Proteòmica del plasma seminal i de les seves vesícules extracel·lulars: nova font de biomarcadors útils en l'estudi de la funcióespermàtica i la infertilitat masculina

Ferran Barrachina Villalonga

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Proteòmica del plasma seminal i de les seves vesícules extracel·lulars: nova font de biomarcadors útils en l'estudi de la funció espermàtica i la infertilitat masculina

Memòria presentada per
Ferran Barrachina Villalonga

Per optar al grau de
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en el
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Treball desenvolupat sota la direcció de
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Als meus pares,
A les meves germanes,
A la meva meitat

"Science is not only a discipline of reason, but, also, one of romance and passion"
— Stephen Hawking

La present tesi doctoral és el resultat del treball realitzat durant els darrers anys on tantíssimes persones m'han acompanyat, animat i donat forces. La participació de tots vosaltres, ja sigui des d'un punt de vista científic o personal, m'ha resultat essencial i ha fet que aquesta tesi sigui tant meva com vostra. A totes les persones que em rodegen els agraeixo el seu suport, ànims i, sobretot, afecte. Sense vosaltres res d'això hauria sigut el mateix.

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1

INTRODUCCIÓ

1.1. L’APARELL REPRODUCTOR MASCULÍ

La reproducció és el procés biològic mitjançant el qual un organisme genera descendència. Tant l’aparell reproductor masculí com l’aparell reproductor femení estan involucrats en aquest procés, i resulten en la producció d’espermatozoides o oòcits, respectivament, amb el potencial de donar lloc a un embrió. Tanmateix, ambdós aparells reproductors difereixen significativament en estructura i funcionament.

Els òrgans reproductors masculins inclouen el testicle, l’epidídim, les glàndules sexuals accessòries, tals com la pròstata, les vesícules seminals i les glàndules bulbouretrals, i, finalment, el penis (**Figura 1**). La producció d’espermatozoides té lloc en els testicles, tot i això, els espermatozoides resultants són cèl·lules immadures i immòbils incapaces de fertilitzar els oòcits pels seus propis medis.

La maduració posttesticular dels espermatozoides succeeix en l’epidídim, i durant l’ejaculació, on els espermatozoides passen al conducte deferent i contacten amb els fluids secretats per les diverses glàndules sexuals accessòries. Aquest contacte dels espermatozoides amb el conjunt de secrecions provinents dels diversos òrgans que es troben al llarg del tracte reproductor masculí, que es coneix com a plasma seminal, permet a l’espermatozoide adquirir la seva total funcionalitat i la capacitat per poder fertilitzar l’oòcit femení, permetent la generació d’un embrió que donarà lloc a la seva descendència. Tant la composició molecular del plasma seminal, com el seu alt contingut en vesícules extracel·lulars (VEs) secretades principalment per la pròstata i l’epidídim, juguen un rol primordial en la transformació funcional dels espermatozoides.

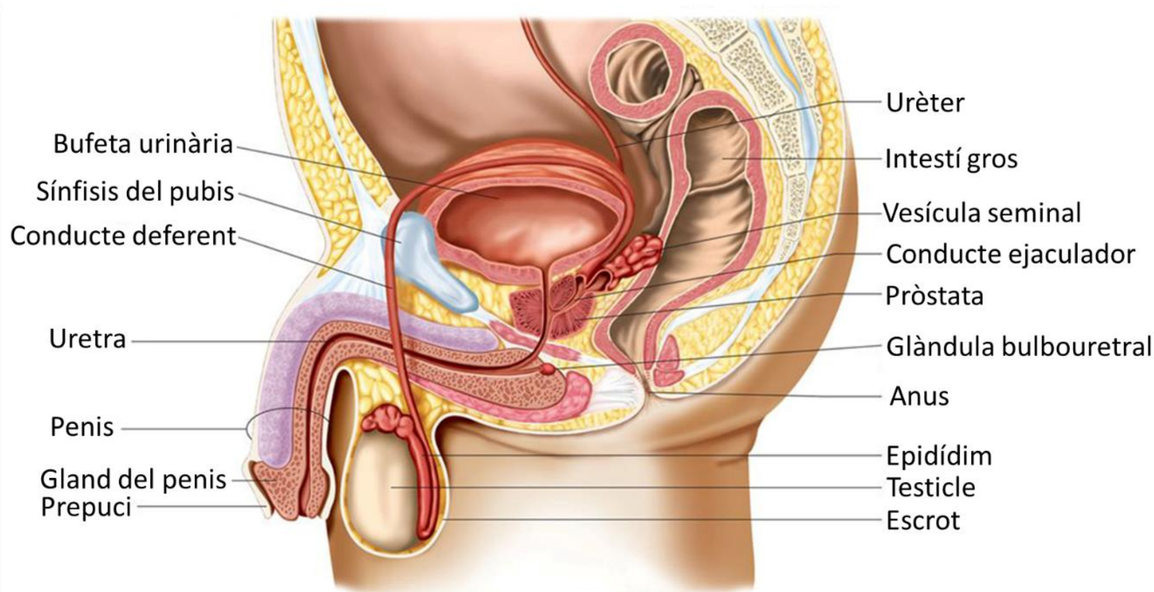

Figura 1. Esquema de l’aparell reproductor masculí. Font: Modificat de (Longenbaker, 2006).

1.2. L'ESPERMATOGÈNESI I L'ESPERMATOZOIDE

El testicle és l'òrgan de l'aparell reproductor masculí on es produeixen els gàmetes masculins, anomenats espermatozoides. L'espermatogènesi és el procés mitjançant el qual es produeixen els espermatozoides a partir de cèl·lules germinals primordials, anomenades espermatogònies, que es localitzen en els túbuls seminífers dels testicles (Hess and Renato de Franca, 2008).

Els testicles tenen una forma ovalada i mesuren, aproximadament, 4-5 cm de llargada. Els testicles es troben dins l'escrot, i estan envoltats per dues capes distintes de teixit connectiu protector, la túnica vaginal i la túnica albugínia (**Figura 2**) (Betts *et al.*, 2013). La túnica albugínia, a més de recobrir externament el testicle, també forma unes invaginacions dins el testicle anomenades septes, que divideixen el testicle en uns 300-400 lòbuls espermàtics (Holstein *et al.*, 2003; Betts *et al.*, 2013). Dins d'aquests lòbuls és on es troben els túbuls seminífers, lloc de producció dels espermatozoides.

Funcionalment, el testicle es subdivideix en dos compartiments, els túbuls seminífers i el teixit intersticial. Els túbuls seminífers representen el 95% del volum testicular i es troben revestits de capes de cèl·lules germinals en diverses etapes de desenvolupament (espermatogònies, espermatòcits primaris, espermatòcits secundaris, espermàtides i espermatozoides) i cèl·lules de Sertoli (de Kretser *et al.*, 1998). Les cèl·lules germinals es troben disposades en els túbuls seminífers de forma concèntrica, estant les cèl·lules immadures (espermatogònies) més properes a la làmina basal, mentre que les cèl·lules més madures (espermatozoides) es troben properes a la llum del túbul (Sutovsky and Manandhar, 2006; Hess and Renato de Franca, 2008). A més, en els túbuls seminífers també hi ha les cèl·lules de Sertoli, les quals donen suport i nodreixen a les cèl·lules germinals, produeixen substàncies endocrines i paracrines que regulen l'espermatogènesi i secreten l'hormona inhibina (Holstein *et al.*, 2003; Luisi *et al.*, 2005; França *et al.*, 2016; Wu *et al.*, 2020). Addicionalment, entre les cèl·lules de Sertoli s'estableixen unions oclusives (en anglès *tight junctions*) que formen la barrera hematotesticular (Holstein *et al.*, 2003; França *et al.*, 2016; Heinrich and DeFalco, 2019; Wu *et al.*, 2020). Els túbuls seminífers són altament contornejats, d'uns 180 µm de diàmetre, i convergeixen en una xarxa de conductes denominada xarxa testicular o *rete testis* (**Figura 2**) (Holstein *et al.*, 2003). Els espermatozoides abandonen la *rete testis* i, per tant, el testicle, per mitjà d'uns 15-20 conductes eferents que creuen la túnica albugínia i connecten amb l'epidídim (Sutovsky and Manandhar, 2006; Betts *et al.*, 2013). Per altra banda, el teixit intersticial consisteix en el teixit connectiu, vasos sanguinis i limfàtics, i alguns tipus cel·lulars com cèl·lules de Leydig, fibroblasts, macròfags o leucòcits (Holstein *et al.*, 2003; Ge *et al.*, 2009; Heinrich and DeFalco, 2019). Les cèl·lules de Leydig són el major tipus cel·lular en el teixit intersticial del testicle i, generalment, es troben adjacents als vasos sanguinis i als túbuls

seminífers. A més, les cèl·lules de Leydig són la font predominant de testosterona, la qual estimula l'espermatogènesi (Ge *et al.*, 2009; Heinrich and DeFalco, 2019).

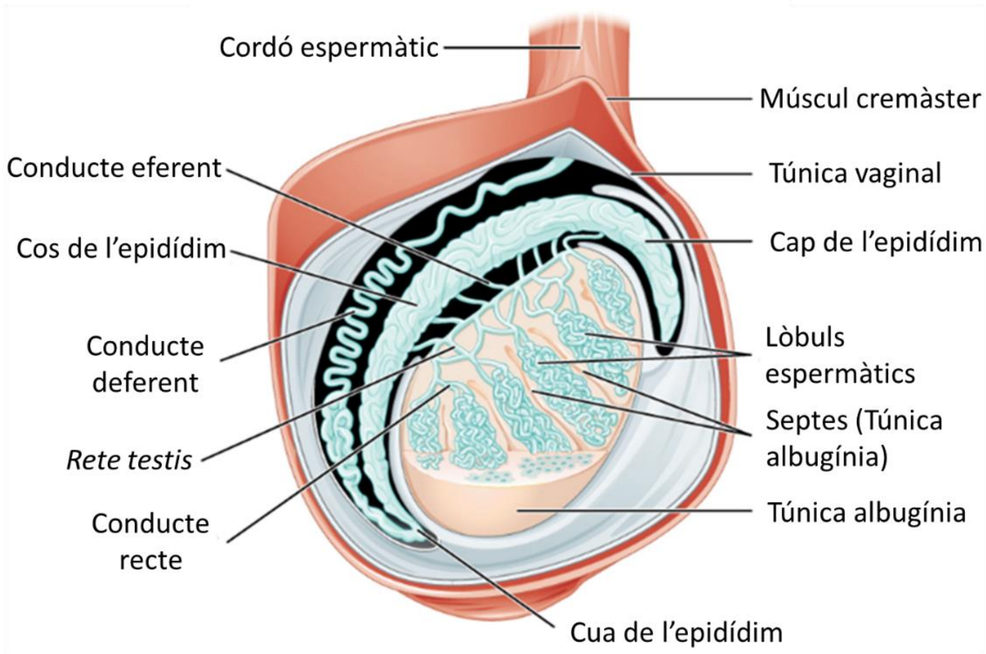

Figura 2. Anatomia del testicle. La producció d'espermatozoides es duu a terme en els túbuls seminífers del testicle. Els espermatozoides testiculars són transferits a l'epidídim, on maduraran i, posteriorment, abandonaran l'epidídim en el moment de l'ejaculació. Font: Modificat de (Betts *et al.*, 2013).

1.2.1. Cèl·lules espermàtiques i fases de l'espermatogènesi

L'espermatogènesi és un procés complex de diferenciació cel·lular que produeix espermatozoides haploides a partir d'espermatogònies diploides (Oliva and Castillo, 2011a). En el testicle humà es produeixen aproximadament entre 100 i 300 milions d'espermatozoides cada dia (Betts *et al.*, 2013). En humans, tot aquest procés és molt llarg, amb una duració aproximada de 64 dies (Betts *et al.*, 2013). Durant la vida fetal, les cèl·lules germinals primordials migren cap a la cresta genital, on donaran lloc als túbuls seminífers. En el moment del naixement, les cèl·lules germinals primordials localitzades dins els túbuls seminífers es transformen en espermatogònies tipus A no proliferatives. No obstant, el procés de l'espermatogènesi es manté quiescent fins a la pubertat, quan s'incrementa la producció de gonadotropines i s'indueix una proliferació mitòtica massiva d'espermatogònies tipus A, conjuntament amb la diferenciació d'espermatogònies tipus A en espermatogònies tipus B, les quals tenen la capacitat d'entrar en meiosi i, després d'un llarg procés, diferenciar-se a espermatozoide (Sutovsky and Manandhar, 2006). La producció d'espermatozoides s'allarga tota la vida fèrtil de l'home, per tant, per tal de mantenir la producció, les espermatogònies tipus A experimenten una auto-renovació i diferenciació contínua (Sutovsky and Manandhar, 2006).

L'espermatogènesi en mamífers es divideix en 3 fases: mitosi, meiosi i espermiogènesi (**Figura 3**) (de Kretser *et al.*, 1998; Chocu *et al.*, 2012; Jodar *et al.*, 2017c; Barrachina *et al.*, 2018b).

- **Fase proliferativa o mitòtica:** En la primera fase, les espermatogònies tipus A és dupliquen contínuament mitjançant mitosis successives. Tan sols una fracció d'espermatogònies, les espermatogònies tipus B, s'embarquen en el procés de diferenciació, i entren a la fase meiòtica per a finalment diferenciar-se en espermatozoides (Plant and Marshall, 2001; Sutovsky and Manandhar, 2006). Aquesta doble habilitat de les espermatogònies d'incrementar la reserva de cèl·lules mare i de diferenciar-se permeten la producció de milions d'espermatozoides al llarg de la vida reproductiva de l'home (Heinrich and DeFalco, 2019). Una particularitat de les espermatogònies que entren en el procés de diferenciació és que aquestes cèl·lules romanen interconnectades entre si per ponts citoplasmàtics (sincitis) que connecten les cèl·lules germinals i permeten el pas de RNAs i de proteïnes.
- **Fase meiòtica:** En la segona fase de l'espermatogènesi es produeixen dues divisions meiòtiques, les quals permeten reduir la dotació cromosòmica de les cèl·lules germinals des de diploide (2n) a haploide (n), i comença amb l'entrada de l'espermatogònia tipus B a la profase de la primera divisió meiòtica. Com a resultat de la primera divisió meiòtica, els espermatòcits primaris es divideixen i converteixen en espermatòcits secundaris. En la segona divisió meiòtica, els espermatòcits secundaris es divideixen i transformen donant com a resultat les espermàtides rodones, les quals són cèl·lules haploides.
- **Espermiogènesi:** La tercera i última etapa de l'espermatogènesi és la fase de diferenciació i maduració, anomenada espermiogènesi. Durant aquesta última fase, tot i que no es produeix cap divisió cel·lular, es produeix una complexa transformació morfològica on les espermàtides rodones esdevindran espermàtides allargades, per a finalment convertir-se en espermatozoides (Plant and Marshall, 2001). Aquesta transformació inclou complexos canvis morfològics i nuclears, com la remodelació de l'estructura de la cromatina pel reemplaçament d'histones per protamines, o la reducció de la mida de l'espermatozoide per la pèrdua del citoplasma, així com la biogènesi de noves estructures tals com l'acrosoma, la peça intermèdia i la cua o peça principal (Sutovsky and Manandhar, 2006; Oliva and Castillo, 2011a).

El resultat de l'espermatogènesi és l'espermatozoide testicular. No obstant, aquest espermatozoide, malgrat i estar morfològicament diferenciat, és una cèl·lula immadura, immòbil i incapaç de fertilitzar l'oòcit per si mateix (Gatti *et al.*, 2004; Jodar *et al.*, 2017c; Barrachina *et al.*, 2019). És per aquest motiu que la cèl·lula espermàtica requereix un període de maduració posttesticular que li conferirà les habilitats que li permetran

ascendir pel tracte reproductor femení i fecundar a l'oòcit (Gatti *et al.*, 2004; Aitken *et al.*, 2007; Guyton and Hall, 2011).

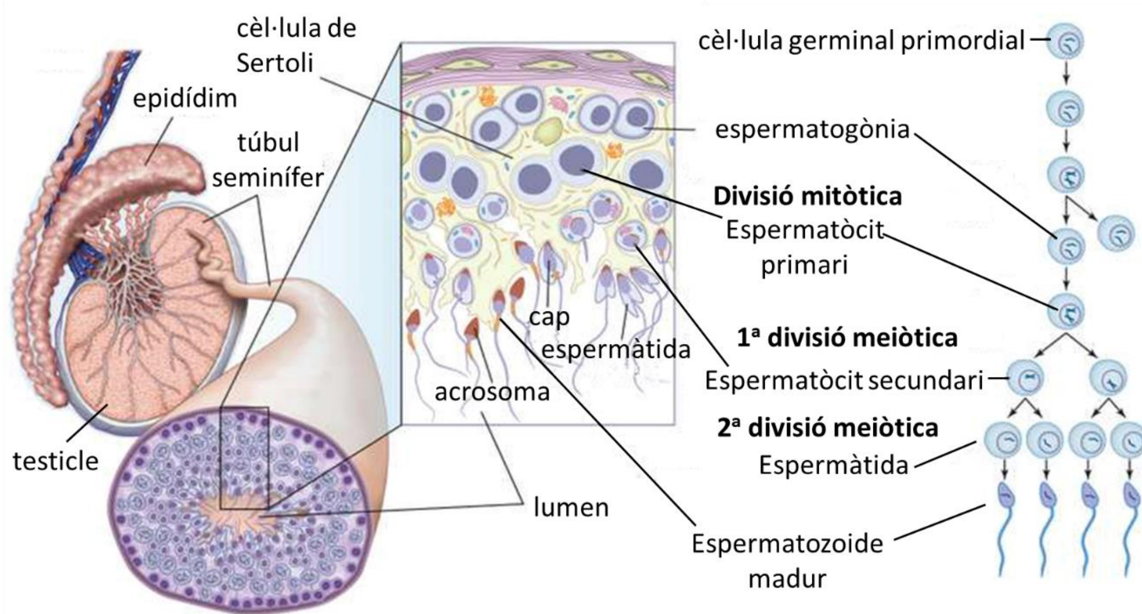

Figura 3. Esquema de la espermatogènesi humana. Font: Modificat de (The Editors of Encyclopaedia Britannica, 2019).

1.2.2. L'espermatozoide

L'espermatozoide en mamífers és una cèl·lula única i altament especialitzada, de morfologia piriforme i amb absència d'activitat transcripcional i traduccional (Oliva and Castillo, 2011b, 2011a; Castillo *et al.*, 2014b; Jodar *et al.*, 2017c). El microscopista Antonie van Leeuwenhoek va ser la primera persona que va descriure l'espermatozoide, l'any 1677 (Karamanou *et al.*, 2010).

L'espermatozoide humà mesura aproximadament uns 60 µm de llargada i està compost principalment de dues estructures (**Figura 4**) (Holstein *et al.*, 2003; Sutovsky and Manandhar, 2006):

- **El cap:** El cap de l'espermatozoide conté el nucli (material genètic patern haploide) summament compacte amb molt poc citoplasma. Aquestes característiques són les responsables de la mida petita del cap de l'espermatozoide, el qual tan sols mesura 5 µm de llargada i 3 µm de diàmetre (Holstein *et al.*, 2003; Toshimori and Ito, 2003). Envoltant les 2/3 parts anteriors del cap de l'espermatozoide es troba l'acrosoma, una vesícula plena d'enzims hidrolítics necessaris per al procés de fertilització. Per tant, el cap de l'espermatozoide es pot subdividir en dues regions, la regió acrosòmica i la regió post-acrosòmica (Toshimori and Ito, 2003).

- **La cua o flagel:** La cua o flagel de l'espermatozoide està compost per 3 subestructures:
    - La **peça intermèdia** (5-7 µm de llargada) està coberta per una baina d'unes 75-100 mitocòndries organitzades formant una espiral, necessàries per produir energia (ATP) i actuar com a motor de propulsió de l'espermatozoide (Sutovsky and Manandhar, 2006).
    - La **peça principal** (45 µm de llargada) és la que proporciona la mobilitat a l'espermatozoide. L'ATP produït per les mitocòndries de la peça intermèdia permetrà el moviment del flagel, el qual s'estén des de la peça intermèdia fins a la peça terminal de la cua, resultant en el moviment de l'espermatozoide.
    - La **peça terminal** és l'extrem del flagel.

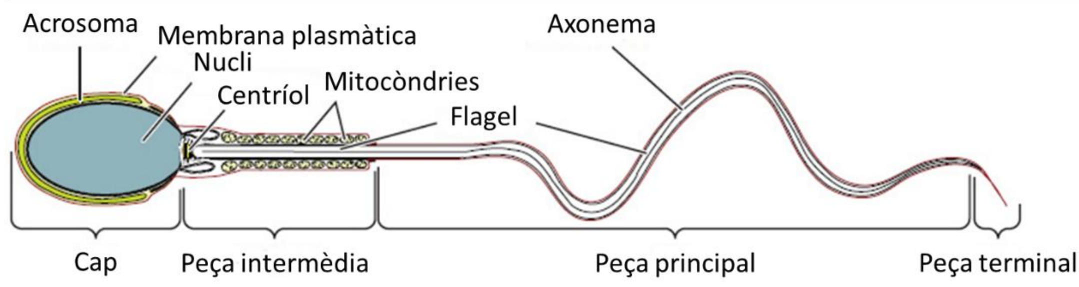

Figura 4. Estructura de l'espermatozoide humà. Font: Modificat de (Betts *et al.*, 2013).

1.2.2.1. El nucli espermàtic

La cromatina espermàtica és diferent en composició i organització a la cromatina nuclear de les cèl·lules somàtiques (Ward and Coffey, 1991). Mentre que en les cèl·lules somàtiques la cromatina està empaquetada per unes proteïnes bàsiques anomenades histones, en l'espermatozoide, durant l'espermatogènesi, la cromatina experimenta una important reorganització. Les histones unides a la DNA són sistemàticament reemplaçades per proteïnes de transició, per a ser finalment reemplaçades per protamines (Mezquita, 1985; Meistrich *et al.*, 2003; Oliva, 2006; Balhorn, 2007). Aquesta substitució progressiva d'histones per protamines és especialment rellevant, ja que fa que el nucli es vagi compactant fortament, el que protegeix el DNA de l'espermatozoide de les nucleases i contribueix a l'adquisició d'una forma hidrodinàmica necessària per a la correcta mobilitat i funcionalitat de l'espermatozoide (Soler-Ventura *et al.*, 2020).

1.2.2.2. La cromatina espermàtica

La remodelació de la cromatina durant l'espermatogènesi s'inicia amb una onada massiva d'acetilació de les histones en la fase d'espermàtida elongada, i és un dels primers signes que desencadenen el reemplaçament d'histones per protamines (Oliva and Mezquita, 1982; Oliva and Dixon, 1991; Hazzouri *et al.*, 2000; Faure, 2003; Oliva, 2006; Govin *et al.*, 2007). La hiperacetilació de les histones relaxa la conformació de la cromatina i redueix l'afinitat que les histones tenen amb el DNA, el que permet la substitució de les histones. Per tant, i de manera progressiva al llarg de l'espermatogènesi, es desassemblen els nucleosomes presents en les espermatogònies, espermatòcits i espermàtides rodones, i les histones són reemplaçades transitòriament per proteïnes de transició i, per últim, per protamines (Mezquita, 1985; Oliva *et al.*, 1987; Meistrich, 1989; Oliva and Dixon, 1991; Oliva, 2006; Balhorn, 2007; Rajender *et al.*, 2011). De fet, l'increment de l'acetilació de la histona H4 (acH4) en els nucleosomes de les espermàtides rodones (rTid) i elongades (eTid) és un prerequisit per al reemplaçament d'histones per protamines (**Figura 5**)

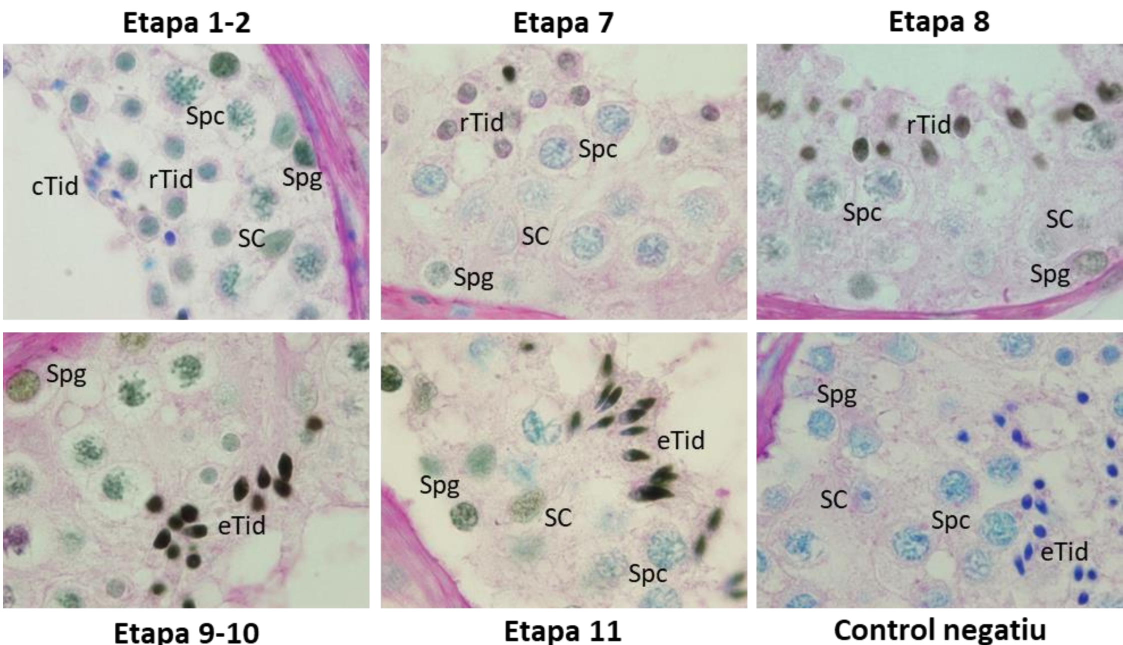

Figura 5. Patró d'hiperacetilació de la histona H4 en les diferents etapes (1-12) de l'espermatogènesi normal en seccions de testicle tenyides amb hematoxilina i eosina. La identificació de les 12 etapes de l'espermatogènesi s'ha basat en la classificació feta per Muciaccia i col·laboradors (Muciaccia *et al.*, 2013). Les cèl·lules positives per acH4 mitjançant immunohistoquímica estan tenyides de color marró. Spg, espermatogònia; Spc, espermatòcits; rTid, espermàtides rodones; eTid, espermàtides elongades; cTid, espermàtides condensants; SC, cèl·lules de Sertoli (Magnificació x 1000). Font: Barrachina F, de la Iglesia A, Soler-Ventura A, Jodar M, Mallofré C, Rodriguez-Carunchio L, Corral JM, Ballescà JL, Castillo J, Oliva R. Deregulation of histone H4 acetylation in testicular tissue adjacent to neoplastic cells (Article en preparació; Resultats no publicats).

(Oliva and Mezquita, 1982; Hazzouri *et al.*, 2000), i un patró d'hiperacetilació de la histona H4 aberrant resulta en una espermatogènesi alterada i deteriorada (Oliva and Dixon, 1991; Sonnack *et al.*, 2002; Faure, 2003; Oliva, 2006).

Com a resultat d'aquest procés de reemplaçament d'histones per protamines, la cromatina de l'espermatozoide presenta una estructura molt compacta i especialitzada, dividida principalment en dos dominis molt ben diferenciats, el majoritari domini nucleo-protamina (NP) i el domini nucleo-histona (NH) (**Figura 6**).

1.2.2.3. Domini nucleo-protamina [1]

En el nucli dels espermatozoides de mamífers, la gran majoria del genoma (85-98%) es troba empaquetat en protamines, formant unes estructures toroidals altament compactes (**Figura 6**) (Gatewood *et al.*, 1987, 1990; Zalensky *et al.*, 2002; Balhorn, 2007; Oliva and Castillo, 2011a). Cada toroide conté aproximadament 60.000 pb de DNA (Hud *et al.*, 1993). Les protamines són unes proteïnes petites i molt bàsiques, altament riques en residus d'arginina de càrrega positiva que permeten una alta compactació de la cromatina del DNA patern, que és de càrrega negativa (Barrachina *et al.*, 2018b). A més, les protamines són riques en residus de cisteïna, el que permet la formació d'enllaços disulfur i ponts de zinc entre protamines, donant lloc al complex de NP toroïdal altament compacte (Balhorn *et al.*, 1987; Björndahl and Kvist, 2010). En mamífers s'han descrit dos tipus de protamines:

- **Protamina 1 (P1):** Aquesta protamina es troba present en totes les espècies de vertebrats estudiades. El gen que codifica per la protamina 1 (*PRM1*) es localitza en el cromosoma 16, i la protamina 1 se sintetitza com a proteïna madura.
- **Família de Protamina 2 (P2):** Aquesta família de protamina només es troba en algunes espècies de mamífers, tals com l'humà o el ratolí. El gen que codifica per la protamina 2 (*PRM2*) també es localitza en el cromosoma 16, però codifica per una forma immadura o precursor de la protamina 2 (pre-P2) que, mitjançant proteòlisi, donarà lloc als diferents components madurs de la família: P2, P3 i P4 (Soler-Ventura *et al.*, 2020).

Avui en dia, s'ha proposat que la remodelació de la cromatina espermàtica gràcies a la participació de les protamines permet: a) Empaquetar el genoma patern en un nucli més compacte i hidrodinàmic, necessari per a una adequada mobilitat de l'espermatozoide,

[1] La informació inclosa en aquesta secció de la Introducció es basa en el Capítol de Llibre ***Barrachina F, Soler-Ventura A, Oliva R, Jodar M (2018)*** **Chapter 2: Sperm Nucleoproteins (histones and protamines). In: A Clinician's Guide to sperm DNA and chromatin damage (Zini A, Agarwal A; eds). Cham (Switzerland): Springer International Publishing AG; p.31-51** que es troba a l'Annex 1.

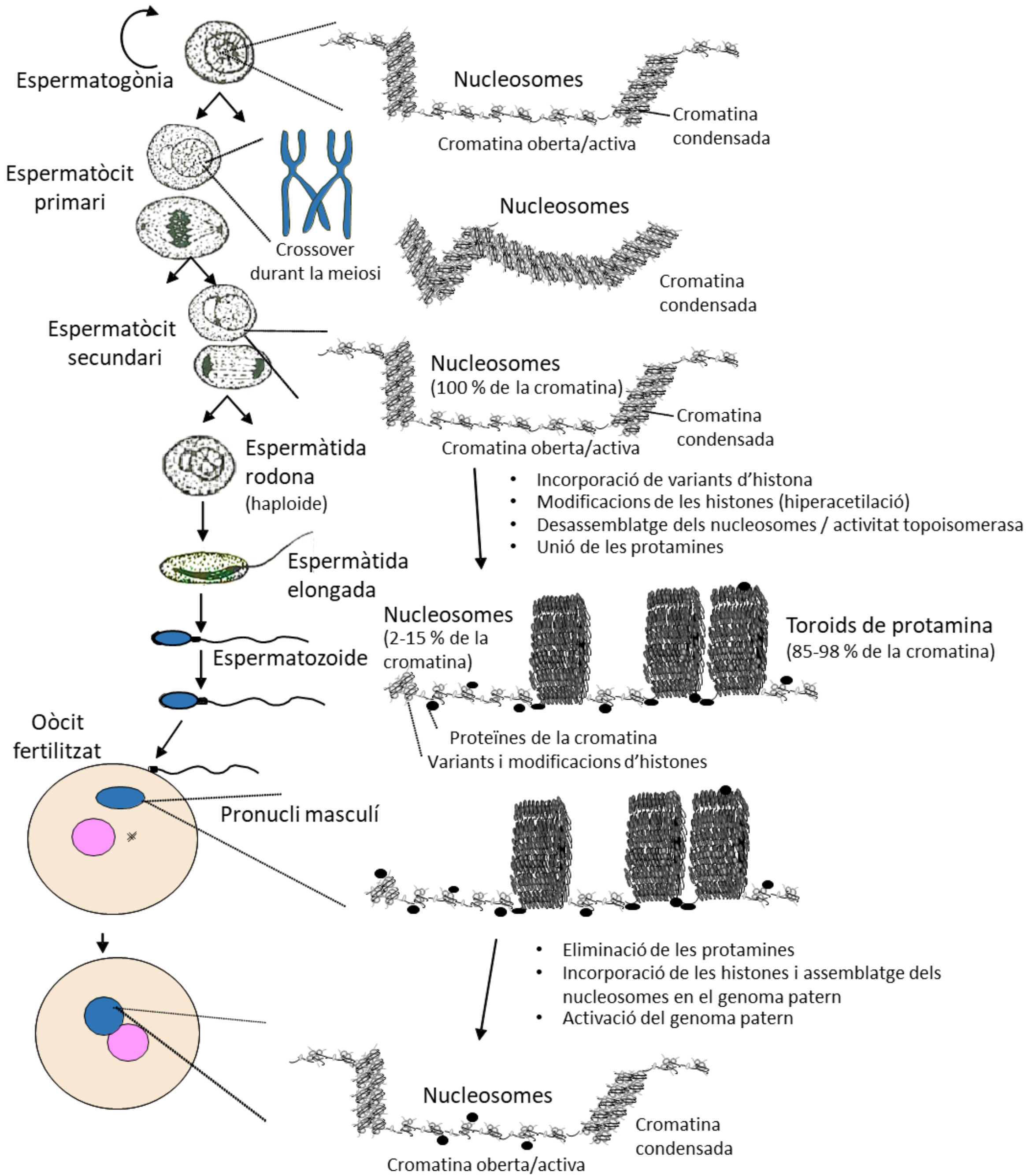

Figura 6. Canvis cel·lulars i en la cromatina durant l'espermatogènesi i en el moment de la fertilització. Font: Modificat de (Barrachina et al. 2019).

b) Protegir el genoma patern de mutàgens exògens o endògens, o de nucleases presents en el tracte reproductor masculí o femení, c) Competir i eliminar factors transcripcionals i altres proteïnes nuclears de la cromatina espermàtica, deixant el genoma patern en un "estat en blanc", de manera que el genoma patern pugui ser reprogramat per l'oòcit, i d) Participar en l'*imprinting* del genoma patern durant l'espermatogènesi i conferir noves marques epigenètiques a determinades zones del genoma de l'espermatozoide, provocant

la reactivació o repressió gènica en les primeres etapes del desenvolupament embrionari (Oliva and Dixon, 1991; Oliva, 2006; Balhorn, 2007; Barrachina *et al.*, 2018b).

El correcte balanç i distribució entre P1 i P2 en el nucli de l'espermatozoide ha sigut altament estudiat com a mesura de la maduració i normalitat de la cromatina de l'espermatozoide, i la desregulació de la relació P1/P2 en espermatozoides de mamífers s'ha associat a la infertilitat masculina (Balhorn *et al.*, 1988; de Yebra *et al.*, 1993; Bench *et al.*, 1998; Aoki *et al.*, 2006; Oliva, 2006; Gázquez *et al.*, 2008; de Mateo *et al.*, 2009; Jodar *et al.*, 2011; Azpiazu *et al.*, 2014; Jodar and Oliva, 2014; Barrachina *et al.*, 2018b).

1.2.2.4. Domini nucleo-histona [1]

No obstant, hi ha un romanent de cromatina espermàtica que es manté unit a histones (2-15%), empaquetant la cromatina en nucleosomes (**Figura 6**) (Gatewood *et al.*, 1987, 1990; Zalensky *et al.*, 2002; Balhorn, 2007; Oliva and Castillo, 2011a). Els nucleosomes de l'espermatozoide són similars als nucleosomes de les cèl·lules somàtiques i consisteixen en un nucli proteic constituït per un octàmer d'histones format per dues molècules de cada histona (H2A, H2B, H3 i H4), el qual està envoltat per 147 pb de DNA. Els nucleosomes adjacents estan interconnectats entre si per una cadena de DNA que pot tenir fins a 80 pb de llargada, la qual està unida a membres de la família d'histones H1. Malgrat l'alt nivell de compactació del DNA que confereixen els nucleosomes, aquesta cromatina és més accessible i dinàmica que la cromatina unida a protamines, i pot ser modulada o regulada degut a la incorporació de variants d'histones, modificacions post-traduccionals d'histones, i factors nuclears que modulin el DNA i les interaccions d'histones. A més, s'ha observat una distribució diferencial dels gens units als dominis nucleo-histona versus als gens units als dominis nucleo-protamina de la cromatina dels espermatozoides (Arpanahi *et al.*, 2009; Hammoud *et al.*, 2009; Samans *et al.*, 2014; Royo *et al.*, 2016), el que suggereix que aquest romanent d'histones paternes unides a la cromatina espermàtica podria actuar com a signatura epigenètica i tenir un rol important durant l'activació del genoma del zigot durant l'embriogènesi primerenca, així com en l'herència epigenètica transgeneracional (Carrell and Hammoud, 2009; Vavouri and Lehner, 2011; Rando, 2012).

1.2.2.5. Conservació entre espècies de les proteïnes espermàtiques relacionades amb la cromatina [2]

Com s'ha mencionat anteriorment, en tots els mamífers la majoria de la cromatina espermàtica es troba condensada per protamines, formant els altament compactes toroides (Oliva and Dixon, 1991; Oliva, 1995; Balhorn, 2007; Oliva and Castillo, 2011b, 2011a; Barrachina *et al.*, 2018b). Aquesta condensació del DNA espermàtic per protamines també es produeix en una proporció substancial de vertebrats (Kossel, 1928;

Felix, 1960; Bloch, 1969; Iatrou *et al.*, 1978; Oliva and Dixon, 1991; Saperas *et al.*, 1993, 1994; Zilli *et al.*, 2005, 2014; Kurtz *et al.*, 2009; Li *et al.*, 2010b; Wu *et al.*, 2011; Nynca *et al.*, 2014; Dietrich *et al.*, 2016; Barrachina *et al.*, 2018a). A més, en espermatozoides de moltes espècies d'invertebrats també s'han trobat protamines o proteïnes bàsiques similars a la protamina (Subirana, 1983; Eirín-López *et al.*, 2006; Martínez-Soler *et al.*, 2007; Dorus *et al.*, 2008; Gärtner *et al.*, 2015; Elnfati *et al.*, 2016; Kimura and Loppin, 2016; Rivera-Casas *et al.*, 2017), així com en algues o en altres organismes eucariotes (Kasinsky *et al.*, 2014).

No obstant, en mamífers, com és el cas de l'home, el ratolí o el toro, s'ha demostrat que la cèl·lula espermàtica aporta a l'embrió un 2-15% del seu DNA organitzat en histones i, per tant, en nucleosomes (Gatewood *et al.*, 1987, 1990; Zalensky *et al.*, 2002; Nazarov *et al.*, 2008; Hammoud *et al.*, 2009; Castillo *et al.*, 2014a, 2014b, 2015; Sillaste *et al.*, 2017), i s'ha observat com hi ha una distribució diferencial dels gens segons si estan units a histones o a protamines (Arpanahi *et al.*, 2009; Hammoud *et al.*, 2009; Samans *et al.*, 2014; Royo *et al.*, 2016). Aquesta distribució diferencial de la cromatina de l'espermatozoide també es troba en espècies de vertebrats que no presenten protamines, però que organitzen el seu DNA espermàtic en histones i variants d'histona, les quals contenen modificacions d'histones i altres marques epigenètiques (Wu *et al.*, 2011). A més, també s'ha descrit la presència de proteïnes relacionades amb la cromatina de l'espermatozoide en espècies d'invertebrats, com la *Drosophila melanogaster* (Dorus *et al.*, 2008; Elnfati *et al.*, 2016; Kimura and Loppin, 2016; Pimenta-Marques *et al.*, 2016).

La caracterització de la cromatina espermàtica mitjançant proteòmica d'alt rendiment ha permès disseccionar la composició molecular de l'espermatozoide (de Mateo *et al.*, 2007; Oliva *et al.*, 2008; Rousseaux and Khochbin, 2012; Carrell *et al.*, 2016; Jodar *et al.*, 2017c, 2017b), demostrant que, en mamífers, els espermatozoides contenen centenars de proteïnes associades a la cromatina, a part de les histones i de les protamines (de Mateo *et al.*, 2011; Chocu *et al.*, 2012; Baker *et al.*, 2013; Amaral *et al.*, 2014a; Azpiazu *et al.*, 2014; Castillo *et al.*, 2014b, 2015; Codina *et al.*, 2015; Vandenbrouck *et al.*, 2016). Per tant, la cromatina de l'espermatozoide proporciona a l'embrió un seguit d'informació epigenètica que inclou la metilació del DNA, modificacions post-traduccionals d'histones, altres proteïnes associades a la cromatina, una estructura única de la cromatina i uns territoris cromosòmics (Zalensky *et al.*, 1995; Zalenskaya *et al.*, 2000; Gawecka *et al.*, 2015; Pantano *et al.*, 2015; Skinner *et al.*, 2018). La caracterització mitjançant espectrometria de masses del proteoma nuclear d'espermatozoides de *Dicentrarchus labrax* (Barrachina *et al.*, 2018a), escollit com a model representatiu d'espècie vertebrada

[2] La informació inclosa en aquesta secció de la Introducció es basa en l'article **Barrachina F, Anastasiadi D, Jodar M, Castillo J, Estanyol JM, Piferrer F, Oliva R (2018) Identification of a complex population of chromatin-associated proteins in the European sea bass (Dicentrarchus labrax) sperm. Syst Biol Reprod Med 64,502–517** que es troba a l'Annex 2.

no mamífer amb protamines, i la seva comparació amb els proteomes d'espermatozoides ja descrits d'altres espècies de vertebrats (*O. Mykiss*, *M. Musculus* i *H. Sapiens*), ha revelat que les proteïnes de la cromatina espermàtica es troben més conservades entre vertebrats del que prèviament es creia (**Taula 1**), el que suggereix el seu potencial paper crític en la funcionalitat de l'espermatozoide. Els resultats extrets d'aquest estudi proporcionen informació filogenèticament estratègica, ja que demostren que la coexistència d'histones, protamines i altres proteïnes de la cromatina espermàtica en l'espermatozoide no és exclusiu dels mamífers, el que suggereix que el marcatge epigenètic de la cromatina espermàtica podria ser més fonamental i conservat del que anteriorment es pensava (Barrachina *et al.*, 2018a).

Taula 1. Conservació de les proteïnes espermàtiques del *Dicentrarchus labrax* relacionades amb la cromatina en altres espècies de vertebrats. El nombre de proteïnes relacionades amb la cromatina en l'espermatozoide del *D. labrax* s'ha dividit per categories i comparat amb els ortòlegs de l'espermatozoide de la truita arc de Sant Martí (*Oncorhynchus mykiss*), ratolí (*Mus musculus*) i l'humà (*Homo sapiens*) Font: Modificat de (Barrachina *et al.*, 2018a)

Nombre de proteïnes espermàtiques de *D. labrax* relacionades amb la cromatina (n = 94)		**Número d'ortòlegs identificats en altres espècies**		
		O. mykiss	***M. musculus***	***H. sapiens***
Histones i variants d'histones	n = 9	3	6	6
Modificadors d'histones	n = 8	3	7	8
Zinc fingers i factors de transcripció	n = 15	2	7	10
Proteïnes centrosòmiques	n = 27	4	17	23
Altres proteïnes relacionades amb la cromatina	n = 35	6	31	33

1.2.3. Regulació hormonal de l'espermatogènesi [3]

Els testicles, a més de produir espermatozoides per mitjà de l'espermatogènesi, també realitzen una funció endocrina molt important, que és la síntesi de les hormones sexuals masculines (andrògens), sent la més abundant la testosterona (Tournaye *et al.*, 2017a). Aquestes dues funcions estan controlades per l'activitat secretora de l'hipotàlem i de la hipòfisi o glàndula pituïtària anterior, que participen en un circuit anomenat eix hipotàlem-hipòfisi-testicular. Aquest eix és el responsable de la regulació de la funció testicular en un context eficient i altament regulat per hormones.

[3] Per a un major aprofundiment veure l'article **Arato I*, Grande G*, Barrachina F*, Bellucci C, Lilli C, Jodar M, Aglietti MC, Mancini F, Vincenzoni F, Pontecorvi A, Calafiore R, Oliva R, Luca G, Mancuso F and Milardi D (2020) "In vitro" Effect of Different Follicle—Stimulating Hormone Preparations on Sertoli Cells: Toward a Personalized Treatment for Male Infertility. Front Endocrinol 11:401** que es troba a l'Annex 3.

L'hipotàlem secreta el factor alliberador de gonadotropines (GnRH), el qual s'uneix als receptors de GnRH presents en la hipòfisi anterior i estimula l'alliberament de l'hormona luteïnitzant (LH) i l'hormona fol·liculoestimulant (FSH) (**Figura 7**) (Pohl and Knobil, 1982; Seeburg *et al.*, 1987; Nieschlag *et al.*, 1999; Jin and Yang, 2014). Aquestes hormones són

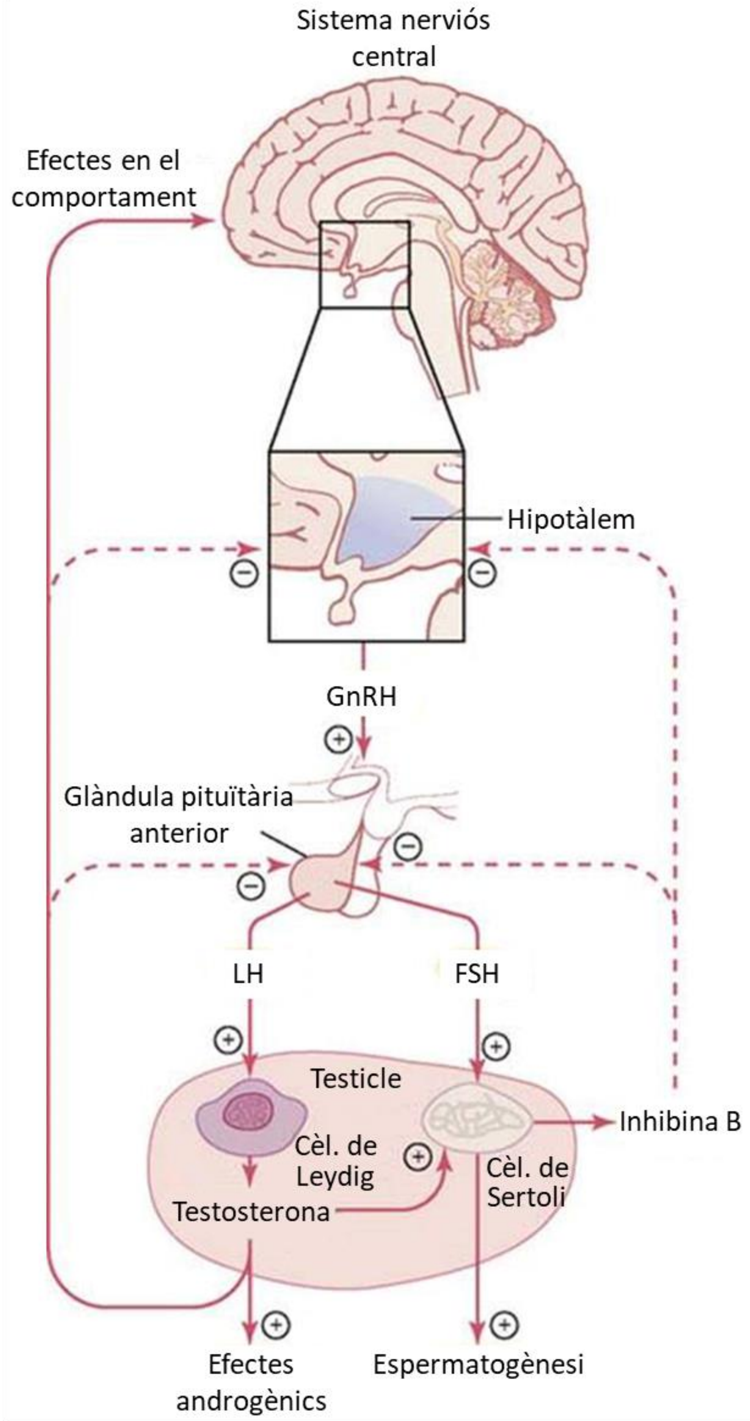

Figura 7. Regulació de la funció testicular per l'eix hipotàlem-hipòfisi-testicular. Els efectes estimuladors es representen amb el símbol "+", mentre que la retroalimentació o feedback negatiu es representa amb el símbol "–". Font: Modificat de (Guyton and Hall, 2011).

glicoproteïnes heterodimèriques compostes per dues subunitats, α i β. La subunitat α és idèntica per a la FSH i la LH, mentre que la subunitat β és la que les diferencia, i la que els hi confereix la capacitat d'interaccionar amb els seus receptors específics (Jin and Yang, 2014).

Ambdues hormones gonadotròpiques (FSH i LH) actuen en l'àmbit testicular (**Figura 7**). Per una banda, l'hormona FSH s'uneix als receptors FSH (FSH-R), els quals només s'expressen en les cèl·lules de Sertoli, i resulta en la transcripció de gens relacionats amb l'espermatogènesi i en la producció de la proteïna transportadora d'andrògens (ABP), la qual s'uneix a la testosterona i l'acumula en el fluid dels túbuls seminífers, i activen l'espermatogènesi (Hansson *et al.*, 1976; Plant and Marshall, 2001; Ruwanpura *et al.*, 2010; Guyton and Hall, 2011). De tal manera que les cèl·lules de Sertoli proporcionen uns factors claus per a la correcta progressió i diferenciació d'espermatogònies cap a espermatozoides. Per altra banda, l'hormona LH s'uneix als receptors específics presents a les cèl·lules de Leydig i promou la producció de testosterona (Ruwanpura *et al.*, 2010; Guyton and Hall, 2011; Jin and Yang, 2014). L'increment de testosterona intratesticular generada per les cèl·lules de Leydig s'uneix als receptors d'andrògens presents en les cèl·lules de Sertoli, i promou la maduració de les cèl·lules germinals. Així doncs, tant la FSH com la testosterona són essencials per iniciar el procés d'espermatogènesi durant la maduració sexual (Spiteri-Grech and Nieschlag, 1993; Plant and Marshall, 2001). A més, fora dels testicles, la testosterona és alliberada a la circulació sanguínia i té efectes en tots els teixits, sent especialment rellevant en el desenvolupament i manteniment dels caràcters sexuals secundaris masculins (Wilson and Davies, 2007; Jin and Yang, 2014).

L'eix hipotàlem-hipòfisi-testicular es troba regulat per la retroalimentació negativa que generen dues hormones secretades pel testicle: la testosterona i la inhibina B (**Figura 7**) (Plant and Marshall, 2001; Tilbrook and Clarke, 2001; Guyton and Hall, 2011). La testosterona és produïda per cèl·lules de Leydig prèviament estimulades per LH. La inhibina B és generada per les cèl·lules de Sertoli sota l'estimulació produïda per la FSH (Spiteri-Grech and Nieschlag, 1993). Ambdues hormones (testosterona i inhibina B) actuen directament a l'hipotàlem per a disminuir la secreció de gonadotropines mitjançant la reducció de producció de GnRH per part de l'hipotàlem, així com la reducció de la sensitivitat de la glàndula pituïtària envers l'estimulació produïda per l'hormona GnRH. A més, la testosterona i la inhibina B circulants en sang regulen negativament la secreció de FSH i LH de la glàndula pituïtària anterior.

1.3. EL PLASMA SEMINAL

El semen és un fluid corporal complex que conté milions d'espermatozoides suspesos en secrecions provinents del testicle i de l'epidídim i que, en el moment de l'ejaculació, es barreja amb secrecions provinents de les glàndules sexuals accessòries com la pròstata, les vesícules seminals i les glàndules bulbouretrals (Jodar *et al.*, 2017c). Aquest conjunt de secrecions s'anomena plasma seminal i constitueix el 95% del volum del semen, mentre que els espermatozoides tan sols representen el 5% del volum restant (Barrachina *et al.*, 2019).

Antigament s'havia assumit que el plasma seminal tan sols era un mitjà de transport i nutrició per a l'espermatozoide (Suarez and Wolfner, 2017). No obstant, actualment s'ha demostrat que el plasma seminal, conjuntament amb la participació d'una alta població de vesícules extracel·lulars (VEs) secretades per les diverses glàndules del tracte reproductor masculí, modulen rols importants tals com la funcionalitat de l'espermatozoide i l'adquisició del seu potencial de fertilització (Bromfield, 2014; Drabovich *et al.*, 2014; Chow *et al.*, 2015; Sullivan, 2016; Jodar *et al.*, 2017c; Björkgren and Sipilä, 2019). De fet, l'espermatozoide resultant de l'espermatogènesi, anomenat espermatozoide testicular, tot i ser morfològicament diferenciat, és una cèl·lula immadura i immòbil que és incapaç de fertilitzar l'oòcit pels seus propis medis (Jodar *et al.*, 2017c). La interacció del plasma seminal amb els espermatozoides és crucial per la maduració de l'espermatozoide, l'adquisició de la mobilitat i el potencial de capacitació, la prevenció d'una reacció acrosòmica prematura i per al reconeixement i fusió de l'espermatozoide amb la zona pel·lúcida de l'oòcit (Poiani, 2006; Sullivan, 2016; Jodar *et al.*, 2017c; Barrachina *et al.*, 2019; Björkgren and Sipilä, 2019). Addicionalment, s'ha observat que el contacte del plasma seminal amb el tracte reproductor femení proporciona un entorn òptim per a la implantació de l'embrió i el seu desenvolupament, i contribueix a l'adquisició dels mecanismes de tolerància immunitària materna envers el fetus semial·logènic (Gillott, 2003; Robertson, 2005; Rodríguez-Martínez *et al.*, 2011; Dorus *et al.*, 2012; Aitken and Baker, 2013; Robertson and Sharkey, 2016; Schjenken and Robertson, 2020).

1.3.1. Composició del plasma seminal

El plasma seminal està conformat per les secrecions de diverses glàndules que es troben al llarg del tracte reproductor masculí (Drabovich *et al.*, 2014; Barrachina *et al.*, 2019). Tanmateix, la seva contribució a la composició del plasma seminal difereix entre si. El plasma seminal està compost per les secrecions dels següents òrgans/glàndules (**Figura 8**):

- **Testicle** (1-2%): La contribució de les secrecions del testicle al plasma seminal és molt escassa, ja que tan sols representa l'1-2% d'aquest. La informació de la

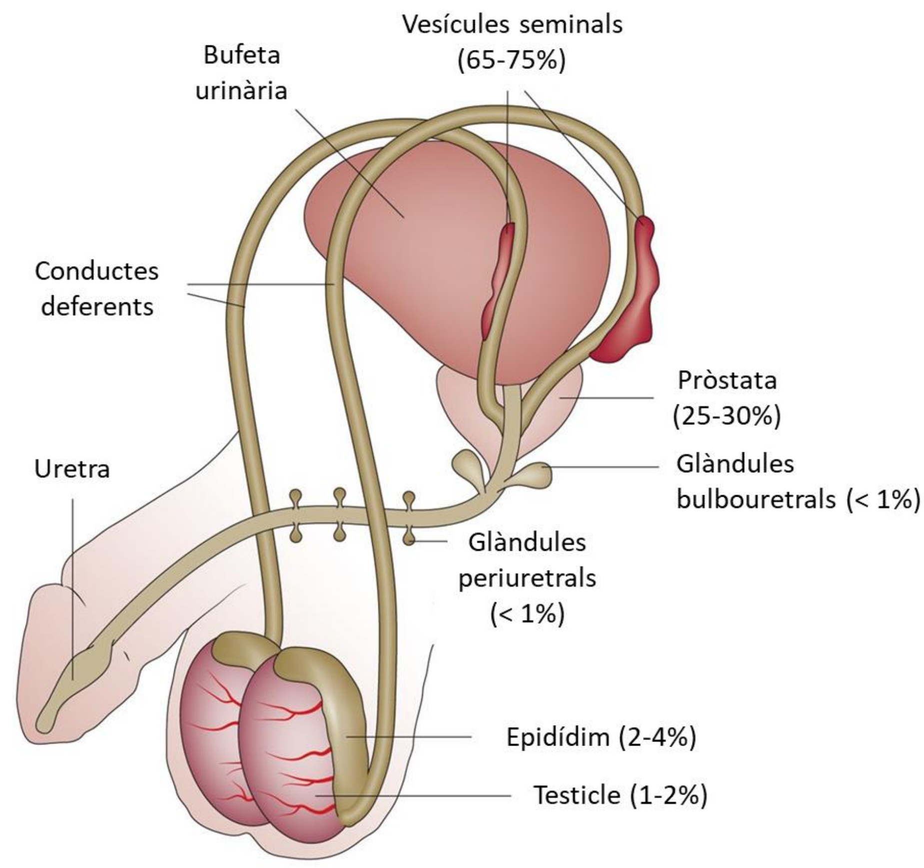

Figura 8. Participació dels diferents òrgans en la composició del plasma seminal en humans. Font: Modificat de (Drabovich *et al.*, 2014).

participació dels components testiculars presents al plasma seminal no són gaire coneguts. Algunes de les molècules identificades al plasma seminal corresponen a marcadors de cèl·lules germinals, el que podria correspondre a detritus o estructures provinents de l'espermatogènesi, o podria provenir de la gota citoplasmàtica de l'espermatozoide.

- **L'epidídim** (2-4%): El rol principal de les secrecions produïdes per les cèl·lules epitelials de l'epidídim és el de crear un ambient òptim per a la maduració i emmagatzematge dels espermatozoides durant el seu trànsit per l'epidídim. Les secrecions de l'epidídim, conjuntament amb les VEs secretades (anomenades epididimosomes) són de gran importància per a la maduració posttesticular de l'espermatozoide.

- **Les glàndules sexuals accessòries**: Les secrecions d'aquest conjunt de glàndules també contribueixen a la composició del plasma seminal.

  - **Pròstata** (25-30%): Les secrecions de la pròstata contenen una gran varietat d'enzims proteolítics i electròlits, i van acompanyades de VEs anomenades prostasomes. Les secrecions prostàtiques participen en la coagulació i la posterior liqüefacció del semen, prevenen una reacció acrosòmica de

l'espermatozoide i una capacitació prematures, i estimulen la mobilitat espermàtica.

- **Vesícules seminals** (65-75%): El principal contribuent del plasma seminal són les secrecions de les vesícules seminals. Les secrecions d'aquesta glàndula són riques en fructosa, una substància altament nutritiva que és una font d'energia per als espermatozoides, i també contenen prostaglandines, components que participen en la coagulació del semen i factors antioxidants.
- **Glàndules bulbouretrals i periuretrals** (<1%): Les seves secrecions estan conformades per substàncies mucoides que recobreixen la uretra per tal de lubricar-la i protegir-la.

1.3.2. Secrecions de l'epidídim: Participació en la maduració posttesticular de l'espermatozoide

L'epidídim és un òrgan tubular altament contornejat, d'uns 6-7 metres de longitud, que es localitza a la part superior dels testicles, i que es troba entre el testicle i el conducte deferent. Després d'haver estat produïts pels testicles, els espermatozoides testiculars, els quals són funcionalment immadurs, s'emmagatzemen fins a dues setmanes a l'epidídim (Gadea *et al.*, 2013; Breton *et al.*, 2016).

Durant el trànsit per l'epidídim, els espermatozoides entren en contacte amb el fluid epididimari, sent crucial per a la maduració posttesticular de l'espermatozoide, ja que contribueix a l'adquisició de la mobilitat espermàtica i del potencial de fertilització que li permetrà interaccionar eficientment amb l'oòcit i fecundar-lo (Sullivan and Belleannée, 2017; Zhou *et al.*, 2018). Aquestes modificacions posttesticulars succeeixen en una cèl·lula que és transcripcionalment i traduccionalment inactiva a causa de l'alta compactació de la cromatina de l'espermatozoide i de l'extrusió de la majoria del seu citoplasma (Dacheux *et al.*, 2012; Castillo *et al.*, 2015; Jodar *et al.*, 2016, 2017c; Barrachina *et al.*, 2018b). Per tant, aquesta transformació funcional posttesticular és atribuïda a l'adquisició per part de l'espermatozoide de components presents en el fluid epididimari, així com per la futura interacció que l'espermatozoide tindrà amb les secrecions de les diverses glàndules sexuals accessòries en el moment de l'ejaculació (Reilly *et al.*, 2016; Jodar *et al.*, 2017c; Zhou *et al.*, 2018; Barrachina *et al.*, 2019; Hernández-Silva and Chirinos, 2019). En els últims anys també estan agafant molta importància la presència d'una alta i heterogènia població de VEs secretades per les cèl·lules epitelials de l'epidídim, les quals són anomenades epididimosomes, com possibles responsables d'aquesta maduració de l'espermatozoide en l'epidídim (Yanagimachi *et al.*, 1985; Sullivan *et al.*, 2007; Sullivan and Saez, 2013; Sullivan, 2015; Gervasi and Visconti, 2017).

1.3.2.1. Anatomia de l'epidídim

Anatòmicament, l'epidídim en mamífers es sol dividir en tres regions principals: el cap (en anglès *caput*), el cos (en anglès *corpus*) i la cua (en anglès *cauda*) de l'epidídim (Belleannée *et al.*, 2013a; Breton *et al.*, 2016). Tot i que l'anatomia de l'epidídim mostra variabilitat entre espècies de mamífers, els tres segments principals es poden visualitzar fàcilment en la majoria de mamífers. Addicionalment, l'epidídim es pot subdividir en 10-19 subcompartiments que presenten unes característiques funcionals diferents, com s'ha prèviament descrit (Turner *et al.*, 2003, 2007a, 2007b; Johnston *et al.*, 2005; Jelinsky *et al.*, 2007; Dacheux *et al.*, 2016). En els humans, l'epidídim sembla estar poc diferenciat, ja que el cap de l'epidídim està format per conductes eferents, i la cua de l'epidídim es troba poc desenvolupada i amb capacitat limitada per emmagatzemar espermatozoides (Sullivan and Mieusset, 2016). En espècies de rosegadors, en la regió proximal del cap de l'epidídim es troba el segment inicial (**Figura 9**) (Cornwall, 2009).

Les diferents regions de l'epidídim presenten unes característiques funcionals molt especialitzades, que contribueixen a crear un perfil luminal diferenciat dins de cada regió, el que és crucial per a la maduració posttesticular de l'espermatozoide a l'epidídim (Cornwall, 2009; Zhou *et al.*, 2018). A causa de la necessitat d'un major nombre d'estudis que permetin un major aprofundiment en la caracterització de la fisiologia de l'epidídim humà, a continuació es descriuen les diferents regions de l'epidídim clarament definides en rosegadors (**Figura 9**):

- **Segment inicial**: En aquesta regió de l'epidídim es produeix l'absorció de la majoria del fluid testicular que arriba a l'epidídim. Aproximadament, més del 95% d'aquest fluid és absorbit en el seu pas pels conductes eferents i el segment inicial (Glover and Barratt, 2004). Aquesta disminució del fluid dóna lloc a un increment de la concentració dels espermatozoides (Abe *et al.*, 1984; Glover and Barratt, 2004; Nicholson and Assinder, 2009; Sullivan and Mieusset, 2016). A més, es creu que el segment inicial és altament actiu en la síntesi de proteïnes i que juga un paper important en la maduració de l'espermatozoide (Cornwall *et al.*, 2002; Sullivan and Belleannée, 2017).
- **Cap de l'epidídim**: Aquesta regió és activa en la síntesi i secreció de proteïnes (Zhou *et al.*, 2018). Els espermatozoides que circulen per aquesta regió comencen a adquirir la mobilitat espermàtica i mostrar l'habilitat de reconèixer els oòcits (Aitken *et al.*, 2007).
- **Cos de l'epidídim**: Tot i ser una regió diferenciada, en el cos de l'epidídim es continua produint la transformació funcional iniciada en el cap de l'epidídim. La mobilitat espermàtica que comença a observar-se en el cap, s'acaba d'adquirir en el cos de l'epidídim (Aitken *et al.*, 2007). A més, la capacitat dels espermatozoides de presentar un moviment hiperactiu en resposta a un estímul cAMP comença a

presentar-se en el cos de l'epidídim, i va augmentant progressivament fins a assolir la cua de l'epidídim (Aitken *et al.*, 2007).

- **Cua de l'epidídim**: Aquesta regió presenta un lumen molt més ample (fins a 100 vegades més ample que en el cap de l'epidídim), preparat per actuar com a reservori d'espermatozoides, els quals han anat madurant durant el seu pas pel cap i el cos de l'epidídim (Cornwall, 2009; Sullivan and Belleannée, 2017). Les cèl·lules epitelials de la cua de l'epidídim mostren una alta activitat d'absorció i endocitosi (Hermo *et al.*, 1988; Robaire *et al.*, 2006). Per contra, en aquest segment de l'epidídim la síntesi de proteïnes i la seva secreció és baixa, el que correlaciona amb el gruix reduït de l'epiteli que revesteix el lumen epididimari.

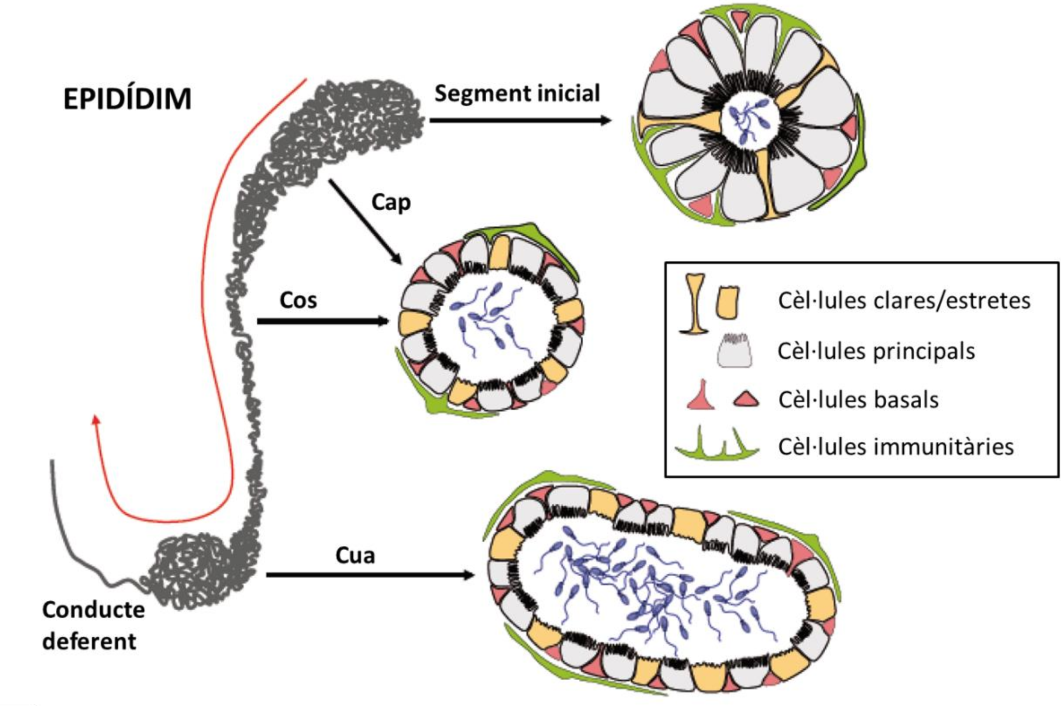

Figura 9. Il·lustració esquemàtica de l'anatomia de l'epidídim on es representen les principals regions i els diferents tipus cel·lulars que componen l'epidídim murí. Font: Modificat de (Shum *et al.*, 2009).

Les diferències funcionals de les diferents regions de l'epidídim s'han confirmat mitjançant estudis de proteòmica i de transcriptòmica, on s'ha pogut comprovar que els diferents segments de l'epidídim, així com el fluid epididimari secretat, presenten uns perfils moleculars diferents (Kirchhoff, 1999, 2002b, 2002a; Dacheux *et al.*, 2003, 2006, 2009; Johnston *et al.*, 2005; Turner *et al.*, 2006; Zhang *et al.*, 2006; Thimon *et al.*, 2007; Dubé *et al.*, 2007; Jelinsky *et al.*, 2007; Li *et al.*, 2010a; Belleannée *et al.*, 2012; Liu and Liu, 2015). Aquesta expressió diferencial dels diferents segments de l'epidídim manté un microambient luminal que canvia progressivament dins del túbul de l'epidídim, i que

impulsa la maduració seqüencial dels espermatozoides durant el seu pas per l'epidídim (Cornwall, 2009; Sullivan and Mieusset, 2016; Sullivan and Belleannée, 2017).

1.3.2.2. Epiteli de l'epidídim

En mamífers, l'epidídim està format per un epiteli pseudoestratificat compost per cèl·lules principals, cèl·lules clares, cèl·lules basals i cèl·lules del sistema immunitari, i cadascun d'aquests tipus cel·lulars contribueix de manera única a la creació del microambient luminal de l'epidídim (Cornwall, 2009; Breton *et al.*, 2016; Zhou *et al.*, 2018). Ara bé, el segment inicial, clarament definit en rosegadors, es diferencia significativament de la resta de l'epidídim, ja que el seu epiteli està format per cèl·lules cuboides (Sullivan and Belleannée, 2017). Les cèl·lules epitelials de l'epidídim es troben adherides unes amb les altres per unions estretes (en anglès *tight junctions*), creant un compartiment intraluminal aïllat i conformat la barrera hematoepididimària (Sullivan and Belleannée, 2017; Breton *et al.*, 2019). La funció dels principals tipus cel·lulars a l'epidídim es troba descrita a continuació:

- **Cèl·lules principals**: Les cèl·lules principals (PC, de l'anglès *Principal cells*) representen aproximadament el 85% de les cèl·lules que constitueixen l'epiteli de l'epidídim (**Figura 10**). En les regions proximals de l'epidídim (cap i cos de l'epidídim), aquest tipus cel·lular és particularment actiu en la biosíntesi i secreció de proteïnes, i és per aquest motiu que les PC tenen una alta abundància de reticle endoplasmàtic, aparell de Golgi i grànuls secretors, el que reflexa la seva alta activitat d'exocitosi (Robaire *et al.*, 2006; Cornwall, 2009; Zhou *et al.*, 2018). A més, en el segment inicial les PC reabsorbeixen bicarbonat. En la regió més distal de l'epidídim (cua de l'epidídim), les PC passen a tenir un rol d'endocitosi essent, conjuntament amb les cèl·lules clares, responsables de la reabsorció del fluid epididimari (Robaire *et al.*, 2006; Zhou *et al.*, 2018), i en aquesta regió les PC tenen la funció tant de secretar bicarbonat com protons (Breton *et al.*, 2019). Les PC també estan implicades en la comunicació intercel·lular amb els espermatozoides, ja que alliberen mitjançant secreció apocrina unes estructures anomenades *apical blebs*, les quals es desintegren en el lumen de l'epidídim i alliberen els epididimosomes (Rejraji *et al.*, 2006; Sullivan, 2016; Gervasi and Visconti, 2017).
- **Cèl·lules clares o cèl·lules estretes**: Les cèl·lules clares (CC, de l'anglès *Clear cells*) o cèl·lules estretes (NC, de l'anglès *Narrow cells*) són cèl·lules que secreten protons al lumen de l'epidídim mitjançant la bomba de protons V-ATPasa, que es localitza en la seva membrana apical, i tenen la funció d'acidificar el fluid intraluminal i regular el pH luminal (**Figura 10**) (Cornwall, 2009; Breton and Brown, 2013; Breton *et al.*, 2016, 2019). Aquesta secreció de H^+ requereix d'hidròlisi d'ATP, i és per aquest motiu que ambdues CC i NC tenen un alt contingut de mitocòndries. A més, ambdues cèl·lules són les cèl·lules responsables de l'absorció selectiva que es

produeix a l'epidídim, ja que tenen una alta capacitat d'endocitosi (Hermo *et al.*, 1988; Brown *et al.*, 1997; Breton *et al.*, 2016; Zhou *et al.*, 2018). Les NC tenen una forma característica de copa de cava i només es localitzen al segment inicial de l'epidídim, mentre que les CC tenen forma cilíndrica i es localitzen al cap, cos i cua de l'epidídim (Breton *et al.*, 2016).

- **Cèl·lules basals**: Les cèl·lules basals (BC, de l'anglès *Basal cells*) es troben a la base de l'epiteli pseusoestratificat de l'epidídim, pel que no estan en contacte directe amb el lumen de l'epidídim (**Figura 10**). No obstant, les BC envien unes projeccions citoplasmàtiques anomenades axopodis que passen entre les cèl·lules epitelials i arriben al lumen, on actuen com a sensors del compartiment intraluminal (Shum *et al.*, 2008; Roy *et al.*, 2016). A més, les BC també estan involucrades en les comunicacions intercel·lulars, ja que es troben en contacte íntim amb la membrana basal i les PC (Zhou *et al.*, 2018). També es pensa que tenen l'habilitat de modificar la seva forma per tal d'ajustar el volum i la pressió del lumen de l'epidídim.
- **Cèl·lules del sistema immunitari**: Les cèl·lules que componen el sistema immunitari de l'epidídim són macròfags, cèl·lules dendrítiques, monòcits i limfòcits tipus B i T (Da Silva et al., 2011; Da Silva and Smith, 2015; Voisin et al., 2018; Breton et al., 2019; Guiton et al., 2019). El correcte funcionament d'aquestes cèl·lules és especialment rellevant per la fertilitat masculina, ja que en l'epidídim aquestes cèl·lules tenen la capacitat de prevenir una resposta immunològica envers l'espermatozoide autoantigènic, mentre que han de mantenir una resposta immune eficient en contra dels patògens o cèl·lules canceroses (Voisin *et al.*, 2018, 2019; Battistone *et al.*, 2019, 2020; Breton *et al.*, 2019).

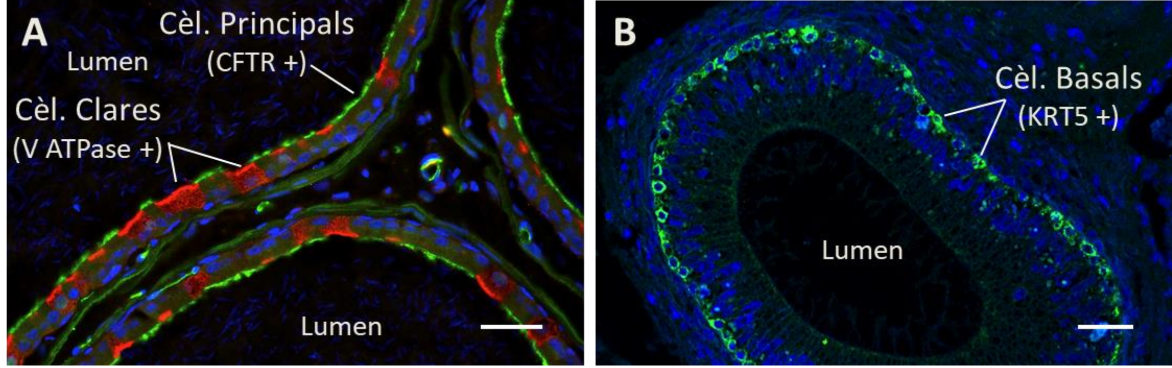

Figura 10. Visualització per microscòpia confocal dels diferents tipus de cèl·lules epitelials que conformen l'epidídim. A) Secció d'epidídim de rata on s'observen cèl·lules principals (en verd; cèl·lules positives en CFTR) i cèl·lules clares (en vermell, cèl·lules positives en V ATPase). B) Secció d'epidídim d'humà on s'observen cèl·lules basals (en verd, cèl·lules positives en KRT5). Barres: 20 µm. Font: A) Breton S (Resultats no publicats), B) Barrachina F, Battistone MA, Castillo J, Mallofré C, Jodar M, Oliva R, Breton S (Resultats no publicats).

El resultat d'aquesta activitat secretora i/o absortiva portada a terme per les cèl·lules epitelials en les diferents regions de l'epidídim resulta en la creació d'un microambient luminal únic i altament especialitzat que promou la maduració seqüencial de l'espermatozoide i el seu transport, concentració, protecció i emmagatzematge (Cornwall *et al.*, 2002; Breton *et al.*, 2016, 2019; Sullivan and Mieusset, 2016).

1.3.2.3. Rol de l'epidídim en la maduració de l'espermatozoide

En el moment en el qual l'espermatozoide testicular, una cèl·lula que encara és immadura i immòbil, entra a l'epidídim, comença el procés de maduració posttesticular que culminarà amb l'adquisició de la mobilitat progressiva i del potencial de fertilització. Aquest procés de maduració es duu a terme en un gàmeta masculí transcripcionalment i traduccionalment inactiu. Durant aquesta maduració al llarg de l'epidídim, els espermatozoides entren en un procés de remodelació on perden o modifiquen algunes de les seves molècules (proteïnes, lípids o RNAs), mentre que n'adquireixen d'altres que es troben en el líquid epididimari o encapsulades en vesícules (Jones, 1998; Bedford, 2004; Dacheux and Dacheux, 2014; Belleannée, 2015; Dacheux *et al.*, 2016; Sullivan, 2016; Sullivan and Mieusset, 2016; Trigg *et al.*, 2019). Com que la majoria dels components del fluid testicular han sigut reabsorbits pel seu pas pel segment proximal de l'epidídim, la majoria dels components presents en el fluid epididimari, incloent-hi tant les molècules com les vesícules, provenen de les cèl·lules epitelials de l'epidídim (Dacheux *et al.*, 2016). Aquestes modificacions en els espermatozoides succeeixen de manera coordinada en les diferents regions de l'epidídim. Com que l'epidídim està altament regionalitzat, els espermatozoides es troben amb el líquid luminal de manera seqüencial, ja que cada regió de l'epidídim té el seu propi microambient (Dacheux and Dacheux, 2014; Sullivan and Mieusset, 2016), i és per aquest motiu que el procés de maduració de l'espermatozoide és progressiu depenent de quina regió de l'epidídim l'espermatozoide es troba (Bedford, 2004).

Aquest intercanvi d'informació entre les secrecions de l'epiteli de l'epidídim i l'espermatozoide que resulta en la seva maduració podria estar relacionat amb el contacte directe de l'espermatozoide amb l'epidídim, a la transferència de complexos proteics o a la interacció amb els epididimosomes alliberats en el fluid epididimari (Martin-DeLeon, 2015; Sullivan, 2015; Dacheux *et al.*, 2016).

Els epididimosomes són una àmplia i heterogènia població de VEs, les quals són secretades per les cèl·lules principals de l'epiteli de l'epidídim mitjançant secreció apocrina (Veure *Secció 1.4.2.1. Vesícules extracel·lulars de l'epidídim: Els epididimosomes*) (Caballero *et al.*, 2010; Sullivan and Saez, 2013; Belleannée, 2015; Martin-DeLeon, 2015; Sullivan, 2016). Les VEs es troben en molts fluids biològics, i semblen estar involucrades en la comunicació intercel·lular. A més, en participar en el trànsit de proteïnes i RNAs, s'està intentant desxifrar el seu contingut molecular per tal saber quin és el seu paper en la

maduració posttesticular i en l'adquisició de les funcions fisiològiques de l'espermatozoide.

Per altra banda, en comparació amb altres fluids corporals, el lumen de l'epidídim té una composició característica i conté nivells baixos de Na^+ i Cl^-, i nivells alts de K^+ (Visconti *et al.*, 2011). A més, aquest fluid epididimari presenta un pH àcid a causa de la secreció de H^+ i a l'absorció de HCO_3^- per les cèl·lules epitelials de l'epidídim. Aquests canvis són deguts a la presència de canals en les cèl·lules epitelials de l'epidídim que permeten el moviment transepitelial d'ions, modificant la composició iònica del fluid epididimari (Da Silva *et al.*, 2006). La primera conseqüència és que s'estableix un microambient luminal àcid que té alta importància per a la maduració de l'espermatozoide, ja que manté a l'espermatozoide funcionalment competent però quiescent, gràcies a la inhibició dels canals o transportadors presents a l'espermatozoide (Navarro *et al.*, 2008; Visconti *et al.*, 2011) i, a més, facilita la transferència de proteïnes des de les cèl·lules epitelials de l'epidídim fins als espermatozoides mitjançant epididimosomes (Zhou *et al.*, 2018). La segona conseqüència és que es produeix una absorció d'aigua del fluid luminal, de tal manera que la concentració d'espermatozoides incrementa de manera espectacular, passant d'una concentració de 10^6 espermatozoides/ml en la *rete testis* a una concentració de 10^9 en la cua de l'epidídim (Dacheux and Dacheux, 2014; Dacheux *et al.*, 2016). La tercera conseqüència és que la composició proteica del medi luminal també varia substancialment com a resultat de la reabsorció d'aigua produïda en la regió proximal de l'epidídim i per l'alta producció i secreció de proteïnes (Abe *et al.*, 1984; Cornwall *et al.*, 2002; Glover and Barratt, 2004; Nicholson and Assinder, 2009; Sullivan and Mieusset, 2016; Sullivan and Belleannée, 2017). Per tant, mentre que el fluid testicular té una concentració proteica de 3,4 mg/mL, en la cua de l'epidídim aquesta és de 30 mg/mL (Hinton *et al.*, 1981; Cooper *et al.*, 1992).

Les funcions principals dutes a terme per l'epidídim i el fluid epididimari són:

- Actuar com a mitjà de transport per als espermatozoides, on el mecanisme responsable de conduir els espermatozoides a través del lumen de l'epidídim són les contraccions musculars rítmiques del múscul llis que recobreix l'epidídim (Robaire *et al.*, 2006; Elfgen *et al.*, 2018).
- La maduració de l'espermatozoide, que permet l'adquisició de l'habilitat de fertilització. Durant el trànsit dels espermatozoides per l'epidídim, aquests es tornen funcionalment madurs, ja que adquireixen l'habilitat de moure's progressivament per ascendir al llarg del tracte genital femení, així com el potencial per a produir la reacció acrosòmica i interaccionar amb els oòcits (unió i penetració dels oòcits). Específicament:
    - Un canvi a causa de la maduració de l'espermatozoide en l'epidídim és l'adquisició del potencial de mobilitat espermàtica, que inclou tant

l'increment quantitatiu del percentatge d'espermatozoides mòbils com la millora qualitativa del patró de mobilitat (Yeung *et al.*, 1993). Mentre que els espermatozoides testiculars són immòbils o presenten un mínim moviment del flagel, els espermatozoides obtinguts del cap de l'epidídim presenten un patró de mobilitat circular, i els espermatozoides de la cua de l'epidídim presenten una mobilitat progressiva i vigorosa (Dacheux and Dacheux, 2014).

- S'adquireix també la capacitat de l'espermatozoide per a realitzar la reacció acrosòmica, reconèixer i unir-se a la zona pel·lúcida i fusionar-se amb la membrana vitel·lina de l'oòcit (Hinrichsen and Blaquier, 1980; Saling, 1982; Moore *et al.*, 1983; Lakoski *et al.*, 1988; Yeung *et al.*, 1997; Burkin and Miller, 2000; Dacheux and Dacheux, 2014).
- Altres canvis durant la maduració espermàtica inclouen canvis estructurals i morfològics en la cèl·lula espermàtica, tals com la migració i desaparició de la gota citoplasmàtica al llarg del flagel, canvis en la membrana plasmàtica de l'espermatozoide i canvis subtils en el contingut de l'acrosoma (Bedford, 2004; Dacheux and Dacheux, 2014). A més, també hi ha canvis en la cromatina del nucli de l'espermatozoide ja que hi ha un augment de ponts disulfur en l'espermatozoide per tal de protegir a la cèl·lula de l'estrès oxidatiu postejaculació (Oliva, 2006), i la condensació de la cromatina espermàtica s'accentua (Haidl *et al.*, 1994). Addicionalment, a causa de l'increment de l'osmolaritat del fluid epididimari, l'espermatozoide es deshidrata i la dimensió del seu cap disminueix (Hinton *et al.*, 1981; Cooper and Yeung, 2003).

- La regió més distal de l'epidídim (cua de l'epidídim) té la particularitat d'actuar com a emmagatzematge dels espermatozoides epididimaris, els quals s'han de mantenir viables però en estat quiescent (Bedford, 2004). En mamífers, el trànsit dels espermatozoides per la cua de l'epidídim dura entre 3 i 10 dies. No obstant això, els espermatozoides poden romandre emmagatzemats a l'epidídim fins a 30 dies (Robaire *et al.*, 2006).
- Un altre rol de l'epidídim és la creació d'una barrera que protegeixi als espermatozoides, l'anomenada barrera hematoepididimària (Sullivan and Belleannée, 2017; Breton *et al.*, 2019). Aquesta barrera permet que es pugui produir i mantenir un microambient luminal especialitzat per la maduració dels espermatozoides. A més, aquesta barrera protegeix als espermatozoides del sistema immune, de xenobiòtics nocius i d'espècies reactives d'oxigen provinents de l'epidídim.

1.3.3. Secrecions de les glàndules sexuals accessòries

En el moment de l'ejaculació, els espermatozoides van des de l'epidídim i a través del conducte deferent fins a arribar a l'ampul·la del conducte deferent, on s'afegiran les secrecions de les vesícules seminals. Posteriorment, el líquid seminal circularà pel conducte ejaculador cap a la uretra, i durant aquest camí s'afegeixen les secrecions de la pròstata i de les glàndules bulbouretrals (glàndules de Cowper) i periuretrals (glàndules de Littre), fins a finalment ser ejaculat fora del cos (Jodar *et al.*, 2017c). Per tant, durant el procés d'ejaculació els espermatozoides estan en contacte amb les secrecions de les glàndules sexuals accessòries, i el fluid resultant serà l'anomenat plasma seminal.

1.3.3.1. Vesícules seminals

Les vesícules seminals són dues glàndules que arriben a produir el 65-75% del volum del plasma seminal, pel que són el principal contribuent del plasma seminal (Aumüller and Riva, 2009; Barrachina *et al.*, 2019). En el moment de l'ejaculació, les vesícules seminals expulsen el seu contingut al conducte ejaculador just quan l'epidídim expulsa els espermatozoides.

El fluid secretat per les vesícules seminals és de color groguenc, viscós i alcalí. Aquest fluid conté grans quantitats de fructosa, que és utilitzada per les mitocòndries dels espermatozoides per tal de generar ATP per permetre el seu moviment a través del tracte reproductor femení fins a arribar a l'oòcit (Gonzales, 1989, 2001). Les secrecions de les vesícules seminals, en ser alcalines, actuen com a tampó envers l'ambient acídic que es troben els espermatozoides en l'aparell reproductor femení (pH de 3,5-4). A més, el fluid de les vesícules seminals també conté altres substancies tals com proteïnes, prostaglandines, fibrinogen, fibronectina, flavoproteïnes i àcid ascòrbic (Gonzales, 2001; Aumüller and Riva, 2009; Flint *et al.*, 2015). Les prostaglandines secretades estimulen les contraccions del múscul llis tant de l'aparell reproductor masculí com del femení, pel que són les responsables de promoure el transport dels espermatozoides (Ruan *et al.*, 2011). Per tant, per una banda les prostaglandines estimulen les contraccions musculars que condueixen a l'ejaculació i, per l'altra banda, causen les contraccions musculars de la vagina i de l'úter que facilitaran el transport dels espermatozoides (Guyton and Hall, 2011; Flint *et al.*, 2015).

Addicionalment, les vesícules seminals secreten un conjunt de components que participen en el procés de coagulació i liqüefacció del semen. Per exemple, el fibrinogen secretat per les vesícules seminals interacciona amb els enzims secretats per la pròstata, i es genera fibrina, el que desencadena en la coagulació del semen (Guyton and Hall, 2011). A més, un dels components principals de les secrecions de les vesícules seminals és la seminogelina, que també és responsable de la coagulació del semen (Aumüller and Riva, 2009), la qual és degradada per les proteases presents en les secrecions de la pròstata (Gonzales, 2001).

Per últim, l'àcid ascòrbic secretat per les vesícules seminals és un antioxidant que protegeix els espermatozoides envers les espècies reactives d'oxigen (Gonzales, 2001).

1.3.3.2. Pròstata

La pròstata és un òrgan glandular de l'aparell reproductor masculí que secreta un fluid que s'uneix al fluid seminal, i aquest passa a anomenar-se semen.

Les secrecions de la pròstata contribueixen aproximadament al 25-30% del volum total del plasma seminal (Aalberts *et al.*, 2014; Barrachina *et al.*, 2019). El fluid secretat per la pròstata és un fluid lletós i alcalí, amb una baixa concentració de proteïnes però que conté una gran varietat d'enzims proteolítics i electròlits (Guyton and Hall, 2011; Flint *et al.*, 2015). Les secrecions prostàtiques estan principalment compostes per àcid cítric, proteases com l'antigen prostàtic específic (PSA, de l'anglès *prostate-specific antigen*), fosfatases com la fosfatasa àcida prostàtica (PPAP, de l'anglès *prostatic acid phosphatase*), zinc, espermina, colesterol, magnesi, fosfolípids, muramidasa, fibrinolisina i fibrinogen (Veveris-Lowe *et al.*, 2007; Graddis *et al.*, 2011; Flint *et al.*, 2015; Suarez and Wolfner, 2017).

Les secrecions de la pròstata són crucials primer per a la coagulació del semen, i després per a la liqüefacció del semen una vegada aquest ja ha sigut ejaculat. Enzims secretats per la pròstata interactuen amb el fibrinogen secretat per les vesícules seminals (Lwaleed *et al.*, 2004), de tal manera que es produeix la fibrina, la proteïna responsable de la coagulació del semen que es produeix en el tracte reproductor femení. Aquesta espessor adquirida pel semen ajuda a retenir el semen dins del tracte reproductor femení, proporcionant temps als espermatozoides perquè aquests puguin utilitzar la fructosa secretada per les vesícules seminals. Per altra banda, la fibrinolisina (enzim proteolític), secretada també per la pròstata, degrada la fibrina, i fa que es produeixi la liqüefacció del semen en uns 15-30 minuts després de la coagulació (Guyton and Hall, 2011). L'enzim PSA també participa en la liqüefacció degradant les seminogelines o fibronectines presents en el semen (Lilja *et al.*, 1987; Gonzales, 2001; Aumüller and Riva, 2009). Per tant, una vegada el semen coagulat entra en liqüefacció, el semen recupera el seu estat fluid i els espermatozoides estan lliures i mòbils per tal de circular dins de l'aparell reproductor femení i fecundar l'oòcit.

A més de tots els components prostàtics, les secrecions de la pròstata també van acompanyades de prostasomes. Els prostasomes són VEs secretades per les cèl·lules epitelials de la pròstata i sembla que la fusió dels prostasomes amb els espermatozoides contribueix a l'estimulació de la mobilitat espermàtica i a la prevenció d'una reacció acrosòmica prematura (Veure *Secció 1.4.2.2. Vesícules extracel·lulars de la pròstata: Els prostasomes*) (Burden *et al.*, 2006; Saez and Sullivan, 2016).

1.3.3.3. Glàndules bulbouretrals o glàndules de Cowper

Les glàndules bulbouretrals, també conegudes com a glàndules de Cowper, són dues glàndules exocrines que alliberen una substància mucosa, viscosa i alcalina (Chughtai *et al.*, 2005). Aquestes secrecions són alliberades abans de l'ejaculació, pel que es coneix com a preejaculat. Generalment, les secrecions de les glàndules bulbouretrals representen menys d'un 1% del volum del plasma seminal. La funció d'aquest fluid mucós i viscós és la de lubrificar la uretra i la vagina abans del pas del semen (Riva *et al.*, 1990; Chughtai *et al.*, 2005). A més, en ser un líquid alcalí neutralitza l'acidesa de la uretra i elimina els residus d'orina presents a la uretra, el que seria perjudicial i danyí pels espermatozoides (Chughtai *et al.*, 2005).

Les secrecions de les glàndules bulbouretrals també contribueixen a la coagulació del semen i a la peculiar constitució gelatinosa del semen (Beil and Hart, 1973; Riva *et al.*, 1990). Per últim, les secrecions d'aquestes glàndules també contribueixen a la defensa immunitària del tracte genitourinari gràcies a la presència de glicoproteïnes, tals com el PSA (Chughtai *et al.*, 2005; Flint *et al.*, 2015).

1.3.3.4. Glàndules periuretrals o glàndules de Littre

Les glàndules periuretrals, també anomenades glàndules de Littre, es troben en la paret de la uretra de l'aparell reproductor masculí i generen substàncies mucoses que s'incorporaran al semen. A més, també secreten glicosaminoglicans per a protegir a l'epiteli envers l'orina (Fahmy, 2017).

En resum, durant l'ejaculació els espermatozoides epididimaris interactuen de manera seqüencial amb les secrecions de les diverses glàndules sexuals accessòries. Aquesta barreja de fluids, anomenat plasma seminal, conté un conjunt de components que protegeixen i nodreixen als espermatozoides, i els permet d'adquirir la mobilitat necessària que permetrà als espermatozoides progressar a través de l'úter i, eventualment, resultarà en una correcta fertilització de l'oòcit. Malgrat que els espermatozoides poden viure diverses setmanes en l'aparell reproductor masculí, una vegada ejaculats, els espermatozoides tenen una esperança de vida de 24 a 48 hores, un temps que hauran d'aprofitar per nedar dins de la vagina, travessar la cèrvix (coll de l'úter) i entrar dins de l'úter, on podran fertilitzar l'oòcit (Guyton and Hall, 2011).

1.4. VESÍCULES EXTRACEL·LULARS DEL PLASMA SEMINAL

1.4.1. Vesícules extracel·lulars

Les VEs són unes vesícules nanomètriques d'entre 40 i 1000 nm de diàmetre, alliberades per les cèl·lules al medi extracel·lular o als fluids corporals. La importància de les VEs recau en la seva capacitat d'actuar com a vehicles en la comunicació intercel·lular, transferint informació entre cèl·lules i, conseqüentment, influenciant la funció de la cèl·lula receptora (Yáñez-Mó *et al.*, 2015; Kalluri and LeBleu, 2020). Aquestes vesícules transporten proteïnes, lípids i RNAs que provenen de la cèl·lula d'origen i tenen un rol fonamental en condicions fisiològiques, mantenint l'homeòstasi i regulació de les funcions, i en condicions patològiques, com en el càncer o en malalties autoimmunitàries (Colombo *et al.*, 2014; Yáñez-Mó *et al.*, 2015).

En l'actualitat, les VEs es categoritzen en 3 subgrups (**Figura 11**):

- **Exosomes**: Aquestes VEs es produeixen mitjançant la via endosomal que inclou la formació de cossos multivesiculars (MVB, de l'anglès *multivesicular bodies*), els quals es fusionen amb la membrana plasmàtica i alliberen el seu contingut en exosomes al medi extracel·lular. Els exosomes tenen una mida entre els 40 i 160 nm de diàmetre (Kalluri and LeBleu, 2020).
- **Microvesícules** (MVs): Són VEs generades a partir l'evaginació de la membrana plasmàtica, un procés que inclou una senyalització depenent de calci i activitat enzimàtica (Lawson *et al.*, 2016). Les MVs tenen una mida entre els 50 nm i 1 µm de diàmetre (Burnett and Nowak, 2016; Kalluri and LeBleu, 2020).
- **Cossos apoptòtics**: Els cossos apoptòtics sorgeixen de la formació de vesícules a partir de la membrana cel·lular de cèl·lules que entren en apoptosi. La mida d'aquests cossos apoptòtics oscil·la entre 1-5 µm de diàmetre (Lawson *et al.*, 2016; Yamamoto *et al.*, 2019), tot i que també es poden trobar fragments de cèl·lules en apoptosi tan petits com els exosomes (Colombo *et al.*, 2014; Burnett and Nowak, 2016).

Tant els exosomes com les MVs transporten en el seu interior un contingut que serà entregat a altres cèl·lules de l'organisme, podent-se aquestes trobar en localitzacions remotes (Lawson *et al.*, 2016). El contingut dels exosomes i MVs inclou lípids, RNAs i proteïnes tals com factors de transcripció, citocines, factors de creixement, receptors de membrana o proteïnes transmembrana (Colombo *et al.*, 2014; Lawson *et al.*, 2016). Aquestes proteïnes localitzades a les membranes de les VEs són específiques de les cèl·lules que les han alliberat, pel que permeten identificar el seu lloc d'origen i, a més,

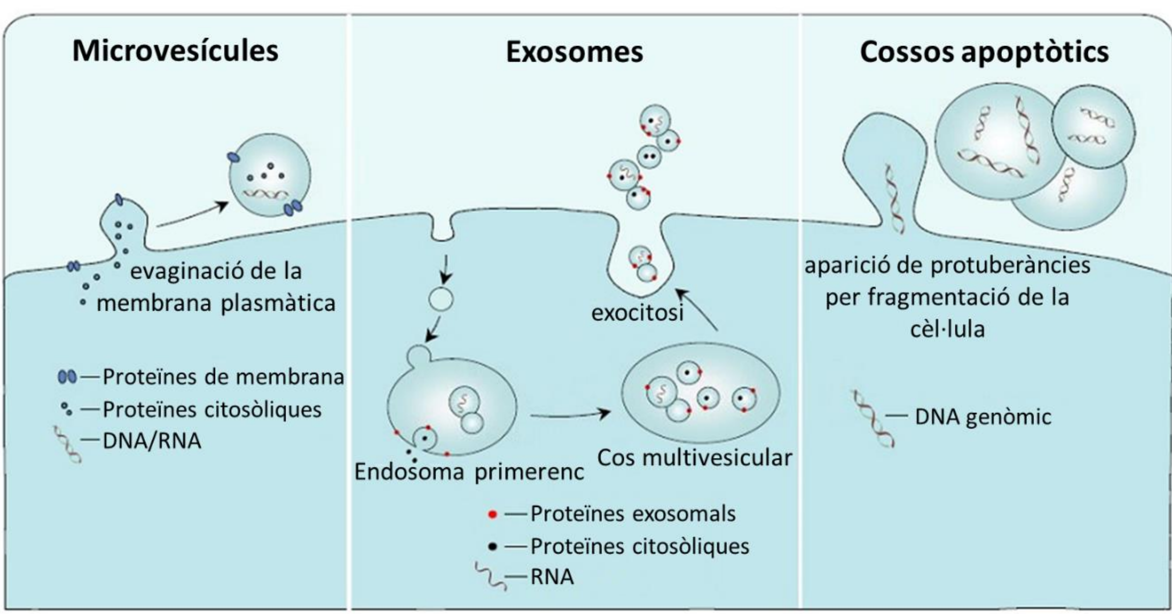

Figura 11. Representació esquemàtica dels diferents subtipus de vesícules extracel·lulars: les microvesícules, els exosomes i els cossos apoptòtics. Font: Modificat de (Lawson *et al.*, 2016).

són unes proteïnes clau, ja que permeten la interacció de les VEs amb unes cèl·lules receptores concretes via interacció amb els seus receptors (Lawson *et al.*, 2016).

De totes les VEs, els exosomes són els que tenen una alta rellevància a causa de la seva funció reguladora i moduladora de processos cel·lulars i, per aquest motiu, són les VEs que tenen un major interès i les que són principalment estudiades. No obstant això, els mètodes actuals per a la purificació d'exosomes resulten en l'obtenció d'una població heterogènia de VEs, pel que la funció dels exosomes encara no està ben definida, ja que els resultats obtinguts avui en dia són d'una barreja d'exosomes i d'altres VEs (Gardiner *et al.*, 2016; Li *et al.*, 2017b; Kalluri and LeBleu, 2020). A més, és necessària una evidència sòlida per a poder parlar d'exosomes, pel que calen diverses metodologies complementàries per a la seva correcta caracterització, el que pot ser complicat d'aconseguir (Colombo *et al.*, 2014).

1.4.1.1. Biogènesi i secreció de les vesícules extracel·lulars

Una gran varietat de tipus cel·lulars poden secretar les VEs, tals com cèl·lules epitelials, plaquetes, cèl·lules dendrítiques, i limfòcits tipus T i B. Les MVs (ectosomes) es generen a partir de l'evaginació de la membrana plasmàtica, contenen proteïnes, DNA, mRNAs i miRNAs, i són alliberades directament al medi extracel·lular (**Figura 12**) (Théry *et al.*, 2009; Raposo and Stoorvogel, 2013; Abels and Breakefield, 2016; Tricarico *et al.*, 2017).

En canvi, els exosomes són vesícules intraluminals (ILV, de l'anglès *intraluminal vesicles*) que es troben dins dels MVB (Colombo *et al.*, 2014; Burnett and Nowak, 2016), els quals es fusionen amb la membrana plasmàtica i expulsen al medi extracel·lular el seu contingut en exosomes (**Figura 12**). Específicament, el primer pas d'aquest procés és la invaginació de la membrana plasmàtica per endocitosis i la formació d'endosomes primerencs (ESE, de l'anglès *Early sorting endosome*) (Abels and Breakefield, 2016). Per invaginació inversa de la membrana d'aquests endosomes es formen les ILV (Stoorvogel *et al.*, 1991; Abels and Breakefield, 2016). El complex ESCRT (ESCRT, de l'anglès *endosomal sorting complexes required for transport*) sembla jugar un rol important en la formació de les ILV i en el reclutament del seu contingut, el qual està format per proteïnes, àcids nucleics i lípids específicament seleccionats (Colombo *et al.*, 2014; Yáñez-Mó *et al.*, 2015). A causa d'un procés de maduració, l'endosoma primerenc passa a anomenar-se endosoma tardà (LSE, de l'anglès *late-sorting endosome*) i, eventualment, genera els MVB (Stoorvogel *et al.*, 1991).

Posteriorment, els MVB poden seguir dues rutes, la degradativa o l'exocítica. En la ruta degradativa, els MVB es fusionen amb els lisosomes o els autofagosomes, el que resulta en la degradació del seu contingut. En la ruta exocítica, els MVB es fusionen amb la

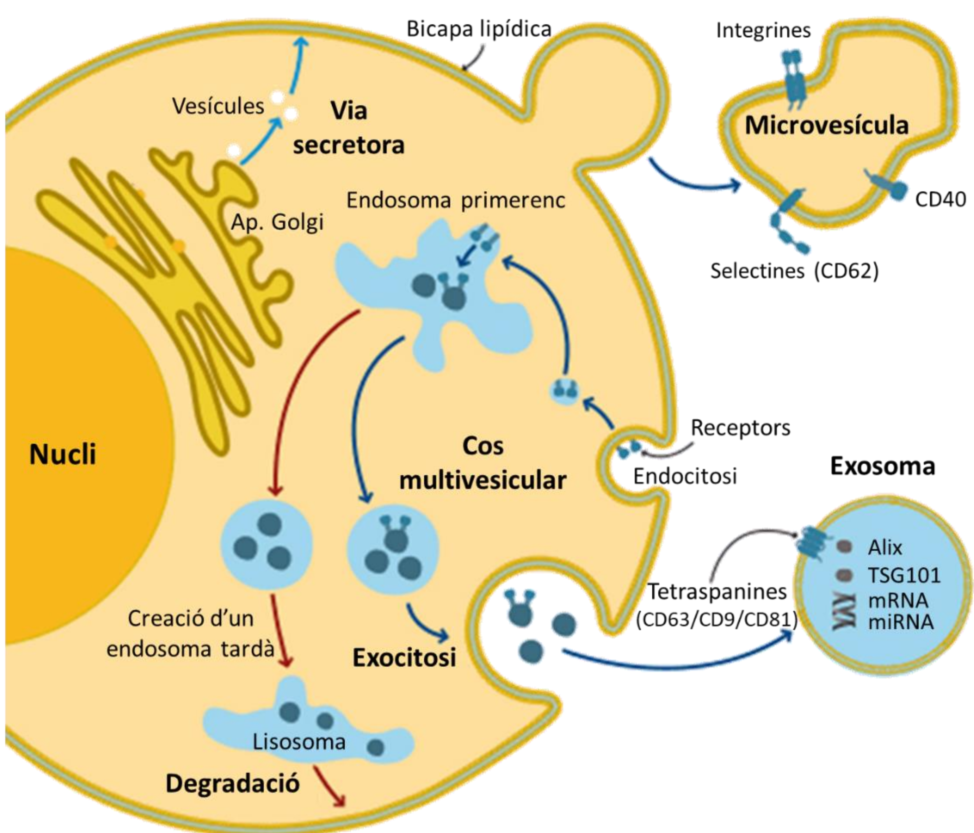

Figura 12. Representació de la biogènesi i secreció de les vesícules extracel·lulars. Font: Modificat de https://www.abcam.com/primary-antibodies/extracellular-vesicles-an-introduction.

membrana plasmàtica i alliberen al medi extracel·lular el seu contingut d'ILV, les quals passen a anomenar-se exosomes (Ludwig and Giebel, 2012; Kalluri and LeBleu, 2020).

La fusió dels MVB amb la membrana plasmàtica i l'alliberació dels exosomes ve regulat per les GTPases, com els receptors tipus Rab (ex.: Rab11 i Rab27) o receptors de proteïnes solubles d'unió al factor sensible a N-etilmaleimida (SNAREs, de l'anglès *soluble N-ethylmaleimide-sensitive factor attachment protein receptors*), tot i que no se sap amb claredat quina és la maquinària cel·lular implicada en la fusió de les MVB amb la membrana plasmàtica (Abels and Breakefield, 2016; Kalluri and LeBleu, 2020). De totes maneres, encara falten més estudis que permetin desxifrar completament el mecanisme responsable per a la formació d'exosomes i el seu empaquetament selectiu de proteïnes, lípids i RNAs concrets.

1.4.1.2. Aïllament de les vesícules extracel·lulars

Les VEs poden ser purificades en medis de cultiu cel·lular, o a partir d'una gran varietat de fluids biològics, com el plasma sanguini, la llet materna, el fluid amniòtic, la saliva, l'orina i el semen, entre d'altres (Ludwig and Giebel, 2012; Lötvall *et al.*, 2014; Yáñez-Mó *et al.*, 2015; Li *et al.*, 2017b; Yamamoto *et al.*, 2019). L'aïllament de les VEs presents en els fluids biològics és complex, ja que, a part de contenir VEs, aquests fluids també contenen proteïnes, lipoproteïnes, carbohidrats, àcids nucleics, restes cel·lulars i cossos apoptòtics, entre d'altres. A més, hem de considerar que són fluids relativament viscosos. No obstant, malgrat que el correcte aïllament de les VEs és complicat degut a la complexitat del medi, és imprescindible per a una correcta caracterització del contingut de les VEs. A més, per tal d'interpretar correctament les funcions biològiques de les VEs, és fonamental confeccionar i utilitzar un mètode d'aïllament que permeti reduir al màxim la contaminació per altres vesícules (Willms *et al.*, 2018).

Actualment, hi ha diferents estratègies que permeten aïllar VEs, malgrat que moltes no aconsegueixen evitar la co-purificació d'altres vesícules amb mides i densitat similar a les VEs (Théry *et al.*, 2006; Vlassov *et al.*, 2012; Li *et al.*, 2017b). La metodologia òptima per al correcte aïllament de VEs hauria de resultar en una alta obtenció de VEs i en l'eliminació de contaminants extravesiculars, mantenint la integritat i les propietats biofísiques de les VEs. Tanmateix, també s'ha de tenir present que les VEs es diferencien en diferents subtipus, com els exosomes i les MVs, els quals tenen unes mides que se solapen, el que dificulta l'aïllament d'una població de VEs determinada. Tot i que s'estan realitzant molts avenços en les metodologies d'aïllament de VEs, avui en dia encara no hi ha un mètode d'aïllament perfecte. Els mètodes d'aïllament més comunament utilitzats es descriuen a continuació:

- **Ultracentrifugació**: Actualment, aquesta és la tècnica més utilitzada per a l'aïllament de VEs (Vlassov *et al.*, 2012; Taylor and Shah, 2015; Gardiner *et al.*,

2016; Li *et al.*, 2017b). Es parteix d'una neteja inicial del fluid biològic d'interès, que generalment inclou centrifugacions a baixes revolucions (ex.: 300 x g i 2.000 x g) i una dilució de la mostra en PBS per a reduir la seva viscositat (Théry *et al.*, 2006). Aquesta neteja inicial de la mostra també sol anar acompanyada d'una filtració per unes membranes de 0,22 µm o de centrifugacions a 10.000 x g per tal d'eliminar vesícules o cossos cel·lulars de diàmetre gran. Per últim, el sobrenedant resultant se centrifuga a 100.000-200.000 x g per tal de precipitar les VEs (Théry *et al.*, 2006). Malgrat i l'alta concentració de material que s'obté utilitzant aquesta metodologia, la ultracentrifugació no és una bona tècnica per a la purificació de VEs, ja que es produeixen agregats, disrupció de les vesícules i co-precipitació d'altres biomolècules no vesiculars. A més, utilitzant aquesta metodologia es precipiten diferents tipus de vesícules de mida similar, i no és possible de diferenciar entre exosomes, MVs o lipoproteïnes (Colombo *et al.*, 2014; Lawson *et al.*, 2016).

- **Ultracentrifugació en gradients de densitat**: Una versió més sofisticada de l'anteriorment mencionada ultracentrifugació és la realització de la ultracentrifugació en gradients de densitat (Théry *et al.*, 2006; Vlassov *et al.*, 2012). En aquest cas, la purificació de les VEs s'optimitza, ja que permet separar les diferents vesícules depenent de la seva densitat. La densitat establerta per a l'aïllament d'exosomes i altres vesícules amb mida i densitat similar va entre 1,13 i 1,19 g/ml de sacarosa (Théry *et al.*, 2009). La ultracentrifugació es pot realitzar en coixins de sacarosa amb aquesta densitat, o en gradients de sacarosa continus o discontinus (Théry *et al.*, 2006; Vlassov *et al.*, 2012). Amb l'ús d'aquesta tècnica, els agregats de proteïnes sedimenten, mentre que les vesícules, al contenir lípids, es mantenen en flotació (Colombo *et al.*, 2014). Tanmateix, la limitació d'aquesta tècnica és la baixa obtenció de VEs, així com el potencial aïllament de vesícules amb mida similar.

- **Cromatografia d'exclusió de mida**: Una altra metodologia popular és l'aïllament de VEs basat en la seva mida, i no per pes molecular. La cromatografia d'exclusió de mida es realitza en unes columnes farcides de partícules poroses (Taylor and Shah, 2015). La funcionalitat d'aquesta tècnica es basa en les diferències de diàmetre d'aquestes porositats. Les vesícules de major mida, com que no poden penetrar en els porus, sortiran de la columna abans que les vesícules més petites, les quals podran penetrar a l'interior del material porós i romandran un major temps en la columna (Li *et al.*, 2017b). No obstant, la tècnica per si mateixa no és escalable i els resultats són difícil de replicar.

- **Precipitació basada en polímers**: Diverses cases comercials ja han desenvolupat algunes tècniques de precipitació basades en polímers per a l'aïllament de VEs, tals com l'ExoQuick™ (System Bioscience) o el Total Exosomes Isolation™ (ThermoFisher Scientific) (Li *et al.*, 2017b). Malgrat que són fàcils d'utilitzar i

permeten l'obtenció de VEs en poc temps, la seva puresa és força baixa i els polímers residuals que romanen en la solució de VEs dificulten la seva posterior anàlisi (Taylor and Shah, 2015; Konoshenko *et al.*, 2018).

- **Aïllament per immunoafinitat**: Altres metodologies més innovadores es fonamenten en l'aïllament de VEs basat en l'expressió de certs marcadors específics de VEs que es troben presents a la superfície de la seva membrana (Clayton *et al.*, 2001; Koliha *et al.*, 2016; Li *et al.*, 2017b), tals com les tetraspanines (CD63, CD9 i CD81). Per tant, aquesta metodologia permet aïllar aquella població de VEs que presenti el marcador específic de membrana d'interès (Vlassov *et al.*, 2012; Taylor and Shah, 2015; Lawson *et al.*, 2016). Tot i que la puresa de les VEs obtingudes per aquesta metodologia és molt alta, l'aspecte negatiu és que tan sols permet purificar aquella subpoblació de VEs que presenta aquella proteïna específica en la seva membrana (Théry *et al.*, 2006; Lawson *et al.*, 2016).

El fet que no s'hagi establert l'ús d'una tècnica rutinària per a l'aïllament dels diferents subgrups de VEs ha impactat directament en la reproductibilitat i la fiabilitat dels resultats obtinguts, complicant la interpretació i integració de diferents estudis (Lötvall *et al.*, 2014). A més, els mètodes d'aïllament de VEs actuals no permeten una purificació específica de les VEs, el que complica la seva investigació (Lötvall *et al.*, 2014). Per tant, encara que molts estudis vulguin estudiar específicament els exosomes, la població de vesícules aïllades per la majoria d'aquestes metodologies no és pura i, a més d'exosomes, probablement també s'aïllen MVs i altres vesícules o lipoproteïnes de mides similars (Lawson *et al.*, 2016). Actualment, un dels principals reptes en l'estudi de les VEs és el de poder definir un mètode que permeti discriminar correctament entre MVs i exosomes, per tal de realment entendre quin és l'origen d'aquestes vesícules i descobrir la seva rellevància biològica (Colombo *et al.*, 2014).

1.4.1.3. Caracterització de les vesícules extracel·lulars

La caracterització de les VEs és un pas essencial per tal de verificar la correcta purificació d'aquestes vesícules i poder obtenir informació verídica sobre les implicacions biològiques de les VEs (Lötvall *et al.*, 2014). Malgrat i no haver-hi uns procediments estandarditzats per a caracteritzar les VEs, habitualment s'avaluen les propietats físiques i moleculars de les VEs purificades per algunes de les metodologies anteriorment mencionades. Concretament, es pot avaluar la mida, morfologia, densitat, concentració i contingut molecular de les VEs aïllades. Com a norma general, s'ha establert que almenys 2 tècniques diferents són necessàries per a la correcta caracterització de les VEs (Lötvall *et al.*, 2014; Konoshenko *et al.*, 2018).

1.4.1.3.1. Caracterització física

Hi ha diferents tècniques físiques per a la caracterització de les VEs. L'ús de la microscòpia electrònica s'ha establert com una de les tècniques *gold standard* per a la caracterització de les VEs, ja que permet acuradament visualitzar la seva dimensió i morfologia (Vlassov *et al.*, 2012; Lawson *et al.*, 2016). Generalment es fa servir la tècnica de tinció negativa i posterior observació per microscòpia electrònica de transmissió. L'aspecte negatiu de la microscòpia electrònica és l'elevat temps requerit per a la seva avaluació i que no proporciona dades quantitatives (Lawson *et al.*, 2016). També s'utilitzen altres tècniques de microscòpia, com la microscòpia electrònica de transmissió estàndard, o en condicions criogèniques, per a visualitzar VEs seccionades (Crescitelli *et al.*, 2013; Lawson *et al.*, 2016), o el microscopi de força atòmica, que permet mesurar la dimensió de les VEs i caracteritzar-les topogràficament (Yuana *et al.*, 2010; Lötvall *et al.*, 2014; Parisse *et al.*, 2017).

Una altra tècnica àmpliament utilitzada és l'anàlisi de seguiment de nanopartícules (NTA, de l'anglès *Nanoparticle Tracking Analysis*), que utilitza les propietats de dispersió de la llum i el moviment brownià per tal d'obtenir la distribució de la mida i la concentració de les VEs en una suspensió líquida (Dragovic *et al.*, 2011; Ludwig and Giebel, 2012). La densitat de flotació en gradients de sacarosa també es pot utilitzar per a separar les diferents subpoblacions de VEs. Per exemple, els exosomes tenen una densitat de flotació entre 1,13 g/m i 1,19 g/ml, mentre que les MVs tenen una densitat entre 1,03 i 1,08 g/ml (Théry *et al.*, 2006, 2009; Ettelaie *et al.*, 2014).

1.4.1.3.2. Caracterització molecular

Les VEs es caracteritzen, a més de per la seva dimensió, morfologia i densitat, per la seva composició molecular, com seria la presència de proteïnes específiques. Per exemple, les MVs es caracteritzen per estar enriquides en integrines, selectines (CD62) i CD40, mentre que els exosomes estan enriquits en tetraspanines (CD63, CD81, CD9), Hsp70, Alix, TSG101, Rab11B i MHCII (**Figura 12**) (Théry *et al.*, 2009; Ludwig and Giebel, 2012; Vlassov *et al.*, 2012; Colombo *et al.*, 2014; Konoshenko *et al.*, 2018; Jeppesen *et al.*, 2019). Aquests marcadors, tot i no ser perfectes i exclusius dels exosomes, es troben altament enriquits en els exosomes, pel que han sigut establerts en consens per tal de caracteritzar aquestes VEs (Lötvall *et al.*, 2014). A més, no tots els exosomes expressen tots els marcadors exosomals, ja que podem trobar diferents subpoblacions d'exosomes expressant un dels marcadors (Théry *et al.*, 2006).

La detecció d'aquestes proteïnes anteriorment mencionades per la tècnica de western blot és àmpliament utilitzada per a la caracterització molecular de les VEs. Per una banda s'ha de validar l'expressió de proteïnes associades amb les VEs (ex.: CD63, CD81, CD9, Hsp70), mentre que, per altra banda, és imprescindible comprovar que hi hagi absència de

contaminants com, per exemple, GM130 (marcador de l'aparell de Golgi), calnexina (marcador de reticle endoplasmàtic), fibronectina (marcador de matriu extracel·lular), histones (marcador de nucli) i annexina A1 (marcador de MVs), entre d'altres (Colombo *et al.*, 2014; Lötvall *et al.*, 2014; Li *et al.*, 2017a; Jeppesen *et al.*, 2019). Com a alternativa a la tècnica de western blot, la detecció de marcadors específics de membrana de VEs també es pot realitzar per citometria de flux, amb l'ús d'unes *beads* múltiplex dirigides envers 39 diferents antígens (Koliha *et al.*, 2016).

1.4.1.4. Composició de les vesícules extracel·lulars

Les VEs són nanovesícules compostes per una bicapa lipídica que conté en el seu interior proteïnes i àcids nucleics. Recentment, s'estan realitzant un gran nombre d'estudis de proteòmica, transcriptòmica i lipidòmica d'aquestes VEs, els quals es poden trobar compilats en catàlegs disponibles en línia tals com Vesiclepedia (www.microvesicles.org) (Kalra *et al.*, 2012), EVpedia (http://evpedia.info) (Kim *et al.*, 2013) i ExoCarta (www.exocarta.org) (Simpson *et al.*, 2012). Aquests estudis han mostrat que malgrat el contingut de les VEs és altament depenent de la cèl·lula d'origen i del seu estat fisiològic/patològic, hi ha unes característiques compartides entre les diferents VEs (Haraszti *et al.*, 2016).

1.4.1.4.1. Composició proteòmica

El contingut proteic dels exosomes i de les VEs en general ha sigut extensament estudiat ja que les proteïnes són un dels principals components de les VEs (Abels and Breakefield, 2016). Les tècniques utilitzades per a la caracterització de la composició proteòmica van des de western blots i immuno-preparació per a microscòpia electrònica, fins a les anàlisis de proteòmica que permeten una identificació a gran escala del contingut proteic de les VEs (Colombo *et al.*, 2014). Aquests estudis han permès identificar un conjunt de proteïnes específiques d'algun tipus cel·lular concret que les ha secretat, però també ha permès identificar proteïnes presents en VEs de diferents orígens, les quals s'estan utilitzant com a marcadors per a la seva caracterització (Lötvall *et al.*, 2014; Haraszti *et al.*, 2016).

1.4.1.4.2. Composició transcriptòmica

El perfil del contingut de RNA de les VEs ha permès identificar que les VEs estan enriquides en tRNAs, mRNAs, miRNAs, snRNAs, snoRNAs, VT-RNAs, Y RNAs, fragments de RNAs i RNAs circulars (Valadi *et al.*, 2007; Michael *et al.*, 2010; Pegtel *et al.*, 2010; Nolte-'t Hoen *et al.*, 2012; Li *et al.*, 2015; Abels and Breakefield, 2016; Konoshenko *et al.*, 2018; Sork *et al.*, 2018). La presència de RNAs en les VEs és de summa importància, ja que evidencia que aquestes vesícules també tenen la capacitat d'actuar com a vectors capaços

de modificar l'expressió gènica de la cèl·lula receptora. Tot i que la presència de RNAs és un reflex de la composició de la cèl·lula d'origen, alguns RNAs es troben més sobrerepresentats que d'altres, el que estaria indicant que hi ha un cert grau d'especificitat en el contingut de RNA de les VEs (Montecalvo *et al.*, 2012; Colombo *et al.*, 2014; Abels and Breakefield, 2016). La presència d'altres àcids nucleics en les VEs, com seria el DNA, o les histones que l'empaqueten, és considerat com artefactes provinents d'un incorrecte aïllament de les VEs (Jeppesen *et al.*, 2019). No obstant, més estudis són necessaris per a desxifrar la presència i rol del DNA en les VEs.

1.4.1.4.3. Composició lipídica

Una de les característiques de les VEs és que estan principalment compostes per fosfatidilserina, fosfatidiletanolamina, esfingomielina, fosfatidilinositol, colesterol i ceramides (Théry *et al.*, 2009; Vlassov *et al.*, 2012; Llorente *et al.*, 2013; Colombo *et al.*, 2014; Abels and Breakefield, 2016; Haraszti *et al.*, 2016; Li *et al.*, 2017b; Skotland *et al.*, 2017). La composició lipídica de la seva membrana es diferencia de la membrana de les cèl·lules a causa de la presència de fosfatidilserina i fosfatidiletanolamina en la part exterior de la membrana de les VEs, el que sembla tenir un rol en la biogènesi dels exosomes (Abels and Breakefield, 2016). Addicionalment, el fet que hi hagi un enriquiment d'esfingomielina i ceramides en les VEs proporciona estabilitat i rigidesa a l'estructura de la membrana d'aquestes vesícules, i també participa en vies de senyalització (Ramstedt and Slotte, 2002). Per tant, aquests estudis evidencien que la composició lipídica de les VEs es diferencia de la composició de les seves cèl·lules d'origen i, per tant, sembla indicar la presència d'un mecanisme que permeti ordenar les espècies de lípids en les membranes de les VEs (Haraszti *et al.*, 2016).

Malgrat que avui en dia hi ha un alt nombre d'estudis focalitzats en la caracterització de les VEs, més estudis són necessaris per a elucidar la funció real de les VEs i la seva participació en algunes malalties.

1.4.1.5. Funcions biològiques de les vesícules extracel·lulars

Recentment, hi ha un interès creixent en l'estudi de les VEs i, en particular, dels exosomes, ja que semblen estar involucrats en una gran quantitat de processos cel·lulars (Colombo *et al.*, 2014). Les funcions de les VEs estan condicionades a la cèl·lula d'origen, i inclouen funcions d'immunomodulació, inflamació, migració cel·lular, proliferació, diferenciació cel·lular, angiogènesi i supervivència cel·lular (Théry *et al.*, 2009; Ludwig and Giebel, 2012; Gaceb *et al.*, 2014; Yáñez-Mó *et al.*, 2015; Li *et al.*, 2017b). Les VEs també s'han vist associades amb malalties cardiovasculars, malalties neurodegeneratives, patogenicitat vírica, l'embaràs i progressió tumoral i metàstasi (Toth *et al.*, 2007; Mincheva-Nilsson and Baranov, 2010; Peinado *et al.*, 2012; Le *et al.*, 2014; Gaceb *et al.*,

2014; Kruger *et al.*, 2014; Ailawadi *et al.*, 2015; Belting and Christianson, 2015; Costa-Silva *et al.*, 2015; Anderson *et al.*, 2016; Baker *et al.*, 2016; Budnik *et al.*, 2016; Burnett and Nowak, 2016; Quek and Hill, 2017; Weidle *et al.*, 2017; Ouattara *et al.*, 2018; Sheller-Miller *et al.*, 2019; Welch *et al.*, 2019; Yuan and Li, 2019; Kalluri and LeBleu, 2020).

Les VEs interaccionen amb les cèl·lules receptores via interacció receptor-lligand, el que facilita que les VEs es fusionin amb les cèl·lules receptores i entreguin el seu contingut de proteïnes, RNAs i lípids, modulant la funció d'aquesta cèl·lula i alterant la seva resposta biològica. La resposta mitjançada per les VEs poden tant promoure la malaltia, com ser un factor protector (Kalluri and LeBleu, 2020). Tenint present que el contingut de les VEs reflecteix l'estatus funcional de la seva cèl·lula d'origen, el qual es pot veure modificat si aquesta cèl·lula es troba en condicions patològiques, ha convertit a les VEs com una font de biomarcadors molt atractius per a la prognosi i diagnosi de diferents malalties, com podria ser el càncer (Vlassov *et al.*, 2012; Cheng *et al.*, 2014; Gaceb *et al.*, 2014; Kruger *et al.*, 2014). A més, en un futur, l'habilitat de les VEs d'interaccionar i alliberar el seu contingut a una cèl·lula receptora específica es podria utilitzar com a una possible eina terapèutica que permeti modular l'activitat de les cèl·lules receptores i, així, evitar la progressió de la malaltia (Colombo *et al.*, 2014; Yamamoto *et al.*, 2019).

1.4.2. Vesícules extracel·lulars del semen

La presència de VEs també s'ha detectat en el semen. Aquestes VEs seminals provenen principalment de les secrecions de l'epidídim o de la pròstata, pel que aquestes VEs s'anomenen epididimosomes o prostasomes respectivament, i representen el 3% del volum total del plasma seminal (Sullivan and Saez, 2013; Samanta *et al.*, 2018; Baskaran *et al.*, 2020).

El rol de les VEs seminals en la reproducció masculina està guanyant importància, ja que hi ha evidencia que les VEs seminals estan implicades en la funció espermàtica, la maduració de l'espermatozoide durant el seu trànsit per l'epidídim, l'estimulació de la mobilitat espermàtica, la prevenció d'una capacitació espermàtica prematura, la modulació del seu potencial de fertilització i la protecció dels espermatozoides envers el sistema immunitari del tracte reproductor femení (Aalberts *et al.*, 2014; Tannetta *et al.*, 2014; Sullivan, 2015; Foster *et al.*, 2016; Machtinger *et al.*, 2016; Jodar *et al.*, 2017c; Simon *et al.*, 2018; Jodar, 2019; Murdica *et al.*, 2019b). La presència d'aquests components en el plasma seminal encara dóna més rellevància a l'important rol que aquest fluid biològic té per a la reproducció masculina.

1.4.2.1. Vesícules extracel·lulars de l'epidídim: Els epididimosomes

Anteriorment, s'ha mencionat la rellevància que tenen les secrecions de l'epidídim en la maduració de l'espermatozoide durant el seu trànsit per les diferents regions de l'epidídim (Veure *Secció 1.3.2. Secrecions de l'epidídim: Participació en la maduració posttesticular de l'espermatozoide*). L'espermatozoide que arriba a l'epidídim és un espermatozoide immadur i immòbil. Com a resultat de la seva interacció amb les secrecions de l'epidídim, els espermatozoides adquireixen la mobilitat progressiva i el potencial de fertilització. Les VEs secretades per l'epidídim, conegudes com a epididimosomes, juguen un paper crucial en aquesta maduració posttesticular de l'espermatozoide (Yanagimachi *et al.*, 1985; Sullivan *et al.*, 2007; Sullivan and Saez, 2013; Sullivan, 2015; Gervasi and Visconti, 2017).

Els epididimosomes són unes vesícules que mesuren entre 50 i 250 nm de diàmetre, tenen un contingut heterogeni ric en proteïnes i RNAs, i la seva membrana està altament enriquida en colesterol (Sullivan *et al.*, 2005; Sullivan, 2016). Aquestes VEs són secretades al compartiment intraluminal de l'epidídim mitjançant secreció apocrina (**Figura 13**) (Hermo and Jacks, 2002; Sullivan, 2016). Aquesta secreció apocrina inclou la formació d'unes estructures anomenades *apical blebs* en el pol apical de les cèl·lules principal de l'epidídim, que s'alliberen en el lumen de l'epidídim (Hermo and Jacks, 2002; Rejraji *et al.*, 2006). A continuació, els *apical blebs* es desintegren i alliberen el seu contingut, el qual conté els epididimosomes (Hermo and Jacks, 2002; Sullivan and Saez, 2013). L'existència de VEs en el fluid epididimari va ser descrit per primera vegada el 1985, quan Yanagimachi i col·laboradors van identificar una població de vesícules adjacents a la superfície de l'espermatozoide epididimari de hàmster xinès (Yanagimachi *et al.*, 1985). Posteriorment la presència d'epididimosomes s'ha confirmat en el fluid epididimari d'altres espècies,

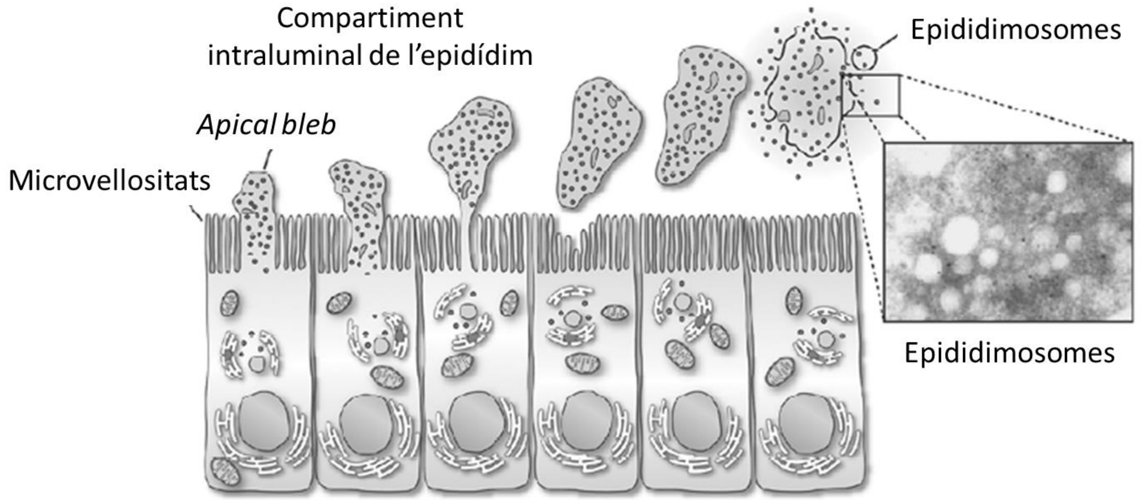

Figura 13. Representació esquemàtica de la secreció apocrina de les cèl·lules principals de l'epidídim. Els epididimosomes són alliberats al compartiment intraluminal de l'epidídim una vegada es desintegren els *apical blebs*. Font: Modificat de (Sullivan *et al.,* 2007).

com en ratolí (Rejraji *et al.*, 2006; Griffiths *et al.*, 2008; Păunescu *et al.*, 2014; Nixon *et al.*, 2019), rata (Grimalt *et al.*, 2000; Fornés *et al.*, 2009), bou (Frenette and Sullivan, 2001; Frenette *et al.*, 2002, 2006) i humà (Thimon *et al.*, 2008).

Diversos grups han realitzat una caracterització del perfil molecular del contingut dels epididimosomes en diverses espècies, amb l'objectiu de desxifrar l'impacte que els epididimosomes podrien tenir sobre la funció dels espermatozoides (Frenette *et al.*, 2006, 2010; Rejraji *et al.*, 2006; Thimon *et al.*, 2008; Girouard *et al.*, 2011; Belleannée *et al.*, 2013a, 2013b; Reilly *et al.*, 2016; Nixon *et al.*, 2019). Estudis de proteòmica en epididimosomes han revelat que aquestes vesícules extracel·lulars contenen centenars de proteïnes, les quals podrien ser crucials per a promoure la maduresa funcional de l'espermatozoide, ja que es troben implicades en la mobilitat espermàtica i en la interacció espermatozoide-oòcit (Girouard *et al.*, 2011; Sullivan, 2015, 2016).

Gràcies a l'ús de proteïnes biotinilades, marcadors fluorescents o ratolins transgènics s'ha demostrat la interacció entre epididimosomes i espermatozoides epididimaris (Frenette et al., 2002, 2010; Schwarz et al., 2013; Reilly et al., 2016; Battistone et al., 2019; Nixon et al., 2019; Zhou et al., 2019). Aquesta interacció dels epididimosomes amb els espermatozoides també s'ha visualitzat en espècies de rosegadors mitjançant microscòpia d'ions d'heli d'alta resolució (Păunescu *et al.*, 2014). A més, avui en dia hi ha evidències que suggereixen que aquests epididimosomes secretats per les cèl·lules epitelials de l'epidídim podrien servir com a vectors que selectivament transporten molècules específiques a l'espermatozoide. Per exemple, s'ha demostrat el tràfic de RNAs des de l'epidídim a l'espermatozoide mitjançant epididimosomes (Reilly et al., 2016; Sharma et al., 2016, 2018). La capacitat dels epididimosomes de transmetre uns RNAs a l'espermatozoide, els quals seran entregats a l'oòcit durant la fertilització, posen en rellevància la seva potencial participació en el control epigenètic que podrien ocasionar durant el desenvolupament embrionari primerenc (Sharma *et al.*, 2016). També hi ha evidències que els epididimosomes podrien estar implicats en la transferència de proteïnes epididimàries secretades cap a l'espermatozoide (Frenette and Sullivan, 2001; Frenette et al., 2010; D'Amours et al., 2012), tot i que hi ha necessitat de més estudis que donin suport a la capacitat dels epididimosomes per a fusionar-se amb la membrana espermàtica i proporcionar a l'espermatozoide un contingut proteic específic.

1.4.2.2. Vesícules extracel·lulars de la pròstata: Els prostasomes

En el moment de l'ejaculació, els espermatozoides també contacten amb el fluid de la pròstata i, conseqüentment, amb l'alta població de VEs presents en aquest fluid, les quals es coneixen amb el nom de prostasomes (Veure *Secció 1.3.3. Secrecions de les glàndules sexuals accessòries*). Els prostasomes són vesícules extracel·lulars d'entre 30 i 500 nm de diàmetre (Foster *et al.*, 2016; Simon *et al.*, 2018) descrits per primera vegada en els anys 70 (Ronquist and Hedström, 1977; Ronquist *et al.*, 1978a, 1978b). Aquestes

VEs són secretades al fluid prostàtic per la fusió dels MVB amb la membrana plasmàtica de les cèl·lules epitelials de la pròstata (Aumüller *et al.*, 1997; Aalberts *et al.*, 2014; Foster *et al.*, 2016). D'igual manera que els epididimosomes i que les VEs d'altres fluids biològics, els prostasomes també presenten un alt contingut de proteïnes, RNAs i lípids.

El perfil lipídic dels prostasomes és inusual, ja que està enriquit en colesterol (la relació colesterol/fosfolípids en prostasomes està al voltant de 2, el que és molt més elevat que la majoria de membranes), i l'esfingomielina representa el ~50% dels fosfolípids (Arvidson *et al.*, 1989; Arienti *et al.*, 1998; Brouwers *et al.*, 2013). Una vegada que els prostasomes es fusionen amb la membrana plasmàtica dels espermatozoides modifiquen la seva composició lipídica i alteren la fluïdesa de la membrana de l'espermatozoide (Simon *et al.*, 2018). Aquesta modificació de la composició lipídica de la membrana de l'espermatozoide resulta en el bloqueig d'una capacitació espermàtica i una reacció acrosòmica prematures (Arienti *et al.*, 1998; Burden *et al.*, 2006; Pons-Rejraji *et al.*, 2011; Brouwers *et al.*, 2013). Els prostasomes també regulen i estimulen la mobilitat espermàtica, ja que proporcionen als espermatozoides el seu contingut en calci i els canals de calci que controlen els seus nivells (Stegmayr and Ronquist, 1982; Park *et al.*, 2011; Aalberts *et al.*, 2014; Tannetta *et al.*, 2014; García-Rodríguez *et al.*, 2018). Altres funcions que també s'han relacionat amb els prostasomes és l'activitat antibacteriana i antioxidant del semen o la immunomodulació per tal de prevenir l'eliminació dels espermatozoides pel sistema immunitari de l'aparell reproductor femení (Kravets *et al.*, 2000; Burden *et al.*, 2006; Aalberts *et al.*, 2014; Saez and Sullivan, 2016; Simon *et al.*, 2018).

Addicionalment, la caracterització del perfil molecular dels prostasomes ha adquirit gran interès per dos motius: Primer, per tal de revelar quines són les funcions fisiològiques dels prostasomes en la reproducció masculina i com aquestes VEs poden influenciar la funció espermàtica (Utleg *et al.*, 2003; Poliakov *et al.*, 2009; Brouwers *et al.*, 2013; Vojtech *et al.*, 2014; García-Rodríguez *et al.*, 2018; Simon *et al.*, 2018) i, segon, per tal d'identificar possibles biomarcadors útils per a la detecció del càncer de pròstata (Velonas *et al.*, 2013; Aalberts *et al.*, 2014). No obstant això, la rellevància clínica dels prostasomes en la infertilitat, prostatitis o càncer de pròstata està encara pendent de determinar (Burden *et al.*, 2006).

1.5. INFERTILITAT MASCULINA

1.5.1. Epidemiologia i aspectes generals de la infertilitat masculina

La infertilitat és un problema freqüent a escala mundial que afecta, aproximadament, al 15% de les parelles en edat reproductiva i que es defineix com la incapacitat d'una parella d'aconseguir una gestació després de 12 mesos de desig gestacional amb relacions sexuals sense utilitzar cap mètode anticonceptiu (Sharlip *et al.*, 2002; World Health Organization, 2010). Actualment, s'ha estimat que al voltant del 40% dels problemes d'infertilitat es deuen a un factor masculí o a un factor femení, mentre que el 20% restant és degut a una combinació d'ambdós factors (Jodar *et al.*, 2017c).

Entre el 40-60% dels pacients amb infertilitat masculina presenten alguna alteració en, almenys, un paràmetre seminal (Dohle *et al.*, 2005). Per tant, l'avaluació inicial de l'home és la realització d'una anàlisi dels paràmetres seminals bàsics, tals com el recompte espermàtic i l'avaluació de la seva mobilitat i morfologia. Aquesta valoració inicial s'ha de complementar amb una curosa anamnesi que reculli els antecedents familiars i personals del pacient, així com amb una exploració general i genital (Sharlip *et al.*, 2002; World Health Organization, 2010). Segons els resultats obtinguts, aquesta primera avaluació podrà fer aconsellable la realització d'altres estudis complementaris, com anàlisis hormonals, genètics, bacteriològics, ecografies del sistema genitourinari o, inclús, una biòpsia testicular. Tots aquests estudis poden ajudar a identificar l'etiologia de la infertilitat masculina i a orientar possibles tractaments.

1.5.2. Anàlisi dels paràmetres seminals

L'anàlisi dels paràmetres seminals, també conegut com a seminograma, és una de les proves inicials a realitzar en l'avaluació de la esterilitat de la parella. Tant el procediment per a realitzar un seminograma, com els valors de referència, es troben estandarditzats en el manual *WHO laboratory manual for the examination and processing of the human semen* publicat i actualitzat per l'Organització Mundial de la Salut (OMS) (World Health Organization, 2010).

Segons l'OMS, les mostres de semen ejaculat es recol·lecten en recipients estèrils, després de 2-7 dies d'abstinència sexual. Una vegada es produeix la liqüefacció de les mostres de semen, la qual triga aproximadament uns 15 minuts, es realitza una valoració de la viscositat seminal i una anàlisi, utilitzant un microscopi amb contrast de fases, de la possible presència de signes de contaminació (ex.: cèl·lules rodones (leucòcits o cèl·lules germinals immadures) o cèl·lules epitelials de descamació) i la presència d'agregació espermàtica. Posteriorment es realitza l'anàlisi dels paràmetres seminals, que inclou l'avaluació de la concentració espermàtica i la seva mobilitat (Dohle *et al.*, 2005). L'anàlisi

de la concentració i mobilitat espermàtica es pot obtenir mitjançant l'ús d'una càmera de l'hemocitòmetre de Neubauer o d'una cambra de Makler, entre altres, tot i que en l'actualitat s'utilitza un sistema informatitzat anomenat *Computer-Assisted Sperm Analysis* (CASA). L'estudi de la mobilitat espermàtica permet distingir diferents graus de mobilitat: progressiva, no progressiva o immobilitat (World Health Organization, 2010). L'anàlisi de la vitalitat dels espermatozoides es basa en l'estudi de la integritat de la seva membrana, i es pot utilitzar un colorant (ex.: Test d'eosina-nigrosina o Test d'eosina) o una solució hipotònica (ex.: test hipoosmòtic). Per últim, es realitza una avaluació de la morfologia dels espermatozoides on, una vegada realitzada l'extensió d'una gota de semen sobre un portaobjectes, els espermatozoides es fixen i tenyeixen amb l'ús de la tinció de Papanicolau, Shorr o Diff-Quick (World Health Organization, 2010).

Els valors de referència normals, establerts per l'OMS el 2010, per a la població d'homes fèrtils es troben descrits en la Taula 2 (World Health Organization, 2010). Els valors seminals que es troben fora d'aquests rangs de referència suggeririen que hi ha un factor masculí afavoridor d'infertilitat, i indicarien la necessitat de realitzar estudis complementaris (Sharlip *et al.*, 2002).

Taula 2. Valors de referència mínims dels paràmetres seminals avaluats en un seminograma establerts per l'OMS. Font: Modificat de (World Health Organization, 2010).

Paràmetres	Límit mínim de referència
Volum seminal (mL)	1,5 (1,4-1,7)
Nombre total d'espermatozoides (10^6 per ejaculat)	39 (33-46)
Concentració espermàtica (10^6 per mL)	15 (12-16)
Mobilitat total (progressiva + no progressiva, %)	40 (38-42)
Mobilitat progressiva (%)	32 (31-34)
Viabilitat (espermatozoides vius, %)	58 (55-68)
Morfologia espermàtica (formes normals, %)	4 (3-4)
pH	≥ 7,2

Els resultats obtinguts en l'anàlisi dels paràmetres seminals permeten classificar als pacients en els següents grups:

- **Pacients normozoospèrmics**: El terme normozoospèrmia significa que les característiques seminals obtingudes en el seminograma compleixen els criteris de normalitat establerts per l'OMS. Tanmateix, el diagnòstic de normozoospèrmia no descarta la possible presència de factors "ocults" d'infertilitat masculina, com podria ser la presència de radicals oxidatius, alteracions genètiques o causes desconegudes.
- **Pacients oligozoospèrmics**: L'oligozoospèrmia significa que hi ha una baixa concentració d'espermatozoides en l'ejaculat. S'ha de considerar tant el total d'espermatozoides en l'ejaculat (Valor mínim: 39 milions d'espermatozoides

totals), com la concentració d'espermatozoides (Valor mínim: 15 milions d'espermatozoides per mL). Aquesta disminució del nombre d'espermatozoides pot ser provocada per diverses causes, sent les més freqüents les alteracions hormonals, aturades en l'espermatogènesi, varicocele o una obstrucció parcial en la via seminal.

- **Pacients azoospèrmics**: L'azoospèrmia, que es defineix com l'absència total d'espermatozoides en l'ejaculat, és un trastorn greu que afecta entre el 5-20% dels homes infèrtils (World Health Organization, 2010). L'azoospèrmia pot ser deguda a una obstrucció de la via seminal (azoospèrmia obstructiva, OA) o deguda a alteracions en l'espermatogènesi que resulten en una no producció d'espermatozoides (azoospèrmia no obstructiva, NOA) (Schlegel, 2004). L'OA representa aproximadament el 40% dels casos d'azoospèrmia i pot ser congènita (absència del vas deferent o obstrucció de la via seminal) o adquirida (a causa d'una infecció, traumatisme o vasectomia) (Willott, 1982; Sharlip *et al.*, 2002). Per contra, la NOA pot ser ocasionada per un problema intrínsec del testicle que es manifesta amb una espermatogènesi deteriorada (fallida testicular primària), o per anomalies endocrines que resulten en una espermatogènesi anòmala (fallida testicular secundària), i representa el 60% de les azoospèrmies (Willott, 1982; Chiba *et al.*, 2016). Mitjançant una avaluació física conjuntament amb estudis genètics i hormonals, que inclouen l'anàlisi dels nivells de FSH, LH i inhibina B en sang, generalment s'aconsegueix el diagnòstic diferencial entre OA i NOA (Chiba *et al.*, 2016). En els casos d'azoospèrmia, l'única possibilitat d'obtenció d'espermatozoides per a la realització d'una tècnica de reproducció assistida és mitjançant un procediment quirúrgic, com seria l'extracció testicular d'espermatozoides (TESE, de l'anglès *Testicular Sperm Extraction*), l'aspiració d'espermatozoides testiculars (TESA, de l'anglès *Testicular Sperm Aspiration*) o la microdissecció testicular per a l'obtenció d'espermatozoides (micro-TESE, de l'anglès *Microdissection Testicular Sperm Extraction*).
- **Pacients astenozoospèrmics**: L'astenozoospèrmia es defineix com la disminució del nombre d'espermatozoides mòbils associat amb una mobilitat espermàtica defectuosa.
- **Pacients teratozoospèrmics**: La teratozoospèrmia és la presència d'una proporció elevada d'espermatozoides amb una morfologia anòmala. L'origen d'aquesta anomalia és en la formació dels espermatozoides en els testicles i pot ser a conseqüència d'un varicocele, de factors tòxics o per causes genètiques, entre altres.

1.5.3. Avaluació endocrina

El correcte funcionament del sistema endocrí és un prerequisit per a la fertilitat masculina. Qualsevol alteració en el delicat i coordinat eix hipotàlem-hipòfisi-testicular pot resultar en hipogonadisme i infertilitat (Veure *Secció 1.2.3 Regulació hormonal de l'espermatogènesi*; **Figura 7**) (Sokol, 2009). L'objectiu de l'avaluació endocrina és determinar si el pacient presenta cap anomalia en la producció d'hormones, i si una teràpia hormonal podria corregir la infertilitat.

L'hipogonadisme és la síndrome clínica que és desencadenada per una alteració de l'eix hipotàlem-hipòfisi-testicular i que resulta en una disfunció dels testicles i en una reducció dels nivells de testosterona en sèrum (Petak *et al.*, 2002). Depenent d'on es localitza l'alteració d'aquest eix, l'hipogonadisme es classifica en primari, secundari o terciari (Darby and Anawalt, 2005). En l'hipogonadisme primari els testicles no funcionen correctament (Darby and Anawalt, 2005; Corona *et al.*, 2012). Aquest hipogonadisme es caracteritza per unes concentracions baixes de testosterona en sèrum, una espermatogènesi danyada, i concentracions elevades de gonadotropines (LH i FSH), pel que també es coneix com a hipogonadisme hipergonadotròpic (**Figura 14**) (Islam and Trainer, 1998; Petak *et al.*, 2002; Nieschlag *et al.*, 2004). Les causes de l'hipogonadisme primari poden ser tan congènites (ex.: síndrome de Klinefelter, síndrome de sol cèl·lules de Sertoli o mutacions en els receptors de LH i FSH), com adquirides (ex.: torsió o traumatisme testicular, orquitis, quimioteràpia, o fallida testicular autoimmune) (Petak *et al.*, 2002; Basaria, 2014). L'hipogonadisme secundari és ocasionat per una alteració en la funció de la glàndula pituïtària anterior, disminuint la secreció de LH i FSH (**Figura 14**) (Darby and Anawalt, 2005; Tournaye *et al.*, 2017b). Aquest tipus d'hipogonadisme es caracteritza per unes concentracions baixes de testosterona en sèrum, conjuntament amb uns nivells baixos o normals de FSH i LH en sèrum, i una espermatogènesi alterada (Petak *et al.*, 2002; Nieschlag *et al.*, 2004; Tournaye *et al.*, 2017a). Per aquest motiu, l'hipogonadisme secundari també és conegut com a hipogonadisme hipogonadotròpic. Les causes de l'hipogonadisme secundari també poden ser congènites (ex.: síndrome de Kallmann, síndrome de Prader-Willi o mutacions en la subunitat beta de les hormones LH i FSH), com adquirides (ex.: dany pituïtari a causa d'un tumor, hiperprolactinèmia, infecció, traumatisme cranial, per fàrmacs (opioides, glucocorticoides, agonistes o antagonismes de GnRH), obesitat mòrbida, diabetis, trastorns de l'alimentació, exercici excessiu o cirrosi) (Petak *et al.*, 2002; Basaria, 2014; Krausz and Riera-Escamilla, 2018; Krausz *et al.*, 2018). Per últim, l'hipogonadisme terciari és degut a un dany a l'hipotàlem, disminuint la secreció de GnRH, i també es classifica com hipogonadisme hipogonadotròpic (Darby and Anawalt, 2005).

L'avaluació endocrina bàsica consisteix en la mesura dels nivells en sang de FSH, testosterona, LH, globulina fixadora d'hormones sexuals (SHBG, de l'anglès *Sex hormone-binding globulin*) i inhibina B, i en alguns pacients es complementa amb els nivells de

prolactina, determinacions que ajuden a diagnosticar quin és l'origen de l'hipogonadisme (Sokol, 2009). L'avaluació dels valors hormonals s'acostuma a realitzar quan els pacients infèrtils presenten una baixa concentració d'espermatozoides, especialment si aquesta és inferior als 15 milions per mL o si hi ha una disfunció sexual (Sharlip *et al.*, 2002). Per una banda, la testosterona és una hormona necessària per al desenvolupament i manteniment de les característiques sexuals secundàries i de la libido, així com de la iniciació i manteniment de l'espermatogènesi (Matsumoto, 1994). Els nivells de testosterona en sèrum són un reflex de l'estat de la funció de les cèl·lules de Leydig en el testicle (Sokol, 2009; Corona *et al.*, 2012). Per altra banda, la mesura dels nivells de LH i FSH en sèrum permeten determinar si la disfunció endocrina prové d'una fallida testicular primària (hipogonadisme primari) o d'una alteració en la glàndula pituïtària anterior o l'hipotàlem (hipogonadisme secundari o terciari, respectivament) (Darby and Anawalt, 2005; Sokol, 2009). Els nivells de FSH en sèrum són un reflex de l'estat de l'epiteli dels túbuls seminífers (Matsumoto, 1994; Corona *et al.*, 2012). Nivells elevats de FSH en sang provenen d'una fallida testicular primària i el conseqüent dèficit en la producció d'inhibina B, una hormona secretada per les cèl·lules de Sertoli. La secreció d'inhibina hauria de regular per retroalimentació negativa l'hipotàlem i la glàndula pituïtària anterior, aturant la secreció de FSH. El fet que aquest circuit no funcioni correctament, i que hi hagi nivells alts de FSH

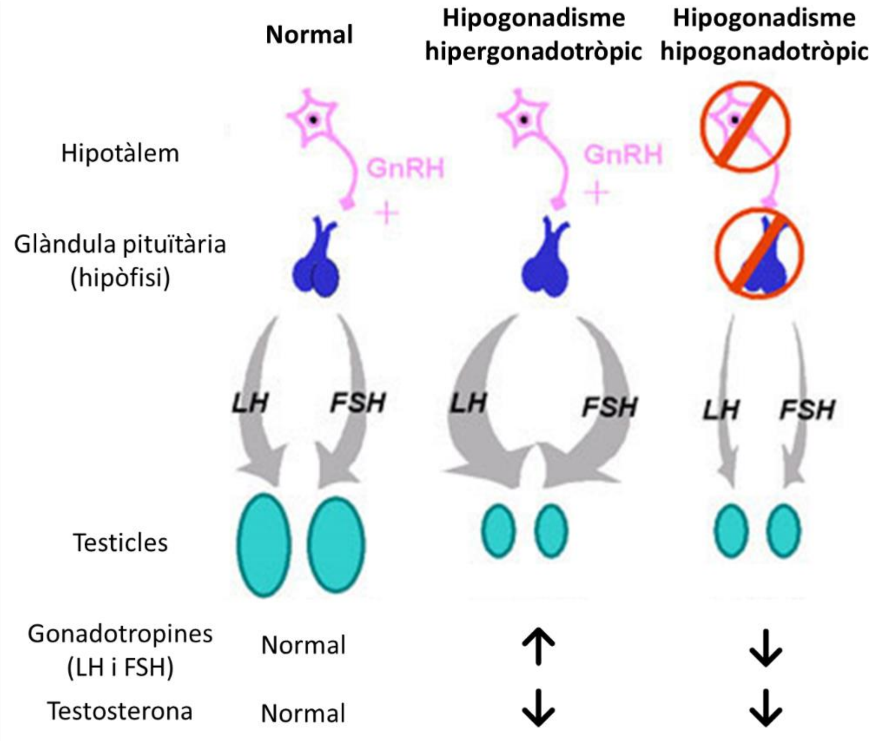

Figura 14. Representació esquemàtica de l'eix hipotàlem-hipòfisi-testicular en un home adult sa, i en el cas d'hipogonadisme hipergonadotròpic (hipogonadisme primari) i hipogonadisme hipogonadotròpic (hipogonadisme secundari i terciari). Font: Modificat de (Hayes *et al.*, 2013).

en sèrum, estaria indicant que hi ha anomalies en l'epiteli dels túbuls seminífers i, per tant, en l'espermatogènesi. Per exemple, pacients amb azoospèrmia no obstructiva per absència de cèl·lules germinals presenten uns nivells de FSH elevats. Per contra, l'hormona LH és secretada per la glàndula pituïtària anterior i estimula la producció de testosterona per les cèl·lules de Leydig (Matsumoto, 1994). La secreció de LH és regulada per retroalimentació negativa per la testosterona en sang, de tal manera que els nivells de LH serveixen per avaluar la funció de les cèl·lules de Leydig (Sokol, 2009). Per tant, els pacients que presenten una fallida testicular presenten nivells elevats de LH i FSH (hipogonadisme hipergonadotròpic), mentre que els pacients amb alteracions en la glàndula pituïtària o en l'hipotàlem presenten una disminució dels nivells de LH i FSH (hipogonadisme hipogonadotròpic).

L'avaluació endocrina del pacient infèrtil i la determinació de l'alteració present en l'eix hipotàlem-hipòfisi-testicular és essencial per a un correcte diagnòstic de l'hipogonadisme i per a la possible l'elecció d'un tractament adequat. Per exemple, els pacients amb hipogonadisme hipogonadotròpic presenten unes característiques sexuals secundàries poc desenvolupades i uns testicles de mida reduïda. La teràpia de reemplaçament de testosterona (TRT) serveix per a recuperar els valors normals de testosterona en sang, i per millorar els símptomes ocasionats per l'hipogonadisme (Nieschlag *et al.*, 2004; Rhoden and Morgentaler, 2004; Darby and Anawalt, 2005). No obstant, si el que interessa és recuperar la fertilitat, s'han de realitzar teràpies de reemplaçament de gonadotropines (LH i FSH) per tal d'estimular el creixement dels testicles i la iniciació de l'espermatogènesi (Corona *et al.*, 2012).

1.5.4. Tècniques -òmiques per a l'estudi de la fertilitat masculina: identificació de biomarcadors

Actualment, l'avaluació de la infertilitat masculina es limita a examinar els paràmetres seminals, els quals tan sols revelen deficiències força evidents en la concentració espermàtica, mobilitat o morfologia (World Health Organization, 2010). Segons els resultats obtinguts en aquest estudi inicial de l'home es poden realitzar estudis complementaris amb l'objectiu d'intentar identificar quin és l'origen de la infertilitat. Tanmateix, les alteracions dels paràmetres seminals tan sols s'han vist associades a un nombre limitat de causes i, a més, l'etiologia de la infertilitat del factor masculí segueix sent en gran part idiopàtica, i això és degut al fet que les eines disponibles per avaluar la fertilitat masculina són limitades i insuficients (Esteves et al., 2011; Jodar et al., 2017a). Per tant, sembla necessari el desenvolupament de noves metodologies per a obtenir una millora en l'estudi i classificació actual de la infertilitat (Weber *et al.*, 2005; Kovac *et al.*, 2013; Oehninger *et al.*, 2014; Bieniek *et al.*, 2016).

En l'era actual, els nous avanços en les tècniques biomoleculars estan permetent un gran aprofundiment en la biologia de la reproducció, i nous tests per a l'avaluació de la infertilitat masculina estan emergent en laboratoris de recerca en l'àmbit de l'andrologia (Esteves *et al.*, 2011; Kovac *et al.*, 2013; Khatun *et al.*, 2018). La revolució de les tècniques -òmiques ha permès aprofundir en el coneixement de la biologia, així com investigar de manera interdisciplinària els processos biològics, el que és conegut com a biologia de sistemes (Horgan and Kenny, 2011; Hasin *et al.*, 2017; Karczewski and Snyder, 2018). El terme -òmiques inclou l'estudi dels gens (genòmica), transcrits (transcriptòmica), proteïnes (proteòmica), metabòlits (metabolòmica) i lípids (lipidòmica).

En el context de la biologia reproductiva i de la infertilitat masculina ens trobem en un moment de creixement incessant d'estudis -òmics (Baker, 2011; Carrell *et al.*, 2016; Jodar *et al.*, 2017b; Yadav, 2017). L'aplicació de les -òmiques en l'àmbit de l'andrologia està permetent el descobriment de nous aspectes de la biologia de l'espermatozoide, així com de noves funcions del plasma seminal (John Aitken and Henkel, 2011; Kovac *et al.*, 2013; Drabovich *et al.*, 2014; Gilany *et al.*, 2015; Bieniek *et al.*, 2016; Jodar *et al.*, 2017b; Yadav, 2017; Barrachina *et al.*, 2018a; Castillo *et al.*, 2018). A més, l'aplicació d'aquestes noves tecnologies en la comparació entre individus fèrtils i infèrtils proporciona informació sobre mecanismes patogènics que podrien estar relacionats amb la infertilitat masculina, i té la potencialitat de derivar en el descobriment de nous biomarcadors amb alta importància clínica (Kovac *et al.*, 2013; Milardi *et al.*, 2013; Jodar *et al.*, 2015; Bieniek *et al.*, 2016; Jodar *et al.*, 2017b; Khatun *et al.*, 2018). L'ús d'aquestes tecnologies en l'àmbit de la reproducció i, en particular, de la fertilitat masculina representa, sens dubte, una veritable promesa per a l'optimització del diagnòstic de la infertilitat i per al seu tractament.

1.6. PROTEÒMICA DEL PLASMA SEMINAL I LES VESÍCULES EXTRACEL·LULARS SEMINALS: IDENTIFICACIÓ DE BIOMARCADORS ÚTILS PER AL DIAGNÒSTIC DE LA INFERTILITAT

1.6.1. La proteòmica [4]

L'inici de l'estudi de les proteïnes en l'àmbit de la reproducció masculina va començar fa més d'un segle, quan Friedrich Miescher el 1874 va aïllar i identificar un component bàsic proteic en els espermatozoides que va anomenar protamina, i va descobrir que aquesta proteïna acompanyava el que ell va anomenar "nucleïna", o el que avui en dia coneixem com a DNA (Miescher, 1874). Malgrat i aquest descobriment inicial, no va ser fins aproximadament 100 anys després que es van desenvolupar mètodes de seqüenciació, separació i detecció de proteïnes que van permetre l'estudi generalitzat de les proteïnes (Engvall and Perlmann, 1971; O'Farrell, 1975; Towbin *et al.*, 1979). Tot i això, aquests mètodes eren encara rudimentaris i les proteïnes s'havien d'estudiar de manera individual. La possibilitat d'estudiar la totalitat o una proporció substancial del proteoma de l'espermatozoide i del plasma seminal ha començat recentment, al voltant del 1995, amb l'aplicació de l'espectrometria de masses per a l'estudi de les proteïnes.

Els passos bàsics en la majoria d'anàlisis de proteòmica actuals són: 1) l'extracció de proteïnes o pèptids de la mostra biològica, 2) la reducció de la complexitat de l'extracte de proteïnes o pèptids, i 3) l'aplicació d'espectrometria de masses i comparacions amb bases per dades per tal d'identificar les diferents proteïnes o pèptids (**Figura 15**) (Codina *et al.*, 2015). El primer pas es realitza mitjançant l'extracció proteica completa de l'espermatozoide o del plasma seminal, o dirigida a compartiments cel·lulars específics (ex.: cap de l'espermatozoide, cua de l'espermatozoide) o subcomponents (ex.: vesícules extracel·lulars del plasma seminal), prèviament purificats (Thimon *et al.*, 2008; Amaral *et al.*, 2014a; Samanta *et al.*, 2018). El segon pas és la reducció de la complexitat inicial de l'extracte proteic o peptídic, el qual es pot aconseguir mitjançant electroforesi en gels de poliacrilamida en una dimensió (1D-PAGE, de l'anglès *One-dimensional Polyacrylamide Gel Electrophoresis*) o en dues dimensions (2D-PAGE, de l'anglès *Two-dimensional Polyacrylamide Gel Electrophoresis*) (**Figura 15**). Tanmateix, un procediment més recent i

[4] La informació inclosa en aquesta secció de la Introducció es basa en el capítol de llibre ***Jodar M, Barrachina F, Oliva R (2017) Chapter 18: The use of sperm proteomics in the assisted reproduction laboratory. In: A practical guide to sperm analysis: basic andrology and reproductive medicine (Garrido G, Rivera R; eds). Boca Raton (FL): CRC press; p.233-244*** que es troba a l'Annex 4***.***

d'alt rendiment és el de convertir l'extracte inicial de proteïnes en pèptids gràcies a la digestió de les proteïnes per tripsina i, posteriorment, fraccionar els pèptids utilitzant l'enfocament isoelèctric (IEF, de l'anglès *isoelèctric focusing*), cromatografia líquida monodimensional (1D-LC, de l'anglès *One-dimensional liquid chromatography*) o cromatografia líquida bidimensional (2D-LC, de l'anglès *Two-dimensional liquid chromatography*) (**Figura 15**).

El pas final en una anàlisi proteòmica s'aconsegueix mitjançant l'aplicació d'espectrometria de masses (MS, de l'anglès *mass spectrometry*) i la identificació de les proteïnes. L'espectrometria de masses es basa en la detecció de molècules ionitzades d'acord amb la seva relació massa-càrrega (m/z). Inicialment, les anàlisis de proteòmica basades en MS implicaven l'ús de MALDI-TOF (MALDI-TOF, de l'anglès *Matrix Assisted Laser Desorption Ionization - Time Of Flight*), el qual es basa en la determinació acurada de les masses peptídiques i en la comparació amb bases de dades peptídiques per a identificar proteïnes. En les anàlisis MALDI-TOF les proteïnes s'escissen d'un gel, es digereixen amb tripsina i es determina la relació m/z dels pèptids resultants. Aquestes masses peptídiques proporcionen una acurada empremta peptídica (PMF, de l'anglès *peptide mass fingerprint*) i es comparen amb bases de dades de proteïnes on la seva seqüència prové de la predicció *in silico* de dades genòmiques. Si diverses masses peptídiques identificades experimentalment coincideixen amb els pèptids teòrics, es considera que s'ha identificat la proteïna (Oliva *et al.*, 2008). Recentment, s'estan aplicant metodologies de major rendiment basades en espectrometria de masses en tàndem (MS/MS, de l'anglès *tandem mass spectrometry*) que també ofereixen la possibilitat de seqüenciar pèptids *de novo* i detectar modificacions posttraduccionals (**Figura 15**) (Codina *et al.*, 2015).

Per a la quantificació de proteïnes també hi ha diferents eines disponibles. Els mètodes inicials es basaven en la tècnica ELISA (Engvall and Perlmann, 1971) o western blot (Towbin *et al.*, 1979) (**Figura 15**). Aquests mètodes són molt robustos i útils, però només són aplicables per a l'estudi d'unes proteïnes concretes i no es poden utilitzar per a estudiar moltes proteïnes de manera simultània. Les tècniques d'alt rendiment disponibles actualment permeten quantificar simultàniament un elevat nombre de proteïnes del proteoma. Els mètodes inicials de quantificació del proteoma es basaven en la mesura de la intensitat de les taques de proteïnes que es trobaven separades en un gel de 2D-PAGE, i posteriorment s'identificava quines eren aquestes proteïnes (Martínez-Heredia *et al.*, 2008; Vilagran *et al.*, 2015). No obstant, les tècniques actuals es basen en la quantificació de pèptids, més que en la quantificació de les proteïnes. Els pèptids poden ser quantificats mitjançant recompte espectral (Batruch *et al.*, 2011) o després de ser marcats per *tandem mass tags* (TMT) (**Figura 15**) (Amaral *et al.*, 2014b; Azpiazu *et al.*, 2014; Castillo *et al.*, 2019; Jodar *et al.*, 2020).

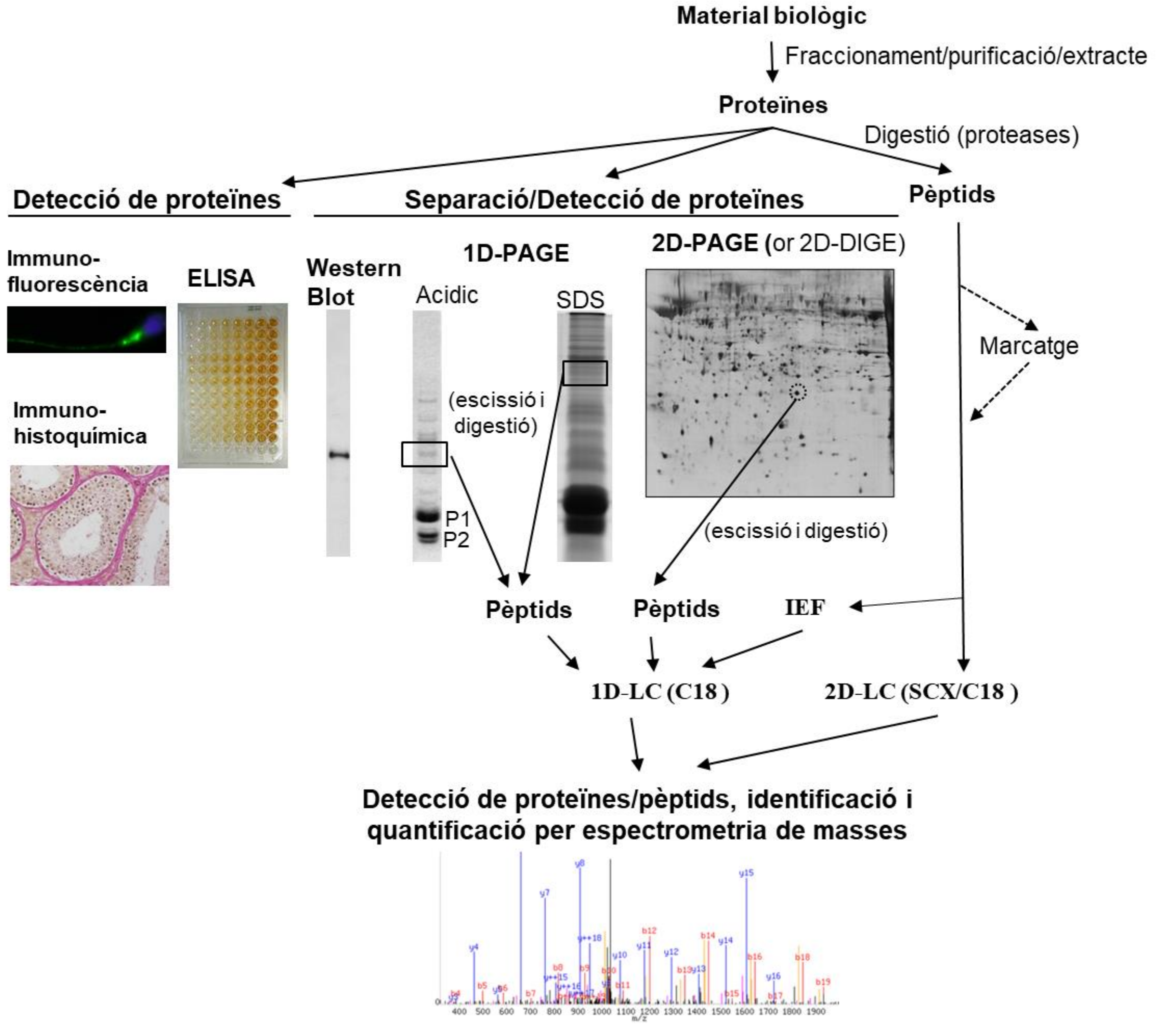

Figura 15. Principals mètodes d'anàlisi de proteïnes disponibles actualment. El material biològic es fracciona o purifica abans de procedir amb l'extracció de proteïnes. La presència de certes proteïnes es pot detectar directament mitjançant immunofluorescència, immunohistoquímica o ELISA (esquerra). Alternativament, les proteïnes es poden separar per electroforesi en gel (centre) i les proteïnes desitjades es poden eluir i digerir en pèptids. Els mètodes actuals d'alt rendiment inclouen la digestió de les proteïnes per proteases per convertir-les en pèptids (dreta). L'última etapa és la separació dels pèptids per cromatografia líquida i procedir a la seva identificació i quantificació per espectrometria de masses. Font: Modificat de (Jodar *et al.*, 2017b).

1.6.1.1. El principi de la LC-MS/MS

En l'actualitat, una de les tècniques analítiques més utilitzades per a l'estudi del proteoma de mostres biològiques complexes és la combinació de cromatografia líquida amb espectrometria de masses en tàndem (LC-MS/MS, de l'anglès *Liquid Chromatography coupled with Tandem Mass Spectrometry*). La cromatografia líquida d'alta resolució (HPLC, de l'anglès *high performance liquid chromatography*) és una tècnica que permet una separació altament eficient dels components d'una mostra biològica. La HPLC consisteix en una fase estacionaria no polar (la columna cromatogràfica, la qual és un cilindre ple de

petites partícules esfèriques amb unes característiques químiques determinades), i una fase mòbil que actua com a portador de la mostra. La mostra en solució és injectada en la fase mòbil i els components de la solució emigren d'acord amb les interaccions no covalents dels compostos amb la columna. Depenent de les interaccions químiques i físiques amb la fase estacionaria, els pèptids es separen al llarg de la columna de cromatografia segons el seu grau de retenció.

A continuació, els pèptids són introduïts a l'espectròmetre de masses (MS) o a dos espectròmetres consecutius (MS/MS). Els espectròmetres de massa consten de tres parts: la font d'ions, l'analitzador de massa i el detector. La font d'ions permet ionitzar el material a analitzar (l'anàlit). Les dues tècniques majorment utilitzades són la ionització per electrospray (ESI, de l'anglès *Electrospray Ionisation*) o per MALDI, tot i que últimament se sol utilitzar una font ESI combinada amb un detector en forma de trampa iònica, ja que augmenta molt la resolució de la tècnica. La font d'ions es troba seguida de dos analitzadors (MS/MS). El primer espectròmetre (MS1) separa els ions segon la seva relació m/z. Aquests ions són posteriorment fragmentats mitjançant dissociació induïda per col·lisió (CID, de l'anglès *collision-induced dissociation*), dissociació per col·lisió d'alta energia (HCD, de l'anglès *higher energy collision dissociation*), dissociació per transferència d'electrons (ETD, de l'anglès *electron-transfer dissociation*) o dissociació per captura d'electrons (ECD, de l'anglès *electron-capture dissociation*). Posteriorment, els fragments d'ions són introduïts a un segon espectròmetre de masses (MS2), que separa els fragments segons la seva relació m/z. L'últim component és el detector, el qual registra la càrrega produïda per l'ió i ho relaciona amb la seva massa (m/z) per generar els espectres. Per tal de predir la identitat de la molècula (pèptid o proteïna) es realitzarà una cerca en bases de dades.

1.6.2. Proteòmica del plasma seminal

Actualment, la identificació d'un total de 2064 proteïnes en el proteoma del plasma seminal i l'anàlisi de les proteïnes identificades ha revelat que, al contrari del que es pensava, el plasma seminal no és tan sols un medi per al transport dels espermatozoides (Jodar *et al.*, 2017c, 2017b). De fet, sembla que el plasma seminal és crucial per a la regulació de la coagulació i liqüefacció del semen, la mobilitat de l'espermatozoide i per a l'adquisició del potencial de fertilització, entre altres funcions (Drabovich *et al.*, 2014; Gilany *et al.*, 2015; Jodar *et al.*, 2017c). A causa de la seva importància per a la reproducció, l'anàlisi del proteoma del plasma seminal i la comprensió de les seves funcions és d'alta rellevància. Durant la dècada passada ja van començar a aparèixer els primers estudis que analitzaven els perfils proteòmics del plasma seminal de pacients sans i de pacients amb alguna alteració reproductiva (Starita-Geribaldi *et al.*, 2001, 2003; Fung *et al.*, 2004; Pilch and Mann, 2006). Posteriorment, i amb la finalitat d'identificar possibles biomarcadors associats amb la infertilitat masculina, s'han

realitzat anàlisis de proteòmica estudiant el plasma seminal de pacients fèrtils (Milardi *et al.*, 2012; Rolland *et al.*, 2013), pacients infèrtils en general (Davalieva *et al.*, 2012; Sharma *et al.*, 2013a; Intasqui *et al.*, 2016), pacients amb azoospèrmia (Yamakawa *et al.*, 2007; Batruch *et al.*, 2011, 2012; Drabovich *et al.*, 2011, 2013; Freour *et al.*, 2013), pacients amb astenozoospèrmia (Wang *et al.*, 2009; Saraswat *et al.*, 2017), oligozoospèrmia (Giacomini *et al.*, 2015), prostatitis (Kagedan *et al.*, 2012), infertilitat induïda per oxidants (Herwig *et al.*, 2013; Sharma *et al.*, 2013b; Agarwal *et al.*, 2015; Intasqui *et al.*, 2015), varicocele (Del Giudice *et al.*, 2016) i pacients hipogonàdics (Milardi *et al.*, 2014). Així doncs, les anàlisis de proteòmica s'han convertit en una eina altament valuosa per a la comprensió de la fisiologia del plasma seminal i per a identificar mecanismes patològics associats amb la infertilitat masculina. A més, la possibilitat de poder comparar milers de proteïnes simultàniament en pacients amb diferents subtipus d'infertilitat podria facilitar la identificació de biomarcadors de fertilitat útils per a les clíniques de reproducció assistida i per al disseny de noves teràpies de fertilitat.

Tot i que s'ha demostrat l'alta utilitat de les tècniques proteòmiques d'alt rendiment com a eina de descobriment de biomarcadors en el plasma seminal, algunes limitacions dificulten l'ús rutinari en les clíniques (Verrills, 2006; Jodar *et al.*, 2017b). Per exemple, diferències en l'obtenció, manipulació i emmagatzematge de les mostres, les tècniques proteòmiques aplicades, i la variabilitat intra- i inter-individual són algunes de les causes que contribueixen a la falta de reproductibilitat dels estudis de proteòmica (Codina *et al.*, 2015; Gilany *et al.*, 2015; Camargo *et al.*, 2018). Addicionalment, després de l'ejaculació, hi ha una alta activitat proteasa durant la liqüefacció del semen, el que podria estar introduint encara més heterogeneïtat en l'estudi de proteòmica del plasma seminal (Laflamme and Wolfner, 2013). Una altra limitació de l'ús rutinari de la proteòmica en la clínica de reproducció assistida és el seu elevat cost, a causa de la necessitat d'una maquinària molt costosa i de professionals altament qualificats. Així doncs, malgrat els últims avenços en l'espectrometria de masses, la proteòmica del plasma seminal fins ara només s'ha utilitzat com a eina de descoberta de biomarcadors. Un cop validat el valor clínic dels biomarcadors candidats detectats a partir d'anàlisis de proteòmica, una opció és desenvolupar tests més econòmics i senzills, com els microarrays de proteïnes o ELISA múltiplex (Verrills, 2006) per al seu ús rutinari en les clíniques de reproducció.

Les eines diagnòstiques per a l'estudi de la infertilitat masculina són força limitades i, actualment, principalment se centren en l'anàlisi dels paràmetres seminals (concentració espermàtica, mobilitat i morfologia). Per tant, el seminograma continua sent l'eina base per a l'avaluació del potencial fèrtil de l'home, malgrat que no aconsegueix una correcta estratificació dels pacients infèrtils i que no permet desemmascarar les causes de la infertilitat d'un gran nombre de pacients. Així doncs, sembla molt prometedora l'aplicació de les tècniques proteòmiques en l'àmbit de la reproducció i, particularment, en la fertilitat masculina per tal d'estudiar àmpliament la fisiologia i la fisiopatologia de la reproducció masculina (Milardi *et al.*, 2013). L'aplicació de la proteòmica en l'anàlisi del

plasma seminal obre la porta a la identificació de nous biomarcadors d'infertilitat útils per a una millora en l'avaluació de la infertilitat masculina, actualment principalment centrada en l'estudi dels paràmetres seminals, i per al diagnòstic i la presa de decisions terapèutiques (Drabovich *et al.*, 2014).

1.6.3. Proteòmica de les vesícules extracel·lulars seminals

En els últims anys, l'estudi de les VEs està guanyant importància gràcies a la seva funció d'actuar com a vehicles en la comunicació entre cèl·lules, i a la seva capacitat d'influenciar la cèl·lula receptora (Yáñez-Mó *et al.*, 2015; Kalluri and LeBleu, 2020). A més, el fet que les VEs es troben en fluids biològics i que proporcionen informació de la cèl·lula d'origen i, per tant, són un reflex de l'estatus de la malaltia, ha resultat ser d'alt interès per a la identificació de biomarcadors útils per al diagnòstic i pronòstic (Yamamoto *et al.*, 2019). A causa de la seva importància en la fisiologia de l'espermatozoide i la importància que tenen per a la reproducció, les vesícules extracel·lulars presents en el plasma seminal (epididimosomes i prostasomes) són altament atractives per al desenvolupament de biomarcadors per a la infertilitat masculina. La millora en les tècniques proteòmiques d'alt rendiment ha contribuït enormement a la nostra comprensió de la composició molecular de les VEs (Simpson *et al.*, 2008).

El primer estudi de proteòmica en VEs seminals es va realitzar l'any 2003 per Utleg i col·laboradors (Utleg *et al.*, 2003) i va resultar en la identificació de 139 proteïnes. A partir d'aquest moment, s'han dut a terme altres estudis de proteòmica de les VEs seminals per tal de desxifrar quin és el contingut d'aquestes vesícules i elucidar l'impacte que tenen en la funció espermàtica i en la reproducció masculina. La composició proteica de les VEs seminals s'ha estudiat en pacients sans (Poliakov *et al.*, 2009; Yang *et al.*, 2017), i també comparant pacients normozoospèrmics amb pacients amb paràmetres seminals alterats (García-Rodríguez *et al.*, 2018) i amb pacients astenozoospèrmics (Lin *et al.*, 2019; Murdica *et al.*, 2019a) amb la finalitat de determinar possibles alteracions en les VEs que podrien estar associades amb la infertilitat masculina i que, conseqüentment, podrien resultar en la identificació de biomarcadors de l'estat fèrtil del pacient. L'estudi de les VEs també s'ha dut a terme de manera individual pels epididimosomes, una de les VEs presents en el plasma seminal. Durant el procediment quirúrgic de vasovasostomia, Thimon i col·laboradors van poder obtenir fluid epididimari d'humà i aïllar els seus epididimosomes, sent els primers a caracteritzar el contingut proteic dels epididimosomes en humans (Thimon *et al.*, 2008). També s'ha estudiat el proteoma dels epididimosomes en altres espècies, tals com l'espècie bovina (Frenette *et al.*, 2006, 2010; Girouard *et al.*, 2011) i murina (Nixon *et al.*, 2019), gràcies al fet que la recol·lecció de fluid seminal en animals d'experimentació és més senzilla. Les estratègies que han seguit aquests grups de recerca per a aïllar les VEs i posteriorment analitzar el seu contingut proteic s'han basat en l'aïllament de les VEs per ultracentrifugació (Thimon *et al.*, 2008; García-Rodríguez *et al.*,

2018; Murdica *et al.*, 2019a), ultracentrifugació acompanyada d'una cromatografia d'exclusió de mida (Frenette *et al.*, 2006; Girouard *et al.*, 2011) i gradients de densitat (Poliakov *et al.*, 2009; Frenette *et al.*, 2010; Yang *et al.*, 2017; Lin *et al.*, 2019; Nixon *et al.*, 2019). Tot i que la informació proporcionada per tots aquests estudis és d'un valor incalculable, i han permès una primera caracterització del proteoma de les VEs seminals, els resultats s'han de prendre amb cautela, doncs les metodologies utilitzades per aïllar les VEs no asseguren l'eliminació de microvesícules o d'altres contaminants. Aquest fet reflecteix la necessitat actual de nous estudis que realitzin una purificació més específica de les VEs, el que permetrà descobrir quina és la veritable rellevància biològica de les VEs seminals i elucidar l'impacte que tenen les VEs en la funció espermàtica i en la reproducció.

2

HIPÒTESI I OBJECTIUS

HIPÒTESI I OBJECTIUS

La infertilitat és un problema freqüent a escala mundial cada cop més comú. No obstant això, la manca de comprensió de la biologia reproductiva masculina i dels mecanismes moleculars alterats en pacients infèrtils resulta en una disponibilitat limitada i insuficient d'eines diagnòstiques i pronòstiques per a l'avaluació de la fertilitat masculina, així com de tractaments per a la infertilitat. Tot i que l'espermatozoide és la peça clau en la transmissió de la informació paterna a l'embrió, hi ha evidències que els fluids secretats per l'epidídim i les glàndules sexuals accessòries, incloent-hi les vesícules extracel·lulars, juguen un paper molt més important en la reproducció masculina que el de simplement ser el mitjà de transport dels espermatozoides. La hipòtesi d'aquesta tesi és que l'aplicació de tècniques proteòmiques d'alt rendiment en l'estudi del plasma seminal de pacients infèrtils podria permetre la identificació de nous potencials biomarcadors clínics no invasius que permetin una millora en la classificació actual de la infertilitat i en l'optimització del seu diagnòstic i tractament, i que ens apropin al diagnòstic personalitzat de la infertilitat masculina. A més, l'estudi de la comunicació intercel·lular entre vesícules extracel·lulars i espermatozoides, i l'aplicació de noves metodologies per a l'obtenció de poblacions específiques de diferents tipus de vesícules extracel·lulars, podrien revelar la veritable importància de les vesícules extracel·lulars de l'epidídim i de les glàndules sexuals accessòries en la funció espermàtica, el que proporcionaria les bases per a futurs estudis orientats a identificar nous biomarcadors diagnòstics i pronòstics, així com desenvolupar noves teràpies per a millorar la fertilitat masculina.

Per tant, els objectius d'aquesta tesi doctoral són els següents:

1. Analitzar els perfils proteòmics del plasma seminal de pacients amb diferents tipus d'infertilitat amb la finalitat d'identificar nous potencials biomarcadors de fertilitat/infertilitat útils per a l'assistència clínica.
    - 1.1. Caracteritzar el proteoma del plasma seminal de pacients infèrtils categoritzats segons els paràmetres seminals (normozoospèrmics, astenozoospèrmics, oligozoospèrmics i azoospèrmics).
    - 1.2. Caracteritzar el proteoma del plasma seminal de pacients infèrtils afectes d'hipogonadisme hipogonadòtropic, abans i després de la teràpia de reemplaçament de testosterona.
2. Explorar la possible comunicació intercel·lular entre les vesícules extracel·lulars seminals i l'espermatozoide, i determinar el seu impacte en la funció espermàtica.
    - 2.1. Demostrar la participació dels epididimosomes (vesícules extracel·lulars secretades per l'epidídim) en la transferència de proteïnes entre l'epidídim i els espermatozoides.
    - 2.2. Determinar l'impacte de les vesícules extracel·lulars seminals CD63+ en la funció espermàtica mitjançant el seu aïllament amb tècniques d'immunoafinitat, i caracteritzar el seu contingut mitjançant proteòmica d'alt rendiment.

HYPOTHESIS AND OBJECTIVES

Infertility is a common and rising problem worldwide. However, the lack of understanding of male reproductive biology and the molecular mechanisms altered in infertile patients results in a limited and insufficient availability of male infertility diagnostic and prognostic tools, as well as fertility treatments. Although the sperm is the key piece in the transmission of paternal information to the embryo, there is evidence that the fluids secreted by the epididymis and male accessory sex glands, including the seminal EVs, play a pivotal role in male reproduction more than simply being a medium to carry the spermatozoa. The hypothesis of this thesis is that the use of high-throughput proteomic techniques for the study of the seminal plasma of infertile patients could allow the identification of novel and non-invasive clinical biomarkers, which could result in an improvement of the current male infertility stratification and an optimization of their diagnostic and treatment, moving towards a personalized evaluation of male infertility. Also, the study of the intercellular communication between seminal EVs and sperm, and the application of new methodologies to obtain specific populations of the different types of EVs, could contribute to revealing the real biological roles of seminal EVs from the epididymis and the male accessory sex glands in sperm function. These findings could provide the basis for future studies aimed at identifying new diagnostic and prognostic biomarkers, as well as developing new therapies to improve male fertility.

Therefore, the objectives of this doctoral thesis are:

1. *To analyze the seminal plasma proteomic profile of patients with different types of infertility in order to identify new potential biomarkers of fertility/infertility useful for clinical care.*
    - 1.1. *To define the seminal plasma proteome signatures of infertile patients categorized according to their seminal parameters (normozoospermia, asthenozoospermia, oligozoospermia, and azoospermia).*
    - 1.2. *To characterize the seminal plasma proteome of infertile patients with hypogonadotropic hypogonadism, before and after testosterone replacement therapy.*
2. *To explore the potential intercellular communication between seminal EVs and sperm, and to determine the impact of this on sperm function.*
    - 2.1. *To demonstrate the participation of epididymosomes (EVs secreted by the epididymis) in protein transfer between epididymis and sperm.*
    - 2.2. *To determine the impact of CD63+ seminal EVs on sperm function by isolating the EVs with immunoaffinity techniques, and to characterize their protein content by high-throughput proteomics.*

3 TREBALLS

TREBALL 1

L'ANÀLISI DE PARELLS DE PROTEÏNES ESTABLES COM A NOVA ESTRATÈGIA PER IDENTIFICAR PERFILS PROTEÒMICS: APLICACIÓ AL PLASMA SEMINAL DE PACIENTS INFÈRTILS

Molecular & Cellular Proteomics

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L'anàlisi de parells de proteïnes estables com a nova estratègia per identificar perfils proteòmics: aplicació al plasma seminal de pacients infèrtils

Objectiu: L'objectiu d'aquest treball és definir els perfils proteòmics del plasma seminal de pacients infèrtils classificats segons els seus paràmetres seminals (normozoospèrmics, NZ; astenozoospèrmics, AS; oligozoospèrmics, OS; i azoospèrmics, AZ) utilitzant un marcatge proteic amb *tandem mass tags* (TMT) seguit d'una anàlisi de proteòmica per LC-MS/MS.

Materials i Mètodes: S'ha obtingut plasma seminal de pacients NZ (n=4), AS (n=4), OZ (n=4) i AZ (n=4) provinents de la clínica de reproducció assistida de l'Hospital Clínic. Les proteïnes presents en el plasma seminal dels diferents pacients s'han digerit, i els pèptids s'han marcat amb TMT (TMT 10-plex) i s'han identificat i quantificat per LC-MS/MS. L'anàlisi de les dades proteòmiques s'ha realitzat per mètodes convencionals (ANOVA i correlació de Pearson), i mitjançant una nova metodologia dissenyada estratègicament basada en l'anàlisi de parells de proteïnes estables (PPE). Les validacions experimentals s'han realitzat en extractes proteics de plasma seminal d'un set independent de pacients (n=18, 6 NS, 6 OZ, 6 AZ) mitjançant western blot, i en epidídim (n=1) i testicle (n=1) humà mitjançant immunohistoquímica.

Resultats: Un total de 349 proteïnes s'han identificat i quantificat sota estrictes criteris de qualitat en el proteoma del plasma seminal de 16 pacients infèrtils. S'han caracteritzat els perfils proteòmics de cadascun dels individus i aquests s'han comparat agrupant els diferents individus segons els paràmetres seminals (4 grups: NZ, AS, OZ, AZ). Mitjançant anàlisis convencionals s'han identificat 5 proteïnes positivament correlacionades amb la concentració d'espermatozoides, el que s'ha validat posteriorment per western blot per la proteïna epididimària *Extracellular matrix protein 1* (ECM1). Estudis *in silico* han mostrat que 3 de les 5 proteïnes del plasma seminal correlacionades amb la concentració d'espermatozoides s'expressen específicament en l'epidídim i no en testicle. Aquesta localització específica en l'epidídim humà s'ha validat mitjançant immunohistoquímica per 2 de les 3 proteïnes (ECM1 i *NPC intracellular cholesterol transporter 2* (NPC2)). No obstant, a causa de l'alta heterogeneïtat del plasma seminal i a la presència de fragments proteolítics, modificacions post-traduccionals de proteïnes i diferents isoformes, la correlació de l'abundància de proteïnes del plasma seminal amb la concentració d'espermatozoides no era tan clara quan les anàlisis eren realitzades a nivell de pèptids, i només NPC2 mantenia la correlació de la majoria dels seus pèptids amb la concentració espermàtica. Per tal de reduir l'heterogeneïtat de les dades proteòmiques s'ha desenvolupat una nova anàlisi basada en la determinació dels parells de proteïnes estables que ha permès identificar, en la població control (NZ), un total de 182 correlacions estables que incloïen 24 proteïnes. Per contra, el número de PPE s'ha reduït dràsticament en pacients que presenten paràmetres seminals alterats (18 en AS, 0 en OZ i 3 en AZ), indicant la presència d'una heterogeneïtat molt més elevada dins del grup de

pacients classificats segons l'alteració dels paràmetres seminals. La repetició de l'anàlisi de PPE en la població control mitjançant l'addició d'un pacient infèrtil cada cop ha permès identificar parells de proteïnes estables desregulades en pacients individuals.

Conclusions: El disseny d'un nou mètode per a l'anàlisi de les dades proteòmiques ha demostrat que l'actual estratificació de la infertilitat masculina basada en paràmetres seminals no és suficient per a l'obtenció d'un bon diagnòstic i suggereix l'existència d'etiologies múltiples que desencadenen en un mateix fenotip alterat. L'aplicació d'aquesta nova metodologia per a l'estudi de mostres individuals permet la identificació de noves proteïnes o mecanismes alterats en la infertilitat masculina, i obre la porta a un diagnòstic personalitzat.

Stable-protein Pair Analysis as A Novel Strategy to Identify Proteomic Signatures: Application to Seminal Plasma From Infertile Patients

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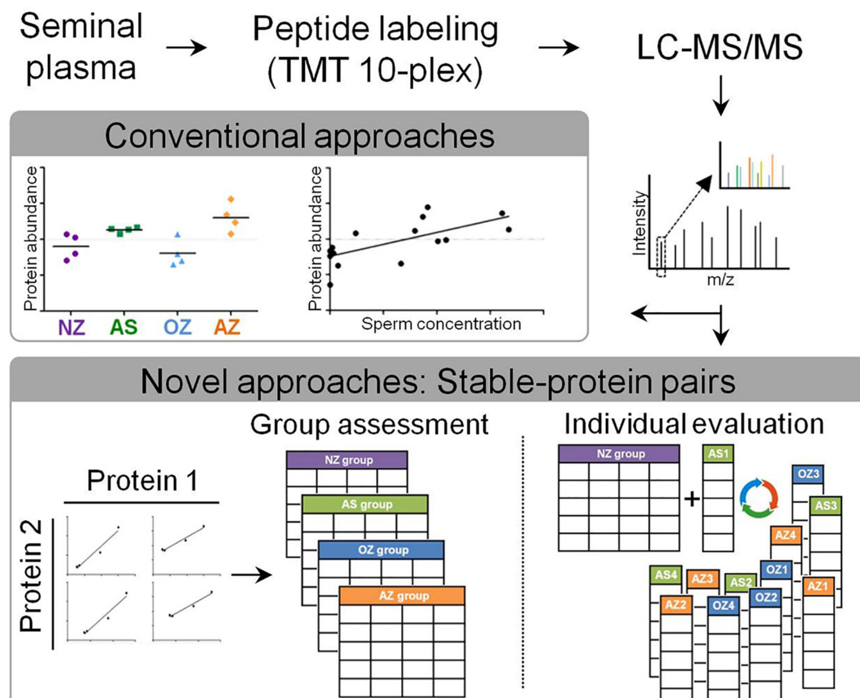
In Brief

The seminal plasma proteome from infertile patients differing in seminal parameters was determined using quantitative MS-based proteomics. Conventional analyses together with a new strategy based on the identification of stable-protein pairs were conducted. A stable-protein pair pattern was established for normozoospermic individuals but not for patients-groups with altered seminal parameters, reflecting multiple causes affecting seminal parameters. Moreover, the evaluation of stable-proteomic pattern in control population, adding an individual patient once a time, opens a window to personalized male infertility diagnosis.

Graphical Abstract



Highlights

- Differences in the analysis of quantitative proteomics data at protein or peptide level.
- Stable-protein pairs as a new approach to analyze quantitative proteomic data.
- Seminal plasma proteome in patients with altered seminal parameters is heterogeneous.
- Stable-protein pairs may open a window to personalized male infertility diagnosis.

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Stable-protein Pair Analysis as A Novel Strategy to Identify Proteomic Signatures: Application To Seminal Plasma From Infertile Patients*

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Our aim was to define seminal plasma proteome signatures of infertile patients categorized according to their seminal parameters using TMT-LC-MS/MS. To that extent, quantitative proteomic data was analyzed following two complementary strategies: (1) the conventional approach based on standard statistical analyses of relative protein quantification values; and (2) a novel strategy focused on establishing stable-protein pairs. By conventional analyses, the abundance of some seminal plasma proteins was found to be positively correlated with sperm concentration. However, this correlation was not found for all the peptides within a specific protein, bringing to light the high heterogeneity existing in the seminal plasma proteome because of both the proteolytic fragments and/or the post-translational modifications. This issue was overcome by conducting the novel stable-protein pairs analysis proposed herein. A total of 182 correlations comprising 24 different proteins were identified in the normozoospermic-control population, whereas this proportion was drastically reduced in infertile patients with altered seminal parameters (18 in patients with reduced sperm motility, 0 in patients with low sperm concentration and 3 in patients with no sperm in the ejaculate). These results suggest the existence of multiple etiologies causing the same alteration in seminal parameters. Additionally, the repetition of the stable-protein pair analysis in the control group by adding the data from a single patient at a time enabled to identify alterations in the stable-protein pairs profile of individual patients with altered seminal parameters. These results suggest potential underlying pathogenic mechanisms in individual infertile patients, and might open up a window to its application in the personalized diagnostic of male infertility. *Molecular & Cellular Proteomics 18: S77–S90, 2019. DOI: 10.1074/mcp.RA118.001248.*

Infertility is a worldwide frequent problem that affects ~15% of reproductive-aged couples. Around the 50% of the fertility problems is because of a male factor and, from those, the 40–60% of the infertile patients present some alterations in at least one of the seminal parameters assessed by a routine semen analysis (sperm concentration, motility and morphology) (1). According to these seminal parameters, infertile patients are categorized in: (i) patients with low or absent sperm concentration (oligozoospermia or azoospermia, respectively), (ii) patients with defective sperm motility (asthenozoospermia) and/or (iii) patients with abnormal sperm morphology (teratozoospermia) (2). The semen evaluation, together with a complete medical history and physical examination, will determine whether the initial assessment needs to be complemented with genetic and/or hormonal analyses, urinalysis or testicular biopsies. Unfortunately, the current available tools for the evaluation of male fertility are limited and insufficient, and the development of new methodologies to better discern the male factor etiology is required (3–6). Currently, the application of high-throughput proteomics for the study of the human sperm cell has resulted in the identification of 6871 proteins as well as some pathogenic mechanisms involved in male infertility (7–11). For instance, studies on the sperm proteome from asthenozoospermic patients revealed alterations mainly in proteins and pathways related with energy production and cytoskeleton (9, 12). However, because the semen is not just composed by sperm cells, the exploration of seminal plasma could help to better elucidate the causes of male infertility.

The human seminal plasma is a complex and protein-enriched biological fluid that constitutes 95% of the semen volume, whereas only the remaining 5% corresponds to sper-

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matozoa (10). This fluid is composed by secretions from the testis (1–2%), the epididymis (2–4%) and the male accessory sex glands including the seminal vesicles (65–75%), the prostate gland (25–30%) and the bulbourethral glands ($< 1\%$) (13–15). Seminal plasma contains a diversity of molecules including DNA, RNA, microRNAs, lipids, proteins and metabolites, together with a highly abundant population of extracellular vesicles, which are mainly secreted by the prostate (10, 16). So far, the compiled proteome profile of human seminal plasma includes 2064 non-redundant proteins from 9 independent studies, revealing that this fluid is not only a simple medium to carry the spermatozoa through the female reproductive tract (10, 17). In fact, once the spermatogenesis is completed, the testicular spermatozoa, although morphologically differentiated, are immotile germ cells unable to fertilize the oocyte by its own (10). Seminal plasma plays a crucial role for the sperm maturation, motility and capacitation, prevention of premature acrosome reaction and sperm-to-zona pellucida recognition and fusion, thereby providing to the spermatozoa the capability to fertilize the oocyte (18–25). Additionally, the contact of the seminal plasma with the female reproductive tract provides an optimal environment that enhances embryo implantation and development as well as contributes promoting maternal immune tolerance of the semiallogenic fetus (26–29).

The analysis of the seminal plasma proteome is currently undergoing an intense study (14, 30). With the aim to shed light on the physiological role of the seminal plasma and to seek for specific protein biomarkers for male infertility diagnosis and/or prognosis, several groups have studied the seminal plasma proteome in certain subtypes of male infertility and/or alterations associated with oxidative stress and sperm functional traits, among others (31–49). In addition, there is an interest to identify seminal plasma protein biomarkers that could be predictive for the presence of sperm cells in the testis of azoospermic patients, which could avoid unnecessary testicular biopsies (34, 50–57).

Therefore, the analysis of the seminal plasma proteome mimicking the infertility classification according to seminal parameters is warranted, because it might help to decipher potential pathogenic mechanisms resulting in these sperm alterations. However, it is important to consider a wide range of factors may alter both sperm and seminal plasma compositions, leading to a high heterogeneity within patients sharing the same phenotype. This is challenging for the conventional analysis of the quantitative proteomic data based on the search of differential proteins between groups of patients with similar characteristics. Therefore, the main aim of the present study was to define the seminal plasma proteome signatures of infertile patients categorized according to their seminal parameters (normozoospermia, NZ[1]; asthenozoospermia, AS; oligozoospermia, OZ; azoospermia, AZ) by applying conventional and novel approaches for the analysis of quantitative proteomic data to try to better stratify the different subgroups of infertile patients. The results derived from this study suggest that the combination of conventional and novel analytical approaches may be useful toward the identification of pathogenic mechanisms of male infertility and, furthermore, to provide the bases for future studies to design new therapies to improve male fertility and move toward the application of an individual and personalized diagnostic of male infertility.

EXPERIMENTAL PROCEDURES

Biological Material and Sample Collection—Semen samples - Human semen samples ($n = 34$) were obtained from patients undergoing routine semen analysis at the Assisted Reproduction Unit from the Clinic Institute of Gynaecology, Obstetrics and Neonatology, from the Hospital Clínic (Barcelona, Spain), after signed informed consent. The ejaculates were collected by masturbation into sterile containers after 3–5 days of sexual abstinence. The evaluation of the seminal parameters was performed using the automatic semen analysis system CASA (Proiser, Paterna, Spain), and the sperm viability test was assessed using 0.5% (w/v) Eosin Y, following the WHO recommendations (2). The semen samples included in this study were classified according to semen parameters as normozoospermic (NZ, $n = 10$), asthenozoospermic (AS, $n = 4$), oligozoospermic (OZ, $n = 10$), and azoospermic (AZ, $n = 10$) (supplemental Table S1).

Testis and epididymis tissues - Biopsies from normal testis ($n = 1$) and normal epididymis ($n = 1$) were provided by the Department of Pathology from the Hospital Clínic (Barcelona, Spain).

Ethics Statement - All samples were used in accordance to the appropriate ethical guidelines and Internal Review Board, and the biological material storing and processing was approved by the Clinical Research Ethics Committee of the Hospital Clínic of Barcelona (Barcelona, Spain). The written informed consent was obtained from all subjects in accordance with the Declaration of Helsinki.

Purification and Isolation of Proteins from Seminal Plasma—Liquefied semen samples were centrifuged at 500 $\times$ *g* for 10 min and 1500 $\times$ *g* for 10 min, to separate the sperm cells from the seminal plasma. The resulting seminal plasma was filtered (0.45 μm pore size) to remove any cellular leftovers. All cell-free seminal plasma samples were frozen at -80 °C until further processing. After thawing, seminal plasma samples were centrifuged at 16,000 $\times$ *g* for 10 min at 4 °C and the protein concentration from the supernatant of each sample was determined using the BCA protein Assay Kit (Pierce™ BCA protein Assay Kit, Thermo Fisher Scientific, Rockford, IL), following manufacturer's recommendations.

Seminal Plasma Peptide Isotopic Labeling (TMT 10-plex)—A total of 16 seminal plasma samples were selected for the proteomics study, including 4 NZ, 4 AS, 4 OZ and 4 AZ patients (Fig. 1). Differential peptide labeling was performed using TMT 10-plex isotopic label reagent set (TMT 10-plex Mass Tag Labeling; Thermo Fisher

[1] The abbreviations used are: NZ, normozoospermia; AS, asthenozoospermia; OZ, oligozoospermia; AZ, azoospermia; CASA, computer assisted semen analysis; WHO, World Health Organization; BCA, bicinchoninic acid; TMT, tandem mass tag; TEAB, triethyl ammonium bicarbonate; TCEP, tris (2-carboxyethyl) phosphine; IAA, iodoacetamide; RT, room temperature; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; MS/MS, tandem mass spectrometry; HCD, higher energy collision dissociation; FDR, false discovery rate; PSMs, peptide spectrum matches; ANOVA, one-way analysis of variance; HPA, Human Protein Atlas; TBST, TBS with 0.1% (v/v) Tween 20; PAS, periodic acid and schiff's reagent; SEMGs, semenogelins; PTMs, post-translational modifications.

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Scientific), following manufacturer's instructions. Briefly, 60 μg of protein from each seminal plasma sample was adjusted to a final volume of 60 μl with 100 mM TEAB, and protein quantification was repeated to ensure that all samples had the same concentration (1 μg/μl). Proteins were reduced in 9.5 mM TCEP for 1 h at 55 °C, alkylated with 17 mM IAA for 30 min in the dark, and precipitated with 500 μl of cold 100% acetone at −20 °C overnight. Samples were centrifuged at 17,500 × *g* for 10 min at 4 °C and the acetone-precipitated protein pellets were resuspended in 60 μl of 100 mM TEAB. Trypsin was then added at 1:20 protein-to-protease ratio and incubated overnight at 37 °C with constant shaking. Prior to peptide labeling, aliquots with the same volume and concentration were taken out from each of the 16 samples and combined, in order to constitute the internal control. After that, 30 μg of peptides from each individual seminal plasma sample ($n = 16$) and internal control ($n = 1$) were labeled with TMT isobaric tags (reporter ions intensity from *m/z* 127.1 to *m/z* 131.1 (TMT-127N, -127C, -128C, -129N, -129C, -130N, -130C, -131), and *m/z* 126 (TMT-126), respectively; Fig. 1). Specifically, 19.5 μl of the TMT label reagents previously equilibrated at RT and dissolved in ACN (Sigma-Aldrich, St. Louis, MO) were added to the corresponding reduced and alkylated peptides. After 1 h of incubation at RT, the reaction was quenched with 4 μl of 5% hydroxylamine for 15 min. Labeled peptides from each sample were combined at equal amounts constituting two different multiplex pools (Pool A and Pool B; each one consisting of 8 different samples plus the same internal control; Fig. 1), which were dried in a speed-vacuum centrifuge and peptides were resuspended in 20 μl of 0.5% TFA (Sigma-Aldrich) in 5% ACN. Finally, the peptides were cleaned up via reversed-phase C18 spin columns (Pierce C18 Spin Columns, Thermo Fisher Scientific), following manufacturer's instructions.

LC-MS/MS Analysis—Labeled peptides were analyzed by a nano-LC Ultra 2D Eksigent (AB Sciex, Brugg, Switzerland) attached to an LTQ-Orbitrap Velos (Thermo Fisher Scientific). For HPLC separation, peptides were injected onto a C18 trap column (L 0.5 cm, 300 μm ID, 5 μm, 100 Å; Thermo Fisher Scientific). Chromatographic analyses were performed using an analytical column (L 15 cm, 75 μm ID, 3 μm, 100 Å; Thermo Fisher Scientific). Two different buffer systems were used for the analysis: buffer A (97% H_2O-3% ACN, 0.1% Formic acid) and buffer B (3% H_2O/97% ACN, 0.1% Formic acid). The following gradient was applied for peptide separation on the analytical column: from 0–4 min 0% of B to 4% of B, from 4–300 min 4% of B to 35% of B, from 300–305 min 35% of B to 100% of B, at a flow rate of 400 nl/min, and from 305–320 min 100% of B at a flow rate of 400 nl/min. MS/MS analyses were performed using an LTQ-Orbitrap Velos (Thermo Fisher Scientific) directly coupled to a nano-electrospray ion source. The LTQ-Orbitrap Velos settings included one 30,000 resolution at 400 *m/z* MS1 scan for precursor ions followed by MS2 scans of the 50 most intense precursor ions, at 30,000 resolution at 400 *m/z*, in positive ion mode. The lock mass option was enabled, and polysiloxane (*m*/z 445.12003) was used for internal recalibration of the mass spectra. MS/MS data acquisition was completed using Xcalibur 2.1 (Thermo Fisher Scientific). The normalized collision energy for HCD-MS2 was set to 40%.

Protein Identification—LC-MS/MS data was analyzed using Proteome Discoverer 1.4.1.14 (Thermo Fisher Scientific). For database searching, raw mass spectrometry files were submitted to the in-house Homo sapiens UniProtKB/Swiss-Prot database with Sus scrufa Trypsin added to it (HUMAN_Tryp_UP_SP_R_2016_03.fasta; released March 2016; 20155 protein entries) using SEQUEST HT version 28.0 (Thermo Fisher Scientific). For re-scoring, percolator search node was used. Searches were performed using the following parameters: five maximum missed cleavage sites for trypsin, TMT-labeled lysine (+229.163 Da) and methionine oxidation (+15.995 Da) as dynamic modifications, cysteine carbamidomethylation (+57.021 Da) as a static modification, 20 ppm precursor mass tolerance, 0.6 Da fragment mass tolerance, 5 mmu peak integration tolerance, and most confident centroid peak integration method. Percolator was used for protein identification with the following identification criteria: at least one unique peptide per protein with a FDR of 1%.

Proteomic Data Analysis by Conventional and Novel Approaches—Conventional relative protein quantification - Normalized TMT quantitative values for each identified spectrum derived from the ratio of the intensity of reporter ions from HCD MS2 spectra corresponding to each individual samples (TMT-127N to TMT-131) with the internal control (TMT-126), which were obtained using Proteome Discoverer software (supplemental Fig. S1) (12, 58). Different isoforms of the same protein were treated as dissociated or "ungrouped" from their respective families, to avoid any possible ambiguity (12, 58). Only those proteins with at least 1 unique peptide quantified by ≥ 2 PSMs in all the samples and a coefficient of variation < 50% in at least 75% of the samples were considered for further statistical analyses. Significant statistical differences among the different subtypes of infertile patients were evaluated after normalizing the relative proteomic quantification values by log2 transformation using ANOVA combined with Tukey's multiple comparison test. Additionally, the correlations between sperm concentration and normalized relative proteomic quantification values were assessed using Pearson correlation test followed by the adjustment of the *p* values to FDR. An MS expert checked the spectra of all the differential proteins.

Establishment of Stable-Protein Pairs Profile—The intensity values from HCD MS2 spectra corresponding to each individual sample (TMT-127N to TMT-131), but not from the internal control (TMT-126), were used to establish the stable-protein pairs for each group of patients. This strategy has been previously applied to the study of stable-transcript pairs obtained from RNA-seq data (59, 60). In our case, only those proteins with at least 2 unique peptides quantified by ≥ 2 PSMs for all samples with a coefficient of variation < 50% in at least 75% of the samples were considered. Stable-protein pairs were determined by applying the following statistical principle: 2 proteins (with more than 1 peptide quantified for each one) were highly-correlated when ≥ 75% of the possible peptide combinations had a Pearson correlation coefficient ≥ 0.9 (Fig. 2). In order to determine alterations in individual samples, stable-protein pair analysis was repeated for the control group (NZ patients) by adding a patient with altered seminal parameters once at a time.

Functional Enrichment and Expression Analyses Using Public Databases—The seminal plasma proteomic datasets were uploaded to the Gene Ontology Consortium database (http://www.geneontology.org/) (61), based on PANTHER v13.1 database (Release date 2018–02-03), in order to predict the functional involvement of the seminal plasma proteins. The significance of enrichment analyses was calculated by a Fisher's exact test. *p* values < 0.05 after FDR adjustment were considered statistically significant.

The HPA Database (http://www.proteinatlas.org/) (62, 63) was used to assess the expression of specific proteins in different human male reproductive tissues.

Immunoblotting—Protein extracts from cell-free seminal plasma samples from an independent set of patients ($n = 18$; 6 NZ, 6 OZ, 6 AZ; supplemental Table S1) were used for Western blotting validation of ECM1 protein. A total of 40 μg of seminal plasma protein extracts from each sample were separated by SDS-PAGE and transferred onto Immobilon-P PVDF membranes (Merck Millipore, Tullagreen, Ireland) as described elsewhere (7). The membranes were blocked in TBST and 5% (w/v) skim milk for 1 h at RT. For immunostaining, anti-ECM1 antibody (polyclonal rabbit ECM1 antibody, #43263, SAB Signalway Antibody, Baltimore, MD) diluted 1:500 in TBST was used. After washing in TBST, membranes were incubated with an ECL horseradish peroxidase-labeled donkey anti-rabbit IgG antibody (Am-

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ersham Biosciences™, GE Healthcare, Little Chalfont, UK). Finally, the detection was done using WesternBright™ ECL Western blotting detection kit (Advansta, Menlo Park, CA) and a LAS-4000 image analyzer (ImageQuant LAS 4000, GE Healthcare Life Sciences). Optical densities from ECM1 bands were obtained and analyzed using Quantity One software (BioRad, Hercules, CA). The correlation between sperm concentration and the optical densities values from ECM1 was evaluated using Pearson correlation test.

Immunohistochemistry—Cross-sections from normal testis ($n = 1$) and a normal epididymis ($n = 1$) were used to detect the expression pattern of ECM1 and NPC2. Bouin's fixed, paraffin-embedded testicular and epididymal sections (4 μm) were deparaffinized in toluene (3×) and hydrated through graded series of ethanol (100%, 100%, 90%, 70%, H_2O milliQ), with a 0.3% hydrogen peroxide (Sigma-Aldrich) intermediate incubation between the two 100% ethanol incubations. For antigen retrieval, sections were incubated with 10 mM sodium citrate (pH 6.0) at 99.5 °C for 20 min. Sections were blocked with PBS-5% skim milk for 30 min at RT and, then, incubated with Avidin/Biotin Blocking Kit (Vector Laboratories, Burlingame, CA). Afterward, sections were incubated for 16 h at 4 °C with the primary antibody of interest: anti-ECM1 (polyclonal rabbit ECM1 antibody, #EPP12545, Elabscience, Houston, TX) diluted 1:20 in PBS-1% skim milk, and anti-NPC2 (polyclonal rabbit NPC2 antibody, #CQA1207, Cohesion Biosciences, London, UK) diluted 1:100 in PBS-5% skim milk. Negative controls for nonspecific binding of the primary antibodies were included using isotype rabbit IgG (Vector Laboratories). Then, sections were incubated with a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories), and with an avidin-biotin-peroxidase detection kit (Vectastain ABC Elite Kit, Vector Laboratories). Finally, slides were incubated with ImmPACT™ DAB Peroxidase Substrate kit (Vector Laboratories). All slides were then washed and stained with hematoxylin, and testis slides were additionally stained with PAS (Sigma-Aldrich). Sections were dehydrated in 100% ethanol (3×), cleared in toluene (3×) and mounted in Eukitt Mounting Medium (Sigma-Aldrich) to be finally analyzed by a transmission light microscope (Olympus BX50, Olympus, Tokyo, Japan).

Experimental Design and Statistical Rationale—The experimental design of this study is shown in Fig. 1. Specifically, seminal plasma proteome from 16 infertile patients including individuals with normal seminal parameters (NZ; control group) and infertile patients with altered seminal parameters (AS, OZ and AZ patients; supplemental Table S1) was characterized and compared. A total of 4 biological replicates per group were used for proteomic analysis. Significant statistical differences among the different subtypes of infertile patients were evaluated after the normalization of the relative proteomic quantification values by log2 transformation using ANOVA combined with Tukey's multiple comparison test. Because some differential proteins showed a gradual decreasing reliant on sperm concentration, we tested the correlation between sperm concentration and normalized values of the relative proteomic quantification using Pearson correlation test followed by the p value adjustment to FDR. To further validate these results, immunoblotting analysis of one differential protein (ECM1) was performed in an independent set of infertile patients as biological replicates (6 NZ, 6 OZ, 6 AZ). The correlation between sperm concentration and the abundance of ECM1 protein was evaluated using Pearson correlation test.

The expression profiles of the differential proteins in human reproductive tissues were also assessed using the information available at the HPA, in order to discern whether the proteins correlated with sperm concentration were just reflecting a variation in the amount of sperm leftovers in the ejaculate or, in contrast, were the result of some proteomic alterations in the secretions from accessory sex glands. Additionally, immunohistochemistry was performed in testicular ($n = 1$) and epididymal ($n = 1$) biopsies to infer the tissue origin of 2 differentially expressed proteins, ECM1 and NPC2. Finally, the correlation between sperm concentration and normalized relative protein abundance was also explored at peptide level, because discrepancies within our findings and results published by others suggested differences on the specific peptides analyzed for each protein (50–52).

In order to assess the heterogeneity of the seminal plasma proteomic profile within the subgroups of infertile patients characterized according to seminal parameters as well as the specific protein alterations in individual patients, a new approach based on the analysis of the stable-protein pairs was conducted.

Both, statistical analyses and the establishment of stable-protein pairs were carried out using R software version 3.4.4 (http://www.r-project.org) (64). p values < 0.05 were considered statistically significant. All graphs were constructed using GraphPad Prism software version 5.01 (GraphPad Software Inc., San Diego, CA).

RESULTS

Proteomic Analysis of Human Seminal Plasma—LC-MS/MS analysis resulted in the identification of a total of 349 proteins in the seminal plasma proteome from 16 infertile patients, with at least one unique peptide and 1% FDR (supplemental Tables S2 and S3). However, just 60 of the 349 seminal plasma proteins fit our strict quantification criteria (at least 1 unique peptide quantified with ≥ 2 PSMs in all samples with a coefficient of variation $< 50\%$ in at least 75% of the samples) and, therefore, only these proteins were used for subsequent analyses. Detailed information of the quantifiable peptides and corresponding proteins are presented in supplemental Tables S4 and S5, respectively.

With the aim to assess the potential role of seminal plasma proteins in the functionality of spermatozoa, as well as the ability of seminal plasma proteome signatures to reflect disturbances in spermatogenesis and sperm maturation processes that could explain the alteration of seminal parameters, we compared the seminal plasma proteome from infertile patients categorized according to their seminal parameters (NZ, AS, OZ, AZ). Two different strategies were applied in our comparative quantitative proteomics study: (i) A conventional approach based on standard statistic analyses (ANOVA and Pearson correlation test) of relative protein quantification values; and (ii) a novel analysis method based on the establishment of the stable-protein pairs separately in groups of patients classified according seminal parameters, as well as the identification of protein alterations in individual samples based on the variations of the stable-protein pairs defined in NZ patients (Fig. 1). The results of each analysis are shown below.

Altered Seminal Plasma Protein Abundance in Infertile Patients—Comparison of the seminal plasma proteomes from the 4 different subtypes of infertile patients (NZ, AS, OZ, AZ) by conventional data analysis revealed a set of 6 differentially expressed proteins among the groups (p value < 0.05; ANOVA with Tukey's Post Hoc test; Fig. 3, supplemental Table S6). Of interest, the Post Hoc test reflected that the protein abundance of 3 of these 6 differentially expressed

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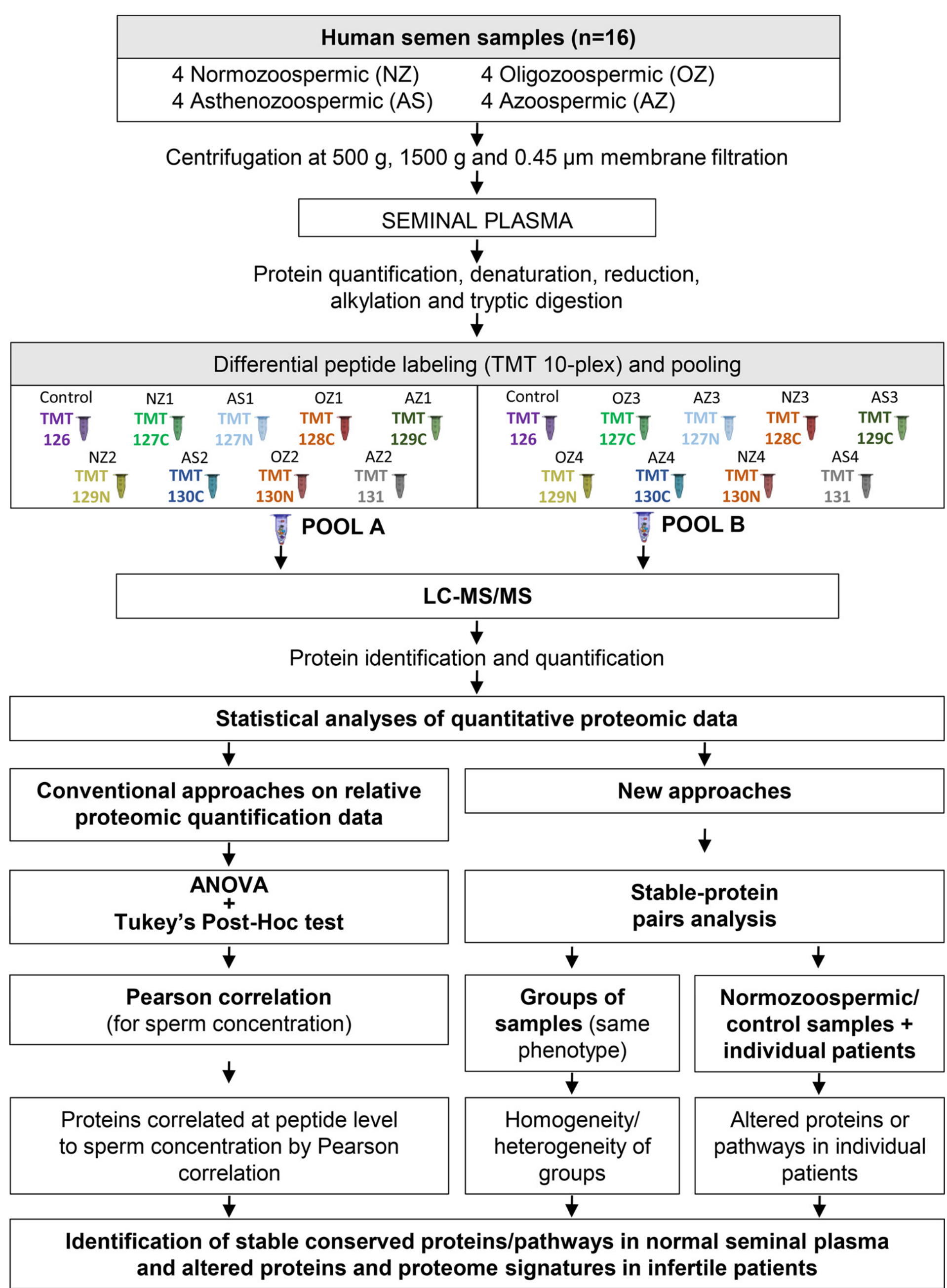

FIG. 1. **Overall strategy used for the identification of proteomic alterations in different subtypes of infertile patients using TMT 10-plex peptide labeling followed by LC-MS/MS.**

proteins (CRISP1, NPC2 and SPINT3) was reduced in patients with low or absence of sperm cells in the ejaculate (OZ and AZ patients, respectively), whereas SCPEP1 protein abundance was increased in AZ patients (Fig. 3). Additionally, only the protein ANPEP displayed reduced protein abundance in patients with decreased sperm motility (AS patients).

Relationship Between Altered Seminal Plasma Proteins and the Sperm Concentration—The correlation between the relative amount of seminal plasma proteins and the sperm concentration parameter was assessed in order to test whether the altered protein abundance detected in OZ and AZ patients relied in the number of sperm cells present in the ejaculate independently of sperm motility rate. Remarkably, the abundance of the proteins SPINT3, NPC2, ECM1, CRISP1, and IGHG2 increased with higher sperm concentration (Table I). To validate these results, a Western blot analysis for ECM1 protein was performed in an independent set of samples not used for MS analysis ($n = 18$; 6 NZ, 6 OZ, 6 AZ). As expected, higher protein abundance for ECM1 protein was found with an increased sperm count (p value < 0.01; Fig. 4). The HPA

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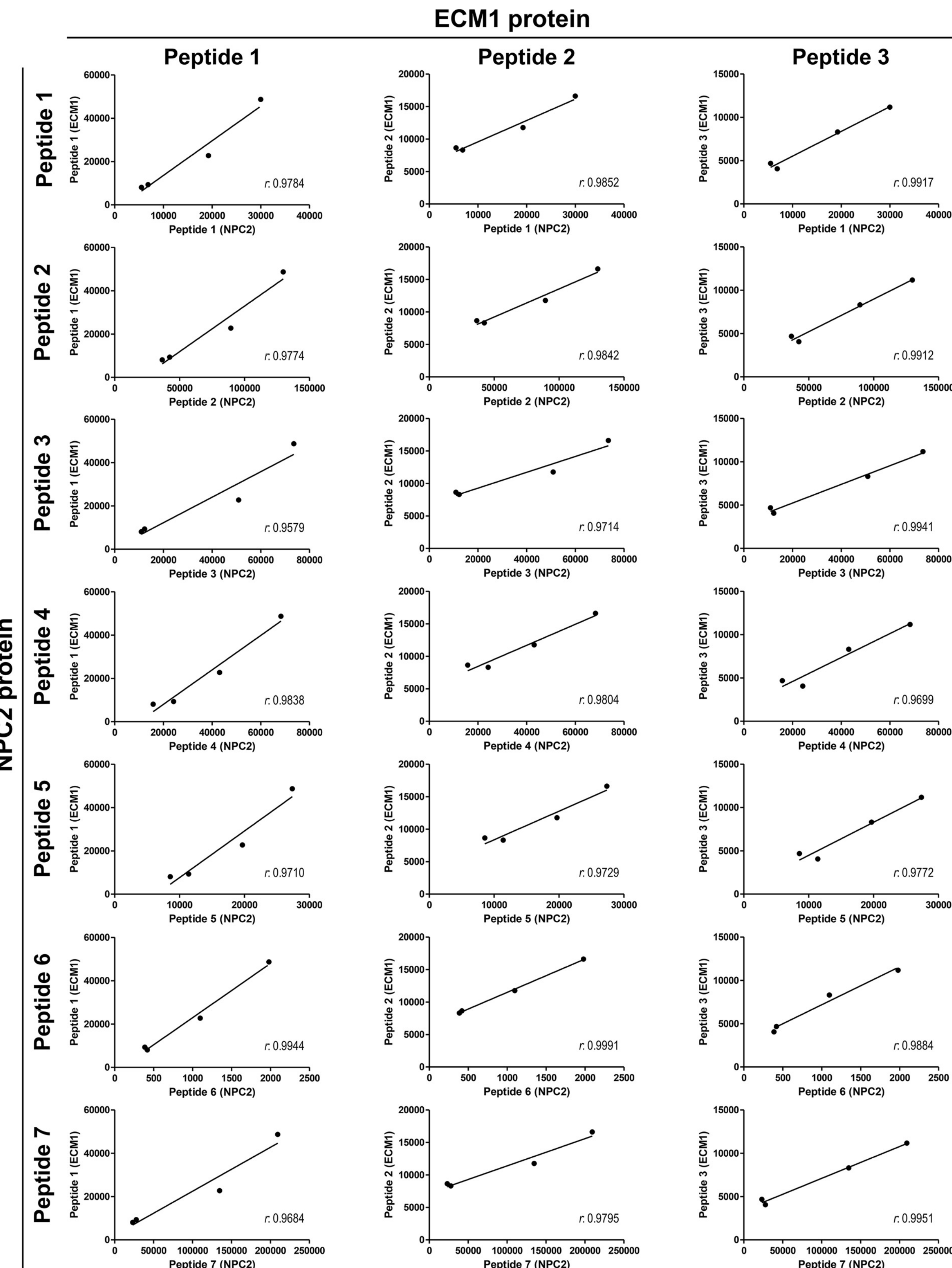

FIG. 2. **Example of ECM1 and NPC2 as stable-correlated proteins in patients with normal seminal parameters (NZ; $n = 4$).** Pearson correlation coefficient (*r*) for ECM1 peptides (3 peptides) and NPC2 peptides (7 peptides) is equal or higher than 0.9 for all the possible peptide-correlations.

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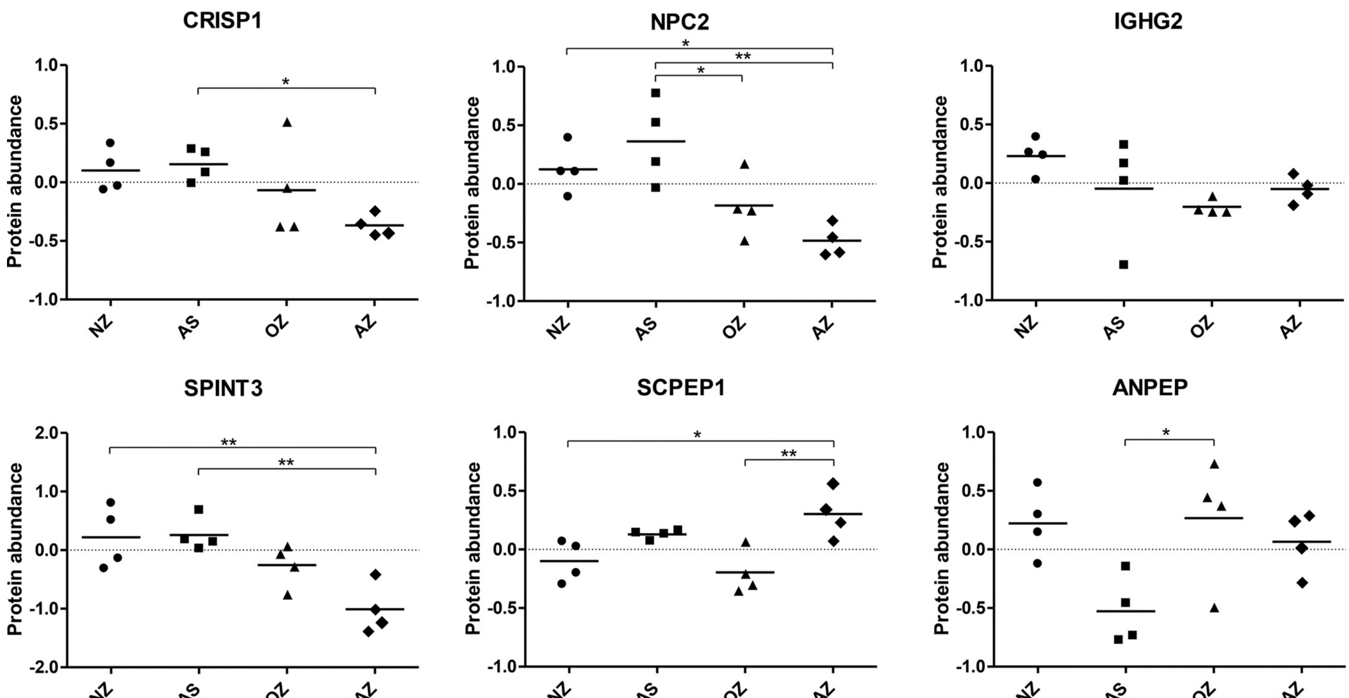

FIG. 3. **Seminal plasma proteins detected with significant different abundance in different subtypes of infertile patients according to seminal parameters.** Statistical comparisons are performed by ANOVA with Tukey's Post Hoc test (* $p < 0.05$; ** $p < 0.01$). Horizontal lines represent mean protein abundance for each group. NZ: Normozoospermic, AS: Asthenozoospermic, OZ: Oligozoospermic, AZ: Azoospermic. The detailed information is found in supplemental Table S6.

TABLE I

Pearson correlation analysis between sperm concentration and seminal plasma protein abundance (LC-MS/MS data). Pearson correlation coefficient, p value and FDR-adjusted p value (q-value) are indicated in the table

Gene name	Uniprot accession number	Pearson correlation (r-value)	Significance (*p* value)	Significance FDR corrected (*q*-value)
SPINT3	P49223	0.746	0.001	**0.03**
NPC2	P61916	0.680	0.004	0.06
ECM1	Q16610	0.683	0.004	0.06
CRISP1	P54107	0.643	0.007	0.08
IGHG2	P01859	0.542	0.030	0.30

database showed that 3 of the 5 seminal plasma proteins positively correlated with sperm concentration are mainly expressed in epididymis, but not detected in testis (Fig. 5*A*). We extended the protein patterns provided by the HPA database by immunohistochemical validation of ECM1 and NPC2 proteins in human testis ($n = 1$) and human epididymis ($n = 1$) biopsies (Fig. 5*B*). However, the analysis at peptide level showed that whereas the majority of peptides quantified for NPC2 protein (6 of 7 peptides) maintain the correlation with sperm concentration, only one peptide quantified for each of the remaining proteins was found correlated (1 of 1 peptide for SPINT3, 1 of 2 peptides for CRISP1 and IGHG2 and 1 of 3 peptides for ECM1; Table II).

Seminal Plasma Stable-Protein Pair Profiles Among Patient Groups and Individuals—A total of 182 stable-protein pairs between 24 different proteins were identified for patients with normal semen parameters (NZ) (Table III). Of note, those 24 proteins are functionally involved in processes already ascribed to seminal plasma, such as the regulation of sperm function, semen coagulation-liquefaction processes, immune system and lipid metabolism, among others. In contrast, very few stable-protein pairs were observed in the different subtypes of patients with altered seminal parameters: 18 stable-protein pairs comprising 16 proteins in AS, 0 in OZ and 3 comprising 5 proteins in AZ (Table III).

In order to assess alterations of stable-protein pairs in individual patients, the analysis of the stable-protein pairs for the NZ patients group was repeated by adding one single patient with an altered seminal parameter at a time (Table IV). This strategy revealed that the asthenozoospermic patient AS2 had a similar seminal plasma proteomic signature to that found in NZ men, because > 75% of the stable-protein pairs established for NZ population were maintained after performing the stable protein-pair analysis with proteomic data from NZ samples ($n = 4$) and patient AS2 (Table IV). In contrast, when adding individually three of the four azoospermic patients (AZ1, AZ2 and AZ4) into the analysis of NZ stable-protein pairs, we detected < 50% stable-protein pairs determined in the NZ individuals, therefore reflecting huge differences in the seminal plasma proteome signature of those AZ patients (Table IV).

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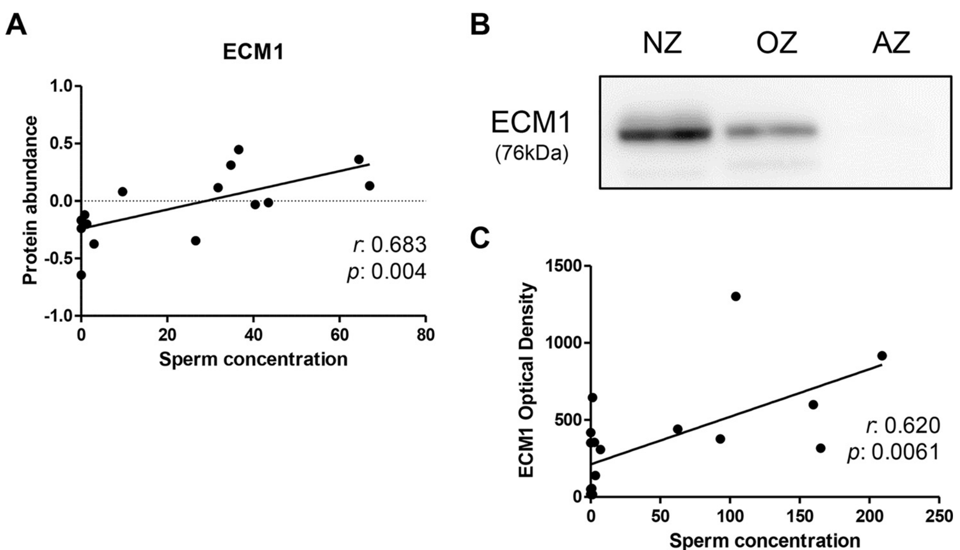

FIG. 4. **Correlation between sperm concentration and seminal plasma ECM1 protein abundance.** *A*, Scatterplot of sperm concentration and ECM1 protein abundance values generated by LC-MS/MS (Table I). The figure describes the significant Pearson correlation coefficient (*r*) and its *p* value (*p*). *B*, Validation of ECM1 protein biomarker by Western blotting in seminal plasma proteins obtained from an independent set of samples ($n = 18$; 6 normozoospermic (NZ), 6 oligozoospermic (OZ) and 6 azoospermic (AZ) patients). One representative example is provided for the results of the Western blotting in a patient of each type. *C*, Quantification of the results in all independent set of samples analyzed. Optical Densities of the bands obtained in ECM1 Western blotting are plotted as a function of the sperm concentration. Correlation coefficient (*r*) and *p* value (*p*) are from Pearson correlation analysis.

DISCUSSION

The heterogeneous composition of the seminal plasma together with the rapid changes that occur in its molecular composition after ejaculation, such as the proteolytic cascade associated to the coagulation-liquefaction process, introduce further complexity to seminal plasma proteomic studies (10, 14). In the present study, a total of 349 proteins were identified in the 16 seminal plasma samples analyzed (supplemental Table S2), which are functionally related to metabolism, response to stress, proteolysis, immune system and energy production. Also, with less extent, these identified human seminal plasma proteins seemed to be involved in processes related to fertilization and embryogenesis (8). This apparently low number of identified seminal plasma proteins could be explained by the detection of the semenogelins I and II (SEMG1 and SEMG2) as the most abundant proteins of the seminal plasma. Specifically, around 40% of the PSMs identified in our proteomic study corresponded to SEMG1 and SEMG2, thus hindering the detection of low abundant proteins. This low number of protein identifications is also observed in other studies assessing the human seminal plasma proteome using MS methods and identification criteria comparable to ours (13, 31, 32, 40–42, 46, 47, 49, 65). For this reason, future studies should consider the incorporation of strategies to deplete SEMGs prior the proteomic characterization of seminal plasma, as for example the use of HPLC columns containing antibodies against SEMGs (10, 13).

Conventional Approach to Analyze Quantitative Proteomics Data—Protein quantification of TMT-labeled peptides using conventional approaches is obtained from the average of relative ion abundance ratios for all peptides encompassing the same protein (12). The conventional statistical analyses conducted in this study showed: (i) The underexpression of the glycoprotein ANPEP in patients with altered sperm motility (Fig. 3), as previously reported by others (33); and (ii) a gradual decline of CRISP1, NPC2, and SPINT3 abundance in infertile patients, ranging from high to low sperm concentration (in decreasing order: NZ-AS, OZ, and AZ) (Table I, Fig. 3). Of note, this gradual declined abundance was also observed for the protein levels of SPINT3, NPC2, ECM1, and CRISP1, independently of sperm motility parameter (Table I). The low abundance of those proteins in seminal plasma from patients with low or absence of sperm cells could reflect either proteomic alterations in the accessory sex glands secretions, or the presence of low amounts of male germ cells remnants coming from apoptotic sperm or from sperm cytoplasmic droplets. This specific question may be elucidated by deciphering the potential tissue origin of these altered seminal plasma proteins. According to the HPA Database, the testicular or extra-testicular origin of NPC2 could not be assessed, because, although NPC2 is a major component of epididymal secretions, it is also expressed in testis (43) (Fig. 5). In contrast, ECM1, SPINT3, and CRISP1 are mainly expressed in the epididymis whereas they are not detected in testis (Fig. 5), suggesting that epididymal secretions could be regulated by the presence of sperm themselves in the epididymis. Interestingly, this potential cross-talk between the spermatozoa and epididymis has also been observed in rat and bovine species

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A

Gene name	Uniprot Accession number	Protein expression overview (HPA)			
		Testis	Epididymis	Seminal vesicles	Prostate
Extra-testicular proteins (n=3)					
ECM1	Q16610	-	High	Low	-
CRISP1	P54107	-	High	-	-
SPINT3	P49223	-	High	-	-
Testicular/ Unknown origin (n=2)					
NPC2	P61916	High	High	High	High
IGHG2	P01859	NA	NA	NA	NA

-: not detected, NA: not available

B

Validation of the HPA data

ECM1 | NPC2

Epididymis (H) | Testis (H + PAS) | Epididymis (H) | Testis (H + PAS)

FIG. 5. **Protein expression pattern in the male reproductive tract.** *A*, Protein expression data obtained from the HPA database of the seminal plasma proteins identified in the present study correlating with sperm concentration ($n = 5$, Table I). Intensity values are based on the immunohistochemical antibody staining score information available in the HPA database. *B*, Results from the subsequent immunohistochemical analysis in human testis ($n = 1$) and epididymis ($n = 1$) sections using ECM1 and NPC2 antibodies assayed in the present study. The obtained protein patterns validate the HPA data. ECM1 is an epididymis-specific protein whereas NPC2 is found in epididymis but also in testes. A lower magnification (×100; images on top) and a higher magnification (×400; images below) from the boxed area are shown. Negative controls with rabbit IgG showed nonspecific staining (data not shown). H: Hematoxylin stain, PAS: Periodic acid-Schiff stain.

TABLE II

Pearson correlation analysis between sperm concentration and seminal plasma peptide abundance (LC-MS/MS data) for the 5 proteins identified as correlated in Table I. Pearson correlation coefficient and p value are indicated in the table

Peptide sequence and modifications	Uniprot accession number	Gene name	Pearson correlation (r-value)	Significance (*p* value)
dLLPNVcAFPMEk N-Term(TMT10Plex); C7(Carbamidomethyl); K13(TMT10Plex)	P49223	SPINT3	0.746	**0.001**
sGINcPIQk N-Term(TMT10Plex); C5(Carbamidomethyl); K9(TMT10Plex)	P61916	NPC2	0.778	**0.0004**
eVNVSPcPTQPcQLSk N-Term(TMT10Plex); C7(Carbamidomethyl); C12(Carbamidomethyl); K16(TMT10Plex)			0.753	**0.001**
aVVHGILMGVPVPFPIPEPDGck N-Term(TMT10Plex); C22(Carbamidomethyl); K23(TMT10Plex)			0.752	**0.001**
dcGSVDGVIk N-Term(TMT10Plex); C2(Carbamidomethyl); K10(TMT10Plex)			0.692	**0.003**
dkTYSYLNk N-Term(TMT10Plex); K2(TMT10Plex); K9(TMT10Plex)			0.595	**0.015**
lVVEWQLQDDk N-Term(TMT10Plex); K11(TMT10Plex)			0.549	**0.028**
nQSLFcWEIPVQIVSHL N-Term(TMT10Plex); C6(Carbamidomethyl)			0.312	0.24
eLPSLQHPNEQk N-Term(TMT10Plex); K12(TMT10Plex)	Q16610	ECM1	0.758	**0.001**
eVGPPLPQEAVPLQk N-Term(TMT10Plex); K15(TMT10Plex)			0.488	0.055
lDGFPPGRPSPDNLNQIcLPNR N-Term(TMT10Plex); C18(Carbamidomethyl)			0.377	0.149
tGVPcEAcPSNcEDk N-Term(TMT10Plex); C5(Carbamidomethyl); C8(Carbamidomethyl); C12(Carbamidomethyl); K15(TMT10Plex)	P54107	CRISP1	0.699	**0.003**
lVTDLPNVQEEIVNIHNALR N-Term(TMT10Plex)			0.411	0.113
sTSESTAALGcLVk N-Term(TMT10Plex); C11(Carbamidomethyl); K14(TMT10Plex)	P01859	IGHG2	0.653	**0.006**
tTPPMLDSDGSFFLYSk N-Term(TMT10Plex); K17(TMT10Plex)			0.319	0.228

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TABLE III
Proteins involved in the stable-protein pairs for the different subtypes of infertile patients according to their seminal parameters. The correlation of two proteins is counted as a single correlation

Proteins	Number of correlations			
	Normozospermic (NZ1–4)	Asthenozoospermic (AS1–4)	Oligozoospermic (OZ1–4)	Azoospermic (AZ1–4)
Sperm function				
CRISP1	21	4	-	1
NPC2	19	-	-	-
CST3	17	3	-	-
PAEP	8	5	-	1
CKB	5	-	-	-
CLU	3	-	-	-
Semen clotting-liquefaction				
MME	19	1	-	-
ACPP	17	1	-	-
KLK3	17	1	-	-
WFDC2	14	-	-	-
ALB	12	-	-	-
TGM4	8	1	-	-
SERPINA5	-	1	-	-
SERPINA1	-	-	-	2
Immune system				
IGKC	21	1	-	-
ANPEP	20	1	-	-
IGHG2	20	2	-	1
ECM1	18	-	-	-
QSOX1	17	-	-	-
LGALS3BP	16	-	-	-
CPE	16	1	-	-
B2M	16	3	-	1
Other				
AZGP1	20	3	-	-
TF	14	-	-	-
IDH1	14	5	-	-
VWA1	12	3	-	-
Total	364	36	0	6
Correlations	**182**	**18**	**0**	**3**
Proteins involved	**24**	**16**	**0**	**5**

TABLE IV
Stable-protein correlations in control population (normal sperm parameters, NZ; n = 4) altered in individual infertile patients

Samples	Number of correlations	Correlations in common with NZ stable-protein pairs group	Proteins involved	Proteins that lost > 75% of correlations
Stable-protein pairs for the NZ group (Control group; n = 4)				
NZ group	182	**-**	**24**	
Alterations in individual infertile patients				
NZ group + AS1	118	116	22	PAEP, CKB, CLU, WFDC2, ECM1, TF
NZ group + AS2	142	138	24	TF
NZ group + AS3	101	99	21	PAEP, CLU, WFDC2, ALB
NZ group + AS4	95	94	22	PAEP, CLU, WFDC2, TGM4, ANPEP, VWA1
NZ group + OZ1	98	96	20	PAEP, CKB, CLU, WFDC2, ECM1, QSOX1, IDH1
NZ group + OZ2	98	96	22	PAEP, CKB, ACPP, KLK3, WVA1
NZ group + OZ3	88	88	22	PAEP, CLU, WFDC2
NZ group + OZ4	115	113	22	CST3, PAEP, CLU, WFDC2, LGALS3BP, VWA1
NZ group + AZ1	89	85	22	CKB, MME, ACPP, ANPEP, CPE
NZ group + AZ2	92	91	19	PAEP, CKB, CLU, ACPP, KLK3, QSOX1
NZ group + AZ3	133	127	23	CLU, ACPP, B2M, VWA1
NZ group + AZ4	87	85	23	PAEP, CLU, WFDC2, ALB, IGKC, QSOX1, VWA1

NZ, normozoospermic; AS, asthenozoospermic; OZ, oligozoospermic; AZ, azoospermic.

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(66, 67). Drabovich and colleagues, in contrast, detected decreased levels of SPINT3, NPC2, ECM1 and CRISP1 only in obstructive azoospermic patients but not in patients with non-obstructive azoospermia (16). Other groups assessing potential seminal biomarkers to discern the different subtypes of azoospermia found other distinct differential proteins, such as LGALS3BP (57) and STAB2, CP135, GNRP, and PIP (34). Altogether, it indicates the presence of some differences between seminal plasma proteomic data from different studies. Of note, a similar lack of concordance has also been observed in quantitative proteomic data of sperm samples from the same type of patients. This is exemplified by the detection of only 17 proteins out the 179 reported as differentially expressed in the sperm cells in at least 2 of the 7 comparative proteomic studies conducted so far for the study of asthenozoospermia (68). Differences in sample collection, handling and storage, proteomic strategies, and the biological intra- and inter-individual variance may be important causes contributing to this lack of reproducibility between studies (14, 17, 69). Moreover, the high and fast protease activity in seminal plasma after ejaculation could introduce even more heterogeneity to the results because of the presence of distinct proteolytic fragments, in addition to the protein PTMs not detected in standard proteomic procedures (70). In order to evaluate this putative heterogeneity of seminal plasma proteome we also assessed the correlation of peptides encompassing the proteins SPINT3, NPC2, ECM1, CRISP1 and IGHG2 with sperm concentration (Table II). Of note, NPC2 is the only protein showing most of its corresponding peptides correlated with the sperm concentration (Table II). In agreement with our findings, Giacomini and colleagues, by using nano LC-electrospray ionization-MS/MS, found decreased levels of NPC2 in seminal plasma from idiopathic oligoasthenozoospermic patients (43). As a summary, there is a need of novel approaches to analyze the results from quantitative shotgun proteomic studies, in order to overcome the limitations produced by the heterogeneity of seminal plasma in the proteolytic fragments, as well as by its conjunction with other variations such as the protein PTMs not detectable by standard proteomic procedures.

Novel Approaches to Analyze Quantitative Proteomics and Identify Patient-specific Alterations—A set of seminal plasma strictly co-regulated proteins was established by following a new approach based on the correlation of the intensities of all unique peptides comprising one specific protein with all the unique peptides quantified for all the other detected proteins in the sample (Fig. 2). This strategy, called stable-protein pairs analysis herein, may contribute to reduce the heterogeneity observed in the seminal plasma proteomic data, because only those proteins displaying a consistent pattern in a specific phenotype are obtained. A total of 182 stable-protein pairs comprising 24 proteins were detected in patients with normal semen parameters (Table III), reflecting the strict co-regulation of these proteins in NZ individuals. These stable-protein pairs include gene products involved in: (i) Sperm function, such as CKB that plays a critical role in the demand of energy necessary for sperm motility (71), CST3, PAEP, and CLU that regulate sperm capacitation (72–74) and CRISP1 and NPC2, which are necessary for sperm-oocyte binding and fertilization (75, 76); (ii) the regulation of semen clotting-liquefaction processes, such as ACCP, KLK3, and MME, which are directly involved in the proteolysis of the SEMGs or other proteins (77, 78), or as WFDC2, ALB, and TGM4, which regulate other components required for clotting-liquefaction process such as proteases, zinc ions and polyamines (79–81); (iii) immunology, including proteins that could participate in the leukocyte-mediated immune response, such as IGKC, IGHG2, ANPEP, LGALSBP3, ECM1, and B2M (82, 83) or in antimicrobial activity, as for example CPE and QSOX1 (84, 85); and finally, (iv) other functions such as lipid metabolism (AZGP1, TF and IDH1) and matrix assembly (VWA1). In contrast to the high number of stable-protein pairs identified in NZ individuals, the stable correlations drastically decreased in the different groups of patients with altered semen parameters (Table III). Indeed, just 18, 3 and 0 stable-protein pairs were detected in AS, AZ, and OZ patients, respectively. The low number of stable-protein pairs observed in AZ patients was not surprising, because this group contains patients indistinctly diagnosed with obstructive and non-obstructive azoospermia, which probably results in different semen protein profiles as previously reported by others (16). However, a high heterogeneity was also observed in the proteomic profile of seminal plasma from AS and OZ patients. The few stable-protein pairs detected in infertile patients with altered seminal parameters indicate that alterations in different proteins may result or be a consequence of the same altered phenotype. Although some hints for the presence of protein-pair correlations was already reported in the sperm proteome using ancillary proteomic methods (86), the present study clearly demonstrates the potential of this approach using proteomics data at peptide level (Table III, Fig. 2).

To assess which protein pairs might be associated to the alterations of the seminal parameters in individual samples, we repeated the analysis of the stable-protein pairs in the NZ population but adding data from one patient with altered parameters at a time. First, we observed that the patient AS2 had a very similar seminal plasma protein signature to the NZ population, because it maintained more than 75% of the NZ stable-protein pairs (Table IV). Of note, one of the proteins that loses more correlations in patient AS2 is the TF, a protein involved in lipid metabolism and sperm protection against oxidative stress (87) (Table IV). It is interesting to note that TF loses more than 75% of the correlations in another asthenozoospermic patient (AS1) (Table IV), suggestive that oxidative stress may be related to the impairment of the sperm motility in both patients AS1 and AS2.

We also observed that proteins involved in the induction of proteolysis of SEMGs and other regulators of the semen clotting-liquefaction process lose $>$ 75% of the correlations in OZ

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and AZ patients (KLK3 in OZ2 and AZ2; ACPP in OZ2, AZ1, AZ2, and AZ3; and MME in AZ1; Table IV), suggesting that the sperm also contribute with regulators for the semen clot proteolysis (88). Whereas some of the protein correlations were lost in the majority of the individual patients, as observed for CLU, PAEP, and WFDC2, suggesting that the correlations established for these proteins in NZ samples are weak, some seminal plasma proteins seem to be altered only in one unique sample such as TGM4 in patient AS4, IDH1 in OZ1, CST3 and LGALS3BP in OZ4, CPE in Z1, B2M in Z3 and IGKC in Z4, although these alterations could not clearly explain the observed phenotype.

Quantitative Proteomics as A Tool to Provide Insights in Seminal Plasma Proteome Signatures Of Infertile Patients—So far, proteomics biomarker discovery experiments have shown a relatively low concordance among different studies. In fact, we demonstrated that the results from relative quantitative proteomics are different if the analyses are performed at protein or at peptide level. These differences could explain the apparent lack of reproducibility of some of the findings, a fact that should be considered also when using antibody-based techniques recognizing specific peptides (such as Western blotting or ELISA) or targeted proteomic approaches to selected specific peptides. Here, we propose introducing a novel complementary approach for the analysis of quantitative proteomic data, which is based on the establishment of stable-protein pairs. This strategy has been previously applied to the study of RNA-seq data (59, 60), but to the best of our knowledge this is the first time it is applied to the proteomic data. The use of this new approach in our seminal plasma proteome dataset has allowed determining highly stable seminal plasma proteome signatures of men presenting normal seminal parameters (NZ). In contrast, we demonstrated that the current classification of infertile patients based on altered semen parameters resulted in a high heterogeneous seminal plasma proteomic profile, thereby suggesting that the current male infertility stratification performed in fertility clinics is not enough to obtain a good diagnosis. Moreover, the stable-protein pairs approach has the potential to pinpoint proteins potentially related to pathogenic mechanisms in individual samples when this strategy is applied for the evaluation of the NZ stable-protein pair alterations in individual infertile patients. Although our study has limitations, the novel data analysis approach proposed herein could be valuable toward the identification of altered proteins and pathogenic mechanisms of male infertility and might open a window to the personalized diagnosis of male infertility in future studies.

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DATA AVAILABILITY

The MS proteomics data generated have been deposited to the ProteomeXchange Consortium via the PRIDE (89) partner repository with the dataset identifier PXD010579. The data files can be downloaded at: https://www.ebi.ac.uk/pride/archive/.

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TREBALL 2

ANÀLISI QUANTITATIVA DEL PROTEOMA DEL PLASMA SEMINAL EN HIPOGONADISME SECUNDARI

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Anàlisi quantitativa del proteoma del plasma seminal en hipogonadisme secundari

Objectiu: L'objectiu d'aquest treball és realitzar una anàlisi quantitativa del proteoma del plasma seminal en pacients amb hipogonadisme hipogonadotròpic (HH), i avaluar l'efecte de la teràpia de reemplaçament de testosterona (TRT) en les secrecions de les glàndules sexuals accessòries.

Materials i Mètodes: Primerament, s'ha obtingut i comparat el perfil proteòmic del plasma seminal d'individus fèrtils (n=10) i de pacients amb HH postquirúrgic (n=10). Posteriorment, el perfil proteic de 5 dels 10 pacients HH s'ha comparat abans i després de 3 mesos de teràpia TRT. Les proteïnes del plasma seminal s'han digerit amb tripsina i cadascuna de les mostres s'ha analitzat per LC-MS/MS. L'abundància de les proteïnes s'ha calculat per cada grup, i aquesta s'ha comparat per a la detecció de proteïnes sobre- o sota-expressades en els pacients HH, i després del tractament TRT. La validació dels resultats de proteòmica s'ha realitzat mitjançant western blot en plasma seminal de controls (n=8) i de pacients HH (n=17) per a les proteïnes *Lactotransferrin* (TRFL) i *Prolactin-inducible protein* (PIP).

Resultats: L'anàlisi quantitativa *label-free* del plasma seminal dels diferents individus ha resultat en la identificació de 110-176 proteïnes per individu. La comparació del plasma seminal entre individus fèrtils i pacients HH ha permès la identificació d'11 proteïnes diferencialment expressades, les quals totes s'han trobat en menor abundància en el plasma seminal de pacients HH. Aquesta reducció proteica és la resposta *in vivo* de les glàndules sexuals accessòries a la deficiència de testosterona. Els resultats de l'anàlisi de proteòmica s'han validat per western blot per les proteïnes TRFL i PIP. Addicionalment, la comparació dels proteomes del plasma seminal de pacients amb hipogonadisme hipogonadotròpic abans i després de la teràpia TRT ha resultat en l'increment de 6 proteïnes, 5 de les quals (PIP, TRFL, *Prostatic acid phosphatase* (PPAP), *Semenogelin-1* (SEMG1), *Semenogelin-2* (SEMG2)) s'havien prèviament detectat en abundància reduïda en pacients HH en comparació amb individus fèrtils, i els seus nivells van incrementar després de TRT. Aquest conjunt de proteïnes representa un panell de proteïnes candidates per al diagnòstic de l'hipogonadisme i per al seguiment de la teràpia TRT.

Conclusions: La identificació d'un conjunt de proteïnes involucrades en l'acció dels andrògens en pacients HH ens poden ajudar a entendre com els andrògens modulen la fisiologia de l'epidídim i de les glàndules sexuals accessòries. A més, els resultats derivats d'aquest estudi han permès la identificació d'unes proteïnes del plasma seminal que podrien ser útils com a nous biomarcadors en el diagnòstic dels pacients amb diferents graus d'hipogonadisme masculí, i en el seu seguiment durant la teràpia TRT.

Article

Quantitative Analysis of the Seminal Plasma Proteome in Secondary Hypogonadism

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Abstract: In the grey zone of testosterone levels between 8 and 12 nmol/L, the usefulness of therapy is controversial; as such, markers of tissue action of androgens may be helpful in adjusting clinical decisions. To better understand the effect of the hypothalamic-pituitary-testicular axis on male accessory secretion, we performed a proteomic quantitative analysis of seminal plasma in patients with secondary hypogonadism, before and after testosterone replacement therapy (TRT). Ten male patients with postsurgical hypogonadotrophic hypogonadism were enrolled in this study, and five of these patients were evaluated after testosterone treatment. Ten men with proven fertility were selected as a control group. An aliquot of seminal plasma from each individual was subjected to an in-solution digestion protocol and analyzed using an Ultimate 3000 RSLC-nano HPLC apparatus coupled to a LTQ Orbitrap Elite mass spectrometer. The label-free quantitative analysis was performed via Precursor Ions Area Detector Node. Eleven proteins were identified as decreased in hypogonadic patients versus controls, which are primarily included in hydrolase activity and protein binding activity. The comparison of the proteome before and after TRT comes about within the discovery of six increased proteins. This is the primary application of quantitative proteomics pointed to uncover a cluster of proteins reflecting an impairment not only of spermatogenesis but of the epididymal and prostate epithelial cell secretory function in male hypogonadism. The identified proteins might represent putative clinical markers valuable within the follow-up of patients with distinctive grades of male hypogonadism.

Keywords: seminal plasma; proteomics; testosterone; testosterone deficiency; hypogonadism; accessory glands

1. Introduction

Male hypogonadism is characterized as "inadequate gonadal function, as manifested by deficiency in gametogenesis and/or secretion of gonadal hormones" [1]. It can be classified as primary, secondary, or tertiary hypogonadism according to the site primarily affected (the testis, the pituitary gland, or the hypothalamus, respectively). Hypogonadotrophic hypogonadism is characterized by low testosterone (T) plasma levels combined with normal or low FSH and LH plasma levels, due to an impairment of the pituitary function. The diagnostic protocol of secondary hypogonadism, also known as hypogonadotropic hypogonadism (HH), includes an accurate medical history, a physical exam, semen analysis, hormone measurements, and instrumental evaluation.

Total testosterone levels of less than 8 nmol/L profoundly bolster a determination of hypogonadism whereas levels greater than 12 nmol/L are likely to be typical. An inadequate androgen status is possible if the total testosterone levels are between 8 and 12 nmol/L. The diagnosis of hypogonadism and the appropriateness of a treatment in the "grey zone" between 8 and 12 nmol/L is debated and should be supported by symptoms, often non-specific. Most patients with a total testosterone between 8 and 12 nmol/L, if asymptomatic, will not be hypogonadal. Patients experiencing symptoms of hypogonadism require further investigations [2]. To date, no markers of androgen tissue action have been suggested and validated in clinical practice. As such, new markers of androgens action might be valuable in orienting diagnosis, decision about testosterone replacement treatment (TRT), and clinical follow-up.

As in all endocrine diseases, the treatment goal for HH is to reestablish the lacking glandular work. Several studies reported that gonadotropin (hCG and FSH) therapy is effective in initiating and maintaining spermatogenesis and moreover should be preferred to testosterone for inducing an increase in vitamin D levels and for being associated with lower concentrations of estrogens [3]. Because of their greater expense and complexity, however, gonadotropins are usually reserved for men with gonadotropin deficiency who desire fertility and in whom spermatogenesis must be initiated and maintained [4]. If fertility is the topic, FSH and LH administration is in fact recommended. In all the other conditions, testosterone replacement therapy (TRT) is the most helpful choice [5,6]. The goals of TRT are to restore the T levels in serum within the mid-normal physiological range associated with the patient's age group, generally considered to be between 13.8 and 24.27 nmol/L, and to improve symptoms in hypogonadal men [7]. Although most of the positive effects of TRT begin to occur between 3 to 6 weeks after initiation, considerable variations have been observed and some patients require up to 1 year to observe clinical effects [8].

Previous data have been provided about the effect of T deficiency and TRT on male fertility, in terms of function of male accessory sex glands (epididymis, prostate, and seminal vesicles). Specifically, it has been reported the effect of orchiectomy on epididymal function. In rats, orchiectomy actuates a diminishment in epididymal weight that is less checked than for prostate or seminal vesicles [9]. Prostate is a highly androgen-dependent tissue. The androgens play a fundamental part in prostate growth and development and within the pathogenesis of prostate diseases, such as benign prostatic hyperplasia (BPH) and adenocarcinoma [10]. In 2009 Ma et al., using a transcriptomic approach, reported the identification of 187 transcripts significantly affected by dihydrotestosterone (DHT) reduction in mice prostate [11]. This finding underpins the speculation that androgen-regulated genes are involved in prostate function [11]. However, few studies have been focused on the androgen action mechanism in epididymal epithelial cells or in prostate tissue, likely due to the need for adequate tools.

One of the opportunities to further elucidate molecular mechanisms of androgen modulation of accessory gland function is offered by proteomics, which captures the overall protein profile instead of the expression of individual genes. Due to the dynamic nature of the proteome, proteomic-based personalized medicine is fluid, adapting to individuals and individual situations [12]. Recent advances in proteomic techniques, proteomic data analysis, and application of proteomic techniques in clinical settings undoubtedly represent a real promise for early disease diagnosis, prognosis, and theragnosis on an individual basis. There is a small question that the next decade will be the time of proteomics [13].

In the area of male fertility, proteomics appears the foremost promising and effective platform recently applied in order to widely study the physiology and pathophysiology of male reproduction [14].

In 2014, we published the first pilot proteomic study, applying high-resolution mass spectrometry before and after 6 months of TRT in human seminal samples of patients affected by hypogonadotropic hypogonadism. We reported the identification of a panel of 14 absent proteins in hypogonadic patients with respect to normogonadic subjects [15].

However, no quantitative proteomic studies have yet been performed to understand how testosterone modulates protein abundance and how the protein abundance is quantitatively regulated by testosterone.

Recent years have in fact witnessed a significant improvement of mass spectrometers. Orbitrap MS was recently improved combining a dual-pressure linear ion trap (Velos Pro) with a new high-field Orbitrap™ mass analyzer to create the ultimate analytical instrument (Orbitrap Elite) [16], which was used in the present study. Furthermore, bioinformatics is more and more capable of supporting proteomics in the interpretation of the data analysis and in the identification of the proteins of clinical interest by means of statistical analysis and use of algorithms [17]. As a consequence high-resolution, quantitative, proteomic approaches may now be useful in resolving clinical questions, such as in this case, the identification of quantitatively regulated proteins by testosterone in seminal plasma or the identification of early markers of response to TRT.

With this aim, we performed a quantitative high-resolution proteomic analysis in seminal plasma samples of patients affected by HH, before and after only 3 months of TRT.

2. Materials and Methods

The study design was approved by the Ethical Committee of Fondazione Policlinico "A. Gemelli", Rome (Italy). A written informed consent in accordance with the Declaration of Helsinki has been given by all subjects.

2.1. Human Subjects

Ten male patients aged between 25 and 55 years with postsurgical HH were selected for this study.

No patients displayed hypogonadism when evaluated before the neurosurgical operation at endocrinological clinic of our tertiary care university hospital ("A. Gemelli", Rome, Italy). All patients underwent andrological evaluation at our clinic 6 months after the neurosurgical operation, which removed a pituitary adenoma or craniopharyngioma. During this time, replacement therapies for thyroidal, adrenal, and somatotropic axes were performed when single or multiple deficits were documented. All patients were also assessed during this time to exclude residual adenoma.

The rationale for studying only men with HH was to select a condition of extreme reduction in blood T levels without other confounding variables. The patients with primary hypogonadism were excluded because their blood T is usually not as low as that of subjects with HH. Moreover, patients with primary hypogonadism often show normal or increased serum estrogen levels, which might represent a confounding factor. Inclusion criteria were as follows: total T less than 8 nmol/L, calculated free T less than 1.6%, and clinical symptoms of hypogonadism. Exclusion criteria for the study included age younger than 20 years and older than 55 years, primary hypogonadism or associated testicular diseases, smoking, residual adenoma, previous androgen replacement therapy, diabetes mellitus, varicocele, and genital tract infections.

Five patients were also evaluated after 3 months of T replacement therapy (TRT) with transdermal testosterone (Tostrex 2% gel), to assess the early impact of androgen substitution treatment on seminal proteome. The evaluation has been performed after 3 months of TRT because it is the proper timing of response to exogenous androgen administration in terms of restoration of seminal vesicle size, which is a sensitive measure of androgen genomic effect [18].

Ten fertile men, whose partners were pregnant when the study started, were selected as a control group in the study. None of them had a history of infertility. All female partners conceived within 3 months before the start of the study.

2.2. Hormonal Study

A blood sample was collected at admission at the andrological clinic 6 months after the neurosurgical operation, at 8:00 a.m. for the determination of sex hormone-binding globulin (SHBG), T, estradiol (E2), luteinizing hormone (LH), and follicle-stimulating hormone (FSH). T and E2 were measured in duplicate by radioimmunoassay (RIA) with the use of commercial kits (Radim, Pomezia, Italy). LH, FSH and SHBG were assayed by immunoradiometric methods on a solid-phase (coated tube), which is based on a monoclonal double-antibody procedure. Reference values of the studied hormones are reported in Table 1.

Testosterone and estradiol levels were then measured in patients receiving TRT after 3 of treatment to confirm the effectiveness of TRT.

The intra-assay coefficients of variation (percentage) were 6.1% for T, 2.3% for E 26.9% for SHBG, 5.6% for LH, and 6.9% for FSH. The inter-assay coefficients of variation were 9.3% for T, 3.5% for E2, 8.5% for SHBG, 9.1% for LH, and 8.4% for FSH.

2.3. Semen Analysis and In-solution Digestion

Complete semen analysis was performed upon admission at the andrological clinic in controls, and in patients 6 months after the neurosurgical operation, according to WHO (2010) classification [19].

Liquefied semen samples were then centrifuged at 3000× *g* for 30 min to get the seminal plasma and to ensure complete expulsion of the cellular components. After the centrifugation, an aliquot was checked under a microscope to confirm that no spermatozoa were displayed. Seminal plasma was divided in 0.5 mL aliquots, which were quickly frozen at −80 °C until mass spectrometry (MS) examination was carried out inside 1 month.

Seminal plasma samples were subjected to solution digestion. Briefly, an aliquot of seminal plasma corresponding to 50 μg of total protein (as measured by Bradford assay) was mixed with 100 mM ammonium bicarbonate pH 8.0 and reduced with 200 mM dithiothreitol (DTT; 10 mM final concentration, Sigma-Aldrich, St. Louis, MI, USA) for 5 min at 100 °C and 15 min at 50 °C, and alkylated with 200 mM iodoacetamide (IAA; 55 mM final concentration, Sigma) in the dark at room temperature for 60 min. The samples were left to digest overnight at 37 °C by adding ammonium bicarbonate solution with sequencing grade modified porcine trypsin (1:50, trypsin: protein concentration, Promega, Madison, WI, USA). To stop the digestion, the samples were acidified with aqueous trifluoroacetic acid (TFA/H_2O 0.2% (*v*/*v*)) and immediately frozen and lyophilized.

2.4. Proteomic Analysis

For proteomic analysis, the samples were suspended in aqueous formic acid [FA/H_2O 0.1% (*v*/*v*)] and equal protein quantity (5 μg) of each sample was analyzed using an Ultimate 3000 RSLCnano HPLC System coupled to a LTQ Orbitrap Elite mass spectrometer (ThermoFisher). Separation was performed using a Zorbax 300SB-C18 column (3.5 mm particle diameter; column dimension 1 mm i.d. 15 cm, Agilent Technologies) and the following eluents: (A) 0.1% (*v*/*v*) aqueous FA and (B) acetonitrile: H_2O 80:20 with 0.1% (*v*/*v*) aqueous FA. We applied a linear gradient from 0 to 55% of solvent B in 60 min, at a flow rate of 50 μL/min. The Elite-Orbitrap mass spectrometer was operated in data-dependent mode in which each full MS scan (60,000 resolving power) was followed by MS/MS scans where the five most intense multiple-charged ions were dynamically selected and fragmented by collision-induced dissociation (CID) at a normalized collision energy of 35% and acquired in linear ion trap at normal scan rate. Samples were analyzed individually; proteomic analysis was performed at the same time for all samples, while data analysis was subsequently performed.

2.5. Data Analysis

Tandem mass spectra were analyzed using the Thermo Proteome Discoverer 1.4.1.14 software based on SEQUEST HT cluster as the search engine (University of Washington, Seattle, WA, USA, licensed by Thermo Electron Corp, Waltham, MA, USA) against *Homo sapiens* UniProtKb/Swiss-Prot protein knowledgebase (release date: 2017-02).

Data were searched for two missed cleavages, cysteine carbamidomethylation as a static modification, and methionine oxidation as a dynamic modification. Criteria utilized to accept protein identification included a false discovery rate (FDR) of 1% and at least 1 unique peptide match per protein. The dissociated or 'ungrouping' of proteins from their respective families was used during the quantification process to avoid the possible ambiguity associated with different isoforms of the same protein.

The label-free quantitative analysis was performed via Precursor Ions Area Detector Node during the bioinformatic analysis using Proteome Discoverer software. This quantification method was used to define the relative quantities of all peptides in a sample. The Proteome Discoverer application calculates peptide areas during processing, using them to automatically calculate protein areas for the proteins in the report. It calculates the area of any given protein as the average of the three most abundant distinct peptides identified in the protein.

Mean ± standard deviation of protein abundance was calculated for each protein in the group of controls (n = 10) and in the group of hypogonadic patients (n = 10). The relative protein level ratios between the group of controls and the group of hypogonadic patients (n = 10) were determined from the respective averages of protein abundances expressed in all patients. We compared for each protein the mean abundance in the group of HH patients with the one reported in the group of healthy males. All the proteins detected with a ratio > 1.5 (less abundant proteins in HH patients) or <0.67 (more abundant proteins in HH patients) have been considered for this study.

In the population of 5 patients who received TRT, we moreover compared, for each patient, the protein abundance in samples obtained before and after TRT therapy and determined the pre-treatment: post-treatment ratio, obtaining a list of under-expressed (ratio > 1.5) or over-expressed (ratio < 0.67) proteins after TRT.

The cut-off values of 1.50 and 0.67 for mean abundance ratios have been selected as previously reported in literature [20–22].

Proteins identified by SEQUEST were then analyzed using the publicly available protein annotation through evolutionary relationship (PANTHER) classification system (http://www.pantherdb.org/). Furthermore, we evaluated the Gene Ontology Molecular Function annotations in the list of the proteins differently expressed in patients versus controls.

2.6. Western Blot

In order to validate the proteomics results, a western blot analysis was performed in seminal plasma samples used for proteomics. Seminal plasma samples used for confirmation analysis included the 10 hypogonadic patients, and 8 of the 10 controls, since 2 control samples were spent for proteomic analysis. Furthermore, to increase the power of the confirmation analysis we added in western blot analysis 7 independent hypogonadic patients samples, furtherly included in the study with the same inclusion and exclusion criteria. Clinical and hormonal parameters of this population of 7 additional hypogonadic patients is reported in Supplementary Table S1. To summarize, the western blot confirmation analysis was performed on seminal plasma samples of 17 hypogonadic patients and 8 controls.

For western blot analysis, seminal plasma were diluted in RIPA buffer (50 mM Tris–Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% Na desoxicholate, 0.1% SDS, 1 mM EDTA) supplemented with a cocktail of protease inhibitors (Boehringer, Ingelheim am Rhein, Germany). Samples were sonicated, quantified by Bradford protein assay, resuspended in SDS Laemmli sample buffer, and boiled for 5 min at 95 °C. Proteins (30 µg) were separated by SDS–PAGE and subsequently transferred onto

PVDF membranes (Millipore, Burlington, MA, USA). Membranes were developed using the enhanced chemiluminescence (ECL Amersham, Little Chalfont, United Kingdom). The following primary antibodies were used: anti-Lactoferrin polyclonal antibody (1:2000) and anti-PIP polyclonal antibody (1:500) (Novus Biologicals, Centennial, CO, USA). Red ponceau staining was used as internal control to check the equal loading of total proteins in western blot analysis [23].

Values were reported as the mean ± S.D. Statistical analysis was performed by the two-tailed unpaired Student's *t*-test using the SigmaStat 4.0 software (Systat Software Inc., San Jose, CA, USA). Differences were considered statistically significant when $p < 0.05$.

3. Results

The results derived from the clinical, hormonal, and seminal data of the 10 hypogonadic patients and the 10 controls are reported in Table 1.

Table 1. Clinical and hormonal parameters in controls and hypogonadotropic hypogonadic (HH) patients—proteomic analysis of seminal plasma; * $p < 0.05$.

	Hypogonadic Patients ($n = 10$)	Controls ($n = 10$)	Range Values
Age	37.5 ± 9.1	39.6 ± 8.3	
Testosterone (T)	1.87 ± 0.64	4.9 ± 0.9 *	2.5–8.4 ng/mL
Estradiol (E2)	23.25 ± 4.72	27.9 ± 9.4	15–44 pg/mL
SHBG	19.67 ± 4.34	38.2± 9.3	16–80 nmol/L
FSH	1.72 ± 1.14	2.6 ± 1.3 *	1.0–8.0 mU/mL
LH	0.90 ± 0.50	2.9 ± 0.8 *	2.5–10.0 mU/mL
Seminal volume	1.75 ± 1.13 mL	2.9 ± 0.9 *	
Sperm concentration	2.61 ± 4.83 × 10^6/mL	74.2 ± 28.3 × 10^6/mL *	
Total sperm motility	8.3 ± 12.90%	52.6 ± 8.3% *	
Normal morphology	3.2 ± 4.1%	8.9 ± 2.0% *	

The mean age of the patients in the two groups did not differ significantly. Total T, FSH, and LH resulted significantly decreased in hypogonadic patients compared with the control group. Seminal volume, sperm count, sperm motility, and normal sperm morphology were significantly reduced compared to hypogonadic patients vs. controls. The T levels in hypogonadic patients were restored after TRT treatment as reported in Table 2.

Table 2. Clinical and hormonal parameters in hypogonadotropic hypogonadic (HH) patients before and after testosterone replacement therapy; * $p < 0.05$.

	Before TRT ($n = 5$)	After TRT ($n = 5$)	
Testosterone (T)	1.24 ± 0.76	3.63 ± 0.48 *	2.5–8.4 ng/mL
Estradiol (E2)	21.47 ± 5.72	29.48 ± 8.73	15–44 pg/mL

We identified—before stringent filters were applied—110 to 175 proteins per individual subject sample in the hypogonadic group, and 117 to 176 proteins in the control group. Furthermore, since samples had not been depleted, semenogelins resulted in the majority of the identified spectra.

As a consequence, the adoption of stringent protein identification criteria (FDR of 1% and at least 1 unique peptide match per protein) resulted in the characterization of 12–17 proteins per individual subject sample in the hypogonadic group and 15–60 in the control group which have been identified with high accuracy and, therefore, finally considered for our study. The comparison of the seminal plasma proteomes of the hypogonadic patients and control groups resulted in the detection of 11 differentially expressed proteins (ratio controls/HH patients < 0.67 or > 1.5; Table 3). Of those, no proteins were found only in the group on HH patients or at higher levels in the group of patients (ratio < 0.67), whereas all the identified 11 proteins were found at lower levels (ratio > 1.5).

Table 3. Reduced seminal proteins in hypogonadotropic hypogonadic patients (HH; *n* = 10) versus controls (C; *n* = 10).

Accession Number	Protein Description	Gene	Ratio C/HH
P05154	Plasma serine protease inhibitor	IPSP	2.89
P12273	Prolactin-inducible protein	PIP	2.32
P54107	Cysteine-rich secretory protein 1	CRIS1	2.21
P20142	Gastricsin	PEPC	2.19
P04279	Semenogelin-1	SEMG1	2.12
P08118	Beta-microseminoprotein	MSMB	2.04
Q02383	Semenogelin-2	SEMG2	1.92
P07288	Prostate-specific antigen	KLK3	1.65
P07602	Prosaposin	SAP	1.64
P15309	Prostatic acid phosphatase	PPAP	1.54
P02788	Lactotransferrin	TRFL	1.52

The analysis of GO annotations for Molecular function of the 11 differentially expressed seminal proteins revealed that the most frequent molecular functions were catalytic activity (among the catalytic proteins the major part were involved in hydrolase activity) and binding (among the binding proteins the major part were involved in protein binding activity).

The comparison of the proteomes of the hypogonadic patients before and after TRT resulted in the detection of 6 differentially expressed proteins, which were found to be increased after TRT (ratio after/before TRT > 1.5; Table 4).

Table 4. Increased seminal proteins in hypogonadic patients after TRT (Ratio after/before TRT > 1.5; *n* = 5).

Accession Number	Protein Description	Gene	Ratio After/Before TRT
P04279	Semenogelin-1	SEMG1	2.42
Q02383	Semenogelin-2	SEMG2	2.15
Q6W4X9	Mucin-6	MUC6	1.87
P12273	Prolactin-inducible protein	PIP	1.76
P15309	Prostatic acid phosphatase	PPAP	1.64
P02788	Lactotransferrin	TRFL	1.51

No proteins have been observed as reduced after TRT.

Western blot analysis confirmed proteomic data and namely the significant reduction in PIP and lactoferrin levels in hypogonadic patients versus controls (Figure 1).

In the Supplementary Material section, we report the picture of all gels after enhanced chemiluminescence, all pictures of membranes after red ponceau staining and western blot membrane with detected bands. Quantification has been furthermore reported as Supplementary Material.

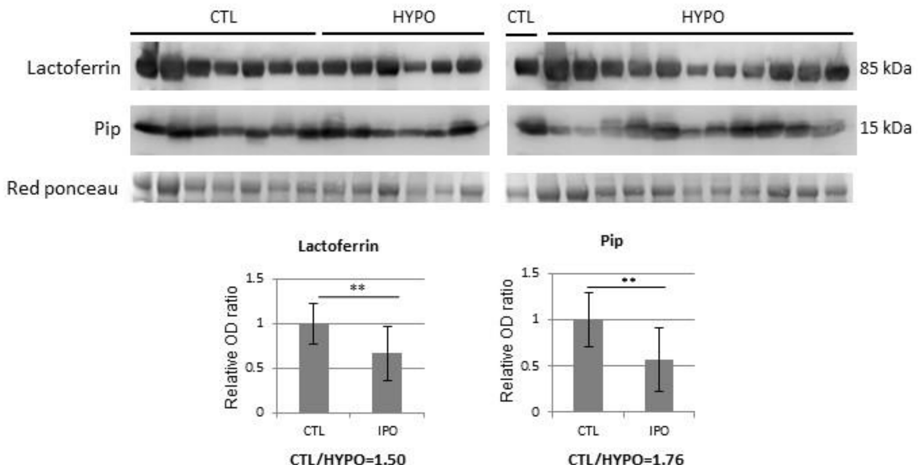

Figure 1. WB analysis of Lactotransferrin (TRFL) and Prolactin-inducible protein (PIP) proteins in seminal plasma samples from controls (CTL) and hypogonadic patients (HYPO). Histograms show the ratio of densitometric values of Lactoferrin and PIP to Red Ponceau. The ratio from controls (CTL) was arbitrarily set to 1. Mean ± SD of patients is shown (** = $p < 0.01$, two-tailed unpaired t-test).

4. Discussion

In this study, we took advantage of the application of high-resolution mass spectrometry technology and a quantitative proteomic approach to decide the in vivo effect of androgen lack on male accessory sexual glands. The full list of the 11 identified decreased proteins in hypogonadism may allow the development of an array of proteins related to T action on normal sexual glands function. From a fundamental perspective it also points to the in vivo tissue response of male accessory sexual glands to testosterone deficiency. Furthermore, the quantitative modulation in the abundance of these proteins, although needing further confirmation, may be used to identify new markers useful in the diagnosis of male hypogonadism, especially in patients with T levels in the "grey zone" between 8 and 12 nmol/L.

The proteins reported as reduced in male hypogonadism are mainly involved in catalytic activity, and namely in hydrolase activity, thus suggesting a homeostasis disorder between proteases and their inhibitor in semen of HH patients, and in protein binding activity. Specifically, the proteins identified with hydrolase activity are plasma serine protease inhibitor (Serpina 5 or IPSP) and gastricsin (PEPC). Serpina 5, also designated as Protein C inhibitor (PCI) or plasminogen activator inhibitor 3, is a member of the serine protease inhibitor (serpin) family that inactivates serine proteases by forming stable, enzymatically inactive enzyme inhibitor complexes [24]. PCI inhibits many proteases such as activated protein C [25,26], thrombin [27], factor Xa [27], factor Xia [27,28], plasma kallikrein [27,28], thrombin–thrombomodulin complex [29], urokinase (uPA) [30,31], tissue plasminogen activator (tPA) [27,31], the sperm protease acrosin [32,33], tissue kallikrein [34,35], and prostate specific antigen (PSA or KLK3) [35,36]. Disruption of the PCI gene, which is highly expressed in the male reproductive tract, comes in infertility of male homozygous PCI-knockout mice [37]. Spermatozoa derived from $PCI^{-/-}$ males were malformed and were not able to bind and to fertilize oocytes, as shown by in vivo and in vitro fertilization experiments [37]. Histological analysis of $PCI^{-/-}$ mice revealed abnormal spermatogenesis associated with damage of Sertoli cells [37]. Apart from the importance of PCI in spermatogenesis and spermiogenesis, previous immunohistochemistry studies together with the malformation of spermatozoa observed in $PCI^{-/-}$ deficient mice show that PCI may play an important role in further sperm cell maturation during the passage through the epididymis as deduced from the presence of PCI in the cytoplasmic droplet (CD) [24]. Serpina 5 is moreover secreted in seminal plasma, where it inhibits many proteases, which plays pivotal roles in male reproduction [35]. The reduction of PCI in hypogonadic patients might reflect an impairment in spermatogenesis or might represent a signature of defective spermatogenesis in HH patients, as well as in epididymal function. Further studies are needed to confirm these hypotheses.

On the other hand, Gastricsin, which in seminal plasma is a prostate-derived protein, was reported to be involved in the degradation of many seminal plasma proteins at low pH in vitro [38]. This role of gastricsin was subsequently confirmed by in vivo studies [39], where it was demonstrated that gastricsin in seminal plasma is activated at low pH in the vagina 2–7 h post-coitus and that gastricsin activity is present more than 24 h thereafter [38]. Thus, gastricsin-mediated cleavage of seminal plasma proteins is a documented in vivo phenomenon. Further studies demonstrated that epididymal protein hCAP-18 is cleaved in vagina by gastricsin into two parts: the cathelin part and ALL-38, which has an antimicrobial activity against a variety of microorganisms [40]. Consequently, in hypogonadic patients, we reported a reduction in this defense mechanism against infection transmission during sexual intercourse. Previous data have been reported for HH patients about the higher incidence in these patients of prostatitis and male tract infection/inflammation (MAGI) [41] and the increased levels of MAGI markers in seminal plasma [42]. The observation of a reduction in gastricsin in our study might represent a molecular mechanism by which hypogonadic patients have more frequently male tract infections. Further studies are needed to confirm this hypothesis.

Binding proteins found to be reduced in hypogonadic patients in our study include beta-microseminoprotein (MSMB), plasma serine protease inhibitor (IPSP) and lactotransferrin (TRFL). MSMB, also known as PSP94, is secreted by the epithelial cells of the prostate [43] and is one of the major constituents display in human seminal plasma [44]. A significant spontaneous acrosome reaction inhibition was observed in a model of guinea pig when epididymal spermatozoa were pre-incubated with MSMB [45]. In addition, previous studies have shown that MSMB binds human immunoglobulin [46,47]. It has been hypothesized that high amounts of MSMB present in the seminal plasma would bind to immunoglobulin and prevent an immune response to spermatozoa in the female reproductive tract [46]. MSMB has been moreover shown to interact with prostatic acid phosphatase (PPAP) and CRISP-3 proteins present in seminal plasma [48]. Similarly, a PPAP-containing zinc-binding multiprotein complex has also been characterized from the human seminal plasma [49], and we previously reported that male hypogonadism is associated with a reduction in seminal PPAP levels [15]. Here we report the reduction of both PPAP and MSMB in seminal plasma of male hypogonadism. The role of PPAP and MSMB as a part of such a functional network needs to be further examined.

Furthermore there is lactoferrin, which has an antioxidative, antibacterial, and immune-modulating role in seminal plasma [50]. Lactoferrin is additionally included in keeping up typical sperm structure and motility, and in tweaking the composition and quality of the semen during sperm maturation and migration through the genital tract [51]. We previously reported that male hypogonadism actuates a noteworthy decrease in lactoferrin [15]. Here, we confirm, through a quantitative proteomic approach, our previous observation.

Interestingly, the effect of testosterone replacement therapy in our hypogonadic patients has induced an increase in 6 proteins (Table 3). Besides, it is important to highlight that SEMG1, SEMG2, PPAP, PIP and TRFL protein levels have been observed as significantly reduced in hypogonadic patients versus controls, but their levels were increased after TRT (Tables 2 and 3), representing a putative panel for the diagnosis of male hypogonadism and the follow-up of the treatment. SEMG1, SEMG2 and PPAP are known androgen-dependent secretory products [52,53]. Of note, PIP is an aspartyl proteinase that ties to numerous proteins, counting human zinc-α-2 glycoprotein [54]. Its capacity to tie an expansive cluster of proteins demonstrates its multifaceted part in various biological processes, such as fertility, antimicrobial activity and immune-regulation [55]. A decrease of seminal PIP was moreover described in asthenospermic patients [56]. We reported in 2014 that PIP was not detectable by qualitative proteomics in seminal plasma by hypogonadic patients [15]. Here we confirm this observation, as PIP was found at lower levels in hypogonadic patients, and, therefore, we suggest that PIP might represent one of the most useful markers for male hypogonadism diagnosis and TRT follow-up.

This study represents the primary application of quantitative high-resolution MS-based proteomics pointed to distinguish an array of proteins in the seminal plasma of hypogonadic patients and reflecting a disability of epithelial cell secretory function in male secondary hypogonadism.

Some limitations of this study are that it was performed on a relatively small sample size. However, the detected proteins and its function are consistent with previous data described in the literature, as discussed above. It was moreover performed in a population receiving only TRT, not compared with patients treated with hCG. Further studies are so needed comparing patients treated with TRT and patients treated with hCG.

Furthermore, the identification of proteins involved in androgen action may represent the basis to clarify how androgens act in the physiology of the sexual accessory glands and could help to explain the link between hypogonadism and infertility. The proteomic approach performed herein in seminal plasma permitted to identify novel molecular markers, which might be translated in clinical practice for the diagnosis of patients with distinctive grades of male hypogonadism and in the TRT follow-up.

Supplementary Materials: The Supplementary Materials are available online at http://www.mdpi.com/2077-0383/8/12/2128/s1. It includes clinical and hormonal parameters of the population of 7 additional hypogonadic patients furtherly added in western blot analysis, the picture of all gels after enhanced chemiluminescence, all pictures of membranes after red ponceau staining and western blot membrane with detected bands, western blot quantification analysis.

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TREBALL 3

ELS ESPERMATOZOIDES ADQUIREIXEN PROTEÏNES ESPECÍFIQUES DE L'EPIDÍDIM A TRAVÉS DELS EPIDIDIMOSOMES

Article en preparació

Els espermatozoides adquireixen proteïnes específiques de l'epidídim a través dels epididimosomes

Objectiu: L'objectiu d'aquest treball és el de validar experimentalment l'adquisició per part de l'espermatozoide de proteïnes específiques de l'epidídim a través d'epididimosomes (vesícules extracel·lulars secretades per l'epidídim).

Materials i Mètodes: En primer lloc, s'ha identificat mitjançant una anàlisi *in silico* quines de les proteïnes espermàtiques humanes prèviament predites com a potencialment adquirides per l'epidídim també podrien ser adquirides per l'espermatozoide murí durant el seu trànsit per l'epidídim. Posteriorment, s'han obtingut seccions d'epidídim i de testicle d'humans i de ratolins, i espermatozoides de la cua de l'epidídim de ratolí, i s'ha avaluat la presència d'algunes de les proteïnes espermàtiques potencialment adquirides en l'epidídim mitjançant microscòpia confocal d'alta resolució. Addicionalment, les vesícules extracel·lulars presents en el fluid epididimari de la cua de l'epidídim de ratolins s'han marcat fluorescentment amb CFSE i, posteriorment, s'han aïllat els epididimosomes (fluorescentment marcats) mitjançant ultracentrifugació en coixins de sacarosa al 25-30%. Aquests epididimosomes marcats fluorescentment s'han incubat amb espermatozoides de ratolí i, mitjançant microscòpia confocal, s'ha avaluat tant la interacció epididimosomes-espermatozoides com la presència de les proteïnes epididimàries en els epididimosomes.

Resultats: A través de l'anàlisi *in silico* s'han identificat 25 proteïnes espermàtiques amb un potencial origen específic en l'epidídim conservat en ambdues espècies (humana i murina). Algunes d'aquestes proteïnes es troben involucrades en funcions consistents amb el procés de maduració posttesticular que té lloc a l'epidídim, tals com la maduració i funcionalitat espermàtica, fertilització, protecció antioxidant i producció d'energia, entre d'altres. L'origen epididimari de 4 d'aquestes 25 proteïnes espermàtiques potencialment adquirides de teixits extratesticulars s'ha validat mitjançant microscòpia confocal en ambdues espècies. Concretament, s'ha demostrat que les proteïnes *very long-chain acyl-CoA synthetase* (SLC27A2/FATP2), *epididymal secretory protein E3-beta* (EDDM3B), *keratin type I cytoskeletal 19* (KRT19) i *WAP four-disulfide core domain protein 8* (WFDC8) s'expressen en l'epidídim d'humà i de ratolí, i no en els respectius testicles, i que també s'identifiquen en espermatozoides de la cua de l'epidídim de ratolí. També s'ha demostrat que els epididimosomes que interactuen amb els espermatozoides *in vitro* contenen les 4 proteïnes espermàtiques d'origen epididimari estudiades.

Conclusions: Els resultats derivats d'aquest estudi posen de manifest la rellevància de les 25 proteïnes espermàtiques conservades evolutivament detectades en l'actual estudi per a la funció de l'espermatozoide, i demostren la participació dels epididimosomes en la transferència de proteïnes entre l'epidídim i els espermatozoides. Aquesta troballa emfatitza el rol que els epididimosomes podrien tenir en la modulació del proteoma de l'espermatozoide durant la seva maduració posttesticular.

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TREBALL 4

DETERMINACIÓ DE L'ENFOCAMENT MÉS ÒPTIM PER AÏLLAR VESÍCULES EXTRACEL·LULARS SEMINALS: UNA ANÀLISI DE PROTEÒMICA

Article en preparació

Determinació de l'enfocament més òptim per aïllar vesícules extracel·lulars seminals: una anàlisi de proteòmica

Objectiu: L'objectiu d'aquest treball és el de comparar, mitjançant anàlisis moleculars i tècniques de proteòmica, diverses metodologies per a la purificació de vesícules extracel·lulars (VEs) seminals a fi d'identificar la metodologia més adequada per al seu aïllament que permeti descobrir l'impacte de les VEs seminals en la funció espermàtica i la reproducció.

Materials i Mètodes: S'han aïllat VEs del plasma seminal de pacients normozoospèrmics (NZ) i azoospèrmics (AZ) per vasectomia mitjançant 3 metodologies: 1) Ultracentrifugació (UC), 2) Ultracentrifugació en coixins de sacarosa al 25-30% (25-30%UC), i 3) Ultracentrifugació en coixins de sacarosa al 25-30% seguit d'una immunoselecció mitjançant l'ús de boles magnètiques unides a l'anticòs específic de VEs CD63 (MBCD63). Posteriorment, les VEs seminals aïllades s'han caracteritzat per microscòpia electrònica, anàlisi NTA, western blot per marcadors específics de VEs (ex.: CD63, CD81) i marcadors de contaminació (ex.: Calnexina, Histona H3) i per proteòmica d'alt rendiment.

Resultats: La comparació mitjançant western blot de les VEs seminals aïllades per les 3 diferents metodologies ha demostrat que les VEs seminals purificades per la tècnica MBCD63 es troben enriquides en VEs CD63+, mentre que es redueixen els marcadors de contaminació. La comparació del perfil proteòmic de les VEs aïllades per 25-30%UC i MBCD63 ha confirmat aquesta troballa, ja que s'ha demostrat que les VEs seminals CD63+ estan enriquides en altres marcadors d'exosomes (ex.: CD81, CD9), i es redueixen altres marcadors de contaminació (ex.: Annexina A1, Annexina A5). També s'ha observat un enriquiment en els processos biològics "glicòlisi" i "reconeixement oòcit-espermatozoide" específicament en VEs seminals CD63+ d'individus NZ, el que confirma la implicació d'aquestes VEs en la mobilitat espermàtica i en l'adquisició del potencial de fertilització. Addicionalment, algunes d'aquestes proteïnes involucrades en els mencionats processos biològics també s'han identificat en les VEs CD63+ d'individus AZ, el que confirma que aquestes proteïnes són secretades per les glàndules sexuals accessòries, en lloc de ser restes de l'espermatogènesi o vesícules derivades d'espermatozoides apoptòtics. S'ha comparat un llistat compilat de proteïnes desregulades en espermatozoides de pacients astenozoospèrmics amb les proteïnes identificades en les VEs CD63+ de NZ, i s'ha observat com un terç (60 de 192) de les proteïnes desregulades en astenozoospèrmics es trobaven presents en les VEs. Per últim, s'ha estudiat el potencial origen tissular de les proteïnes identificades en VEs CD63+ de NZ, i s'han identificat proteïnes específiques de l'epidídim, pròstata i vesícules seminals.

Conclusions: En el present estudi s'han comparat diverses metodologies per aïllar VEs seminals i s'ha utilitzat, per primera vegada, una tècnica d'immunoafinitat per a seleccionar VEs seminals CD63+. La caracterització molecular ha confirmat que la

ultracentrifugació en coixins de sacarosa al 25-30% seguit de l'ús de boles magnètiques unides a l'anticòs específic de VEs CD63 resulta en la millor aproximació per a la purificació de VEs seminals. A més, la potencial implicació de les VEs seminals CD63+ en la mobilitat espermàtica i en la fusió espermatozoide-oòcit emfatitza el paper fonamental de les secrecions de VEs de l'epidídim i de les glàndules sexuals accessòries en la maduració posttesticular de l'espermatozoide, així com en l'adquisició de funcions necessàries per a la correcta fertilització.

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DISCUSSIÓ

La infertilitat és un problema mundial que afecta aproximadament al 15% de les parelles en edat reproductiva, sent el factor masculí el responsable del 50% dels problemes d'infertilitat (Sharlip *et al.*, 2002; Kovac *et al.*, 2013; Jodar *et al.*, 2017c). Malgrat i la seva alta prevalença, l'avaluació de la infertilitat masculina continua sent força limitada principalment a una anàlisi dels paràmetres seminals bàsics i a un examen físic, els quals, segons els resultats obtinguts, es poden complementar amb un estudi del perfil endocrinològic, anàlisis bacteriològics, estudis genètics o ecografia dels testicles i de la pròstata (Weber *et al.*, 2005; AUA Staff, 2010; Kovac *et al.*, 2013; Bieniek *et al.*, 2016). D'entre tots aquests estudis, l'anàlisi dels paràmetres seminals és considerada la pedra angular de l'avaluació de l'home que consulta per infertilitat. No obstant això, les anàlisis seminals tan sols són capaços de revelar alteracions en el recompte espermàtic, la mobilitat o la morfologia espermàtica i, avui en dia, s'han identificat un nombre limitat de causes associades als paràmetres seminals alterats (Esteves *et al.*, 2011; Jodar *et al.*, 2017a). Conèixer la causa de la infertilitat masculina és clau per a poder afrontar la consulta del pacient i, així doncs, prendre la millor decisió mèdica per satisfer el desig gestacional del pacient infèrtil, el que posa de manifest la necessitat de trobar noves eines diagnòstiques, pronòstiques i terapèutiques que permetin una millora en l'avaluació i el tractament de la infertilitat masculina (Kovac *et al.*, 2013). L'avaluació i el tractament del pacient infèrtil masculí és limitat sobretot degut a la falta de coneixement dels mecanismes moleculars implicats en la funció espermàtica i en la contribució real del pare en la generació de la descendència. Aquesta falta de coneixement és encara més evident en el 40% dels pacients que tot i tenir els paràmetres seminals en el rang de la normalitat són infèrtils (Dohle *et al.*, 2005; Hamada *et al.*, 2011).

En l'actualitat, una de les tècniques analítiