also present in the proteome, including IZUMO proteins or CRISP1 and 2 that actually have an epididymal origin (Sutovsky, 2009). Several members of the ADAM (A Disintegrin and A Metalloprotease domain) family are also found but their putative role in sperm–oocyte recognition has been debated, notably in mouse models (Evans, 2002).

On a slightly different note, some proteins that are involved in trafficking may have other roles in sperm. For example, caveolin-1, a main player in caveolae endocytosis has been better studied for its role in lipid rafts and cell signalling during sperm capacitation (Gamboa and Ramalho-Santos, 2005; Baltierrez-Hoyos et al., 2012). Indeed other typical components of lipid rafts (such as flotillins) are also detected (Miranda et al., 2009; Boerke et al., 2013).

RNA metabolism

Although once doubted, the presence of a complex population of RNAs in ejaculated human sperm is now completely established. This include messenger RNAs (mRNAs; Miller, 2000), small non-coding RNAs, such as microRNAs (Ostermeier et al., 2005), piwi-interacting RNAs and repeat-associated small RNAs (Krawetz et al., 2011), as well as mitochondrial RNAs (Jodar et al., 2012). The functional significance of the nuclear-encoded sperm transcripts is yet to be established, especially given the assumed transcriptionally inactivity of sperm chromatin. Actually, the RNAs needed for the haploid phase of spermatogenesis are synthesized at an earlier stage and are stored awaiting translation during spermiogenesis (Steger, 2001). Thus, sperm RNAs were often considered stored leftovers. However, recent advances in the field suggest that at least some sperm transcripts are likely to be functional, with probable roles in paternal genome packaging, early embryogenesis, transmission of paternal epigenetic information or in protein translation (Lalancette et al., 2008; Dadoune, 2009; Johnson et al., 2011). As previously indicated, the overlap between the sperm transcriptome and proteome seems to be only partial (Wang et al., 2013), ruling out a putative role of the sperm transcripts in *de novo* protein synthesis. On the other hand, sperm harbour RNAs that were not found in unfertilized oocytes (Ostermeier et al., 2004). Seeing that human embryonic genome activation occurs between the 4- and 8-cell stages (Braude et al., 1988), an extensive comparison of sperm and metaphase II oocytes both at the transcriptome and proteome levels may add new insights into the functional significance of the sperm RNAs and proteins in early embryonic development.

As expected, and although human sperm possess various transcription factors and proteins involved in RNA metabolism, analysis of the compiled sperm proteome suggests that sperm do not have all of the proteomic machinery needed to perform transcription (i.e. transcription was not a significant sperm pathway). In contrast, sperm seem to harbour proteins involved in RNA metabolism, which includes mRNA degradation pathways, as well as the packaging and processing of small nuclear ribonucleoproteins.

Actually, various sperm proteins known to be involved in nonsense-mediated mRNA decay (NMD; Chang et al., 2007; Kervestin and Jacobson, 2012) were shown to play key roles during spermatogenesis. To this extent, CCR4-associated factor 1 KO mice were viable and healthy but the males were sterile due to early spermatogenesis disruption (Berthet et al., 2004; Nakamura et al., 2004). Although the possibility of RNA surveillance in sperm permits the destruction of transcripts that would be obsolete in sperm, the existence of NMD events in mature sperm is unlikely as, at least in other cells, NDM is a translation-coupled mechanism and as discussed, there is no good evidence that protein synthesis of nuclear-encoded genes takes place in sperm. Thus, these proteins may be mere leftovers from spermatogenesis or, alternatively, could have other non-canonical functions. As some of these proteins were shown to be required for early embryogenesis (McIlwain et al., 2010), it is tempting to suggest that sperm proteins involved in RNA metabolism could be transmitted to the zygote and have a functional role in early embryo development.

Taken together, that sperm possess the machinery to regulate mRNA stability suggests that there are various cellular events not generally taken into account that may potentially be activated in sperm under certain conditions. For instance, incubation of sperm with exogenous nucleic acids can result in their internalization in the nuclei, and activates an endogenous sperm reverse transcriptase, resulting in the production of retro-genes that can be delivered to the oocyte upon fertilization (Spadafora, 2008).

Apoptosis

Apoptosis is the process of programmed cell death responsible for the elimination of unhealthy, old or unnecessary cells. The expression of various apoptotic factors in human sperm and their association with sperm quality has been well documented (Varum et al., 2007; Almeida et al., 2011; Zalata et al., 2011, just to give a few examples), and thus that proteomic data would suggest an implication of the apoptotic pathway (Supplementary data, Fig. S10) in the male gamete was already expected. However, one may ask why sperm should experience apoptosis. There are two main reasons why this process may be relevant: (i) it can act as a cell-selection mechanism, by preventing DNA-damaged sperm to participate in fertilization and (ii) it might be linked to the

removal of sperm from the female reproductive tract *post coitum* (Aitken and Koppers, 2011).

As in many other cellular events, the programmed form of cell death seems to operate in a unique way in the male gamete, with similarities but also dissimilarities to the somatic cell counterpart. Apoptosis is known to be required for normal spermatogenesis (Rodriguez *et al.*, 1997; Russell *et al.*, 2002) and in fact, it has been proposed that the apoptotic markers found in sperm would result from an 'abortive apoptosis' process initiated during spermatogenesis (Sakkas *et al.*, 1999, 2002). One classical apoptotic event is the activation of endonucleases, which may be exported to the nucleus and cause DNA damage. Given the compartmentalization of sperm and the condensed nature of its chromatin, this process may be prevented in the male gamete (at least in the majority of DNA, which is condensed by nucleoprotamines). One of the potential functions of protamines is to protect the sperm DNA (Oliva and Dixon, 1991; Eirin-Lopez and Ausio, 2009; de Mateo *et al.*, 2011b; Jodar *et al.*, 2011; Oliva and Castillo, 2011a). It is also well known that infertile patients with altered protamination have increased DNA damage and decreased fertility (de Yebra *et al.*, 1993; Bench *et al.*, 1998; Torregrosa *et al.*, 2006; de Mateo *et al.*, 2009; Castillo *et al.*, 2011; Simon *et al.*, 2011). Still, it seems that apoptosis results in sperm DNA damages but that this is an oxidative rather than an enzymatic process, and that mitochondria play a major role here (De Iuliis *et al.*, 2009; Aitken and De Iuliis, 2010). It was suggested that sperm undergo a truncated apoptotic cascade, which may be required to stimulate the maternal immune cells to phagocyte sperm without a concomitant inflammatory response, as observed in other cell types (Kurosaka *et al.*, 2003).

The molecular mechanisms that trigger apoptosis in sperm have just started to be determined. It seems that sperm apoptosis is regulated by the phosphoinositidine 3-kinase/protein kinase B (PI3K/AKT) pathway (Aquila *et al.*, 2007; Koppers *et al.*, 2011). Additionally, sperm oxidative stress seems to be able to self-perpetuate by means of a positive feedback mechanism involving the activation of mitochondrial ROS production and apoptosis by electrophilic aldehydes produced in mitochondria (Aitken *et al.*, 2012b). Thus, human sperm may undergo an intrinsic (or mitochondrial) apoptotic cascade and in fact, *Reactome* analysis of the compiled proteome suggests that only the intrinsic apoptotic pathway is active in sperm.

Interestingly, *Reactome* outcomes also suggest that caspase-mediated cleavage of proteins may occur during the sperm apoptotic execution phase. As discussed previously, sperm has all the machinery needed to perform proteolysis and various caspase targets were described in the sperm proteome. In any case, the significance of this cellular process in sperm needs further confirmation. Analysis of the sperm proteome suggests that sperm apoptosis could theoretically be regulated by the proteasome-mediated degradation of a serine/threonine protein kinase (PAK). Seeing that the sperm chromatin is condensed in nature, the significance of this pathway seems doubtful but there is always the possibility that a caspase activated-PAK may stimulate sperm apoptosis via a different mechanism.

At any rate, sperm apoptosis seems to be a highly regulated process that may be prevented by incubating cells with appropriate components. In fact, by stimulating AKT phosphorylation and suppressing caspase activation and capacitation, prolactin was shown to have a prosurvival effect on human sperm (Pujianto *et al.*, 2010). Similarly, incubation of sperm with nucleophilic thiols resulted in rescued sperm motility, by counteracting the action of electrophilic aldheydes (Aitken *et al.*, 2012a). In a normal situation, these and other prosurvival factors may exist both in the epididymis and the female reproductive tract, in order to prevent sperm from undergoing apoptosis.

Other pathways

Unsurprisingly, *cell cycle* and *meiosis* were also detected as likely active pathways in human sperm (Table II). The meiotic proteins might be remnants of spermatogenesis, with no function in mature sperm. Notably, and although only 5–15% of the sperm DNA remains packaged by nucleosomes (Oliva and Castillo, 2011a), the sperm proteome contains an array of histone variants. Some of these are testis specific and their role in the germ cells has been extensively studied (Churikov *et al.*, 2004; Govin *et al.*, 2004). The detailed analysis of all sperm histones (and their PTMs) will be particularly relevant especially given their potential epigenetic role during embryo development (Arpanahi *et al.*, 2009; Hammoud *et al.*, 2009; Oliva and Ballescà, 2012).

Another pathway detected by *Reactome* was *hemostasis* (Table II). How can haemostatic pathways (the processes causing bleeding arrest) be related to sperm function? One of the reasons is that sperm cells undergo a multistep process controlled by a specific set of receptor–ligand interactions, required for cell–cell and cell–extracellular matrix adhesion events, and that many of the proteins involved in this process are identical to those required for the recognition and adhesion processes present in haemostasia, leukocyte recruitment, platelet adhesion and aggregation (Zhou *et al.*, 2004; Preissner and Bronson, 2007; Koyama *et al.*, 2009). The sperm plasma membrane also experiences changes in membrane fluidity, activation of ion channels, rearrangement of surface proteins and calcium induced-acrosomal exocytosis, which are also similar to those required for platelet aggregation. Finally, there are similarities between blood coagulation and fibrinolisis, and the clotting of semen and subsequent liquefaction after ejaculation which involves interactions with proteins present in the sperm cell (Fernandez *et al.*, 1997; Lwaleed *et al.*, 2007; Jonsson *et al.*, 2010).

Comparative and functional sperm proteomics

Proteomics comparisons of sperm of differing quality have suggested novel putative male (in)fertility biomarkers. The most commonly used proteomic strategy in sperm comparative studies combined 2DE with in-gel protein digestion and MS peptide identification (Fig. 1, Table I), despite the relatively low throughput capacity of this approach. Still, interesting outcomes have been obtained. For instance, the 2DE protein pattern of a sperm sample of a patient with failed IVF, and for which female factors were excluded, consistently revealed 20 different protein spots when compared with that of 3 fertile donors (Pixton *et al.*, 2004). This pioneering work resulted in the identification of 4 proteins. The use of a more sensitive proteomic approach (2D fluorescence difference gel electrophoresis) to compare the proteomic mapping of sperm from 3 infertile patients and from 3 donors resulted in the identification of 12 differentially expressed proteins (Frapsauce *et al.*, 2009). Although relevant, the outcomes obtained by these two studies did not demonstrate a clear role of the detected protein changes in sperm function. Therefore, and in order to understand the failure of some IVF treatments, future studies following this line are undoubtedly warranted.

Thus far, most sperm comparative proteomic studies aimed to characterize potential proteomic anomalies in infertile patients with altered spermiogram results. The most studied phenotype has been asthenozoospermia, not only due to the biological importance of finding proteins potentially involved in sperm motility regulation but also because obtaining sufficient protein from asthenozoospermic sperm is relatively straightforward (extracting sufficient protein from oligozoospermic sperm, for instance, would be more difficult). To this extent, analysis of the proteomic profile of sperm from 8 asthenozoospermic patients and 8 normozoospermic fertile donors by 2DE and MALDI-TOF MS resulted in the identification of 10 differentially expressed proteins (Zhao *et al.*, 2007). Using a similar approach but a larger number of samples our group identified 7 proteins down-regulated and 10 up-regulated in asthenozoospermic sperm (Martínez-Heredia *et al.*, 2008). Interestingly, 15 of the proteins identified were reported for the first time to have altered levels in sperm with altered motility. Siva *et al.* added 7 other proteins to the list, some of which were predicted to be phosphorylated by a PTM searching *in silico* approach (Siva *et al.*, 2010). Since phosphorylation of certain proteins plays a role in sperm motility (Luconi and Baldi, 2003), some studies have focused on the differential analysis of the phosphorylation status of sperm proteins. This has been accomplished using either conventional 2DE or by performing immobilized metal affinity chromatography (IMAC) before MS analysis. The first resulted in the identification of 12 proteins, 10 of which were hypophosphorylated in asthenozoospermic patients compared with controls, attesting the important of this PTM for sperm motility (Chan *et al.*, 2009). That the phosphoproteome of sperm with differing motility is different was recently confirmed by the recognition of 68 phosphoproteins either up- or down-regulated in asthenozoospermic samples (Parte *et al.*, 2012).

Taken together, the differential proteomic studies performed so far identified a total of 109 proteins with altered levels in asthenozoospermia (Fig. 3). As expected, given the role of the flagellum in sperm motility and the need for ATP to propel the flagellar dynein arms, a high proportion of the detected up- or down-regulated proteins belong to the cytoskeleton or are enzymes involved in metabolism and energy production. The remaining proteins belong to two additional functional groups: proteins involved in protein metabolism and proteins known to be required for spermatogenesis and sperm function. It is however worth mentioning that only 6% of these proteins have been identified in more than one independent study and that differing results were published, most probably because of the presence of PTMs. This is certainly the case for HSPA2, which was up-regulated in asthenozoospermia in one study (Martínez-Heredia *et al.*, 2008), and down-regulated in two others (Siva *et al.*, 2010; Parte *et al.*, 2012). The root of this apparent inconsistency may be the presence of PTMs, with different protein variants detected in each of the studies. In any case, additional studies will be needed to clarify this issue.

Specific proteomic alterations were also associated with globozoospermia (a severe form of teratozoospermia; Liao *et al.*, 2009) and oligoasthenozoospermia (Botta *et al.*, 2009; Thacker *et al.*, 2011). Moreover, sperm with distinct protamine content and different levels of DNA damage seem to have a different proteome profile (de Mateo *et al.*, 2007). Proteomics data also pointed out possible differences in signalling and metabolic pathways between sperm from normozoospermic men and from healthy donors (Xu *et al.*, 2012). Additionally, sperm protein patterns from diabetic patients, non-diabetic obese patients and healthy donors seem to be dissimilar (Paasch *et al.*, 2011).

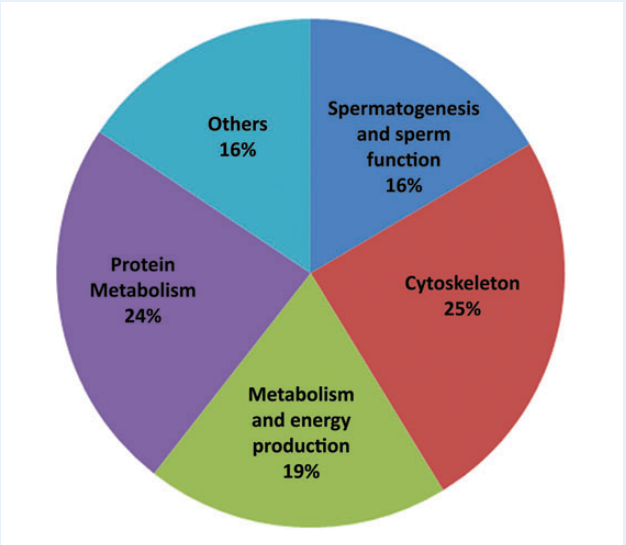

Figure 3 Functions of the proteins reported to be differentially expressed in sperm samples with different motility. The 109 proteins detected as up- or down-regulated in asthenozoospermic compared with normozoospermic samples were classified according to their main function using the information available at the UniProtKB/Swiss-Prot website.

As already described, mammalian sperm capacitation has been widely studied using proteomic approaches, particularly focused on the proteins that are substrates for tyrosine phosphorylation. Using a combination of 2DE and anti-phosphotyrosine immunoblots and immobilized metal ion affinity chromatography phosphopeptides enrichment before MS, 16 proteins undergoing tyrosine phosphorylation in human sperm capacitation were identified (Ficarro *et al.*, 2003). Likewise, Secciani *et al.* (2009) compared the protein profiles of capacitated sperm, swim-up selected capacitated sperm and ejaculated sperm (i.e. not capacitated) using conventional 2DE. Besides phosphorylation, the role of *S*-nitrosylation in sperm capacitation has been also studied. Sperm from normozoospermic donors were incubated with NO and *S*-nitrosylated proteins were detected using the biotin switch assay (Lefievre *et al.*, 2007). Targets of NO include various metabolic proteins and proteins associated with energy generation and movement. More recently, the human sperm sumoylome (i.e. the set of SUMOylated proteins—proteins with Small Ubiquitin-like Modifier) was also described (Vigodner *et al.*, 2013). SUMO targets in sperm include flagellar proteins, proteins involved in sperm differentiation and maturation, metabolic enzymes, several of which are also targets for tyrosine phosphorylation and nitrosylation in sperm. Finally, proteomic analyses also instigated the identification of sperm protein complexes that seem to mediate the interaction with the zona pellucida (Redgrove *et al.*, 2011, 2012, 2013).

To conclude, although many comparative and functional sperm proteomic studies have been published, providing remarkable results, some points remain unclear. More studies are justified in order to define, at the protein level, what makes a functional sperm. The use of higher throughput techniques coupled to various up-to-date options for

differential protein labelling might provide further light towards knowledge of sperm (dys)functions.

Conclusions and future directions

The human sperm cell proteomics field has advanced enormously in the last decade and the studies performed so far have resulted in the identification of 6198 proteins involved in several cellular pathways. Using the *ratios* of Reactome pathways analysis, we can make an estimation of the putative total number of sperm proteins. As an example, we know that the mitochondrial transfer chain comprises several enzymes (all needed for its proper function) but that some of these were never detected in sperm. However, it is logical to believe that they might be there, although they have escaped MS identification, owing to either their physicochemical characteristics or their relatively low abundance in cells. And the same also applies to proteins from many other pathways (Reactome *ratios* ranged from 0.43 to 1). Overall, by calculating the mean of the *ratios* of all Reactome pathways identified, we estimated that around 78% of the sperm proteins might have already been identified. As these pathways are very likely active in sperm, the remaining 22% of proteins might belong to the sperm proteome. Using this rationale, we estimate that the complete human sperm proteome may be composed of at least 7500 different proteins.

MS technologies are constantly being updated and thus more sperm proteins will certainly be identified in the next couple of years. Additional subcellular proteomics studies might help to complete the description of the sperm proteins catalogue. For instance, and although this may be technically challenging, it would be interesting to analyse the proteome of isolated human sperm acrosomes, fibrous sheaths and mitochondrial sheaths. Furthermore, the use of up-to-date techniques to perform comparative proteomics is warranted. Determining how dissimilar, at the protein level, different quality sperm samples (and/or different sperm subpopulations within a sample) are will help in establishing the elements of the sperm proteome that are required for the distinct aspects of sperm function, and ultimately, for sperm fertilization ability.

Describing the human sperm protein repertoire is crucial to define 'what sperm is made of'. Now that we nearly have this complete knowledge, it is time to translate sperm protein lists into functional interactomes. In this review, by determining which cellular pathways are expected to be active in sperm through the analysis of the compiled sperm proteome, we have provided the first step towards this goal. However, the functional relevance of some of the sperm proteins, such as those related to RNA metabolism and protein synthesis, for example, remains unclear. Future experiments should aim to determine what the roles of these sperm proteins (if any) are. Additionally, we should try to investigate the hypothesis that specific paternal proteins may be key protagonists in early embryonic development.

Supplementary data

Supplementary data are available at http://humupd.oxfordjournals.org/.

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Authors' roles

A.A.: participation in study design, execution, analysis, manuscript drafting, critical discussion. J.C. and J.R.-S.: participation in study design, manuscript drafting, critical discussion. R.O.: participation in study design, execution, manuscript drafting, critical discussion.

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Conflict of interest

None declared.

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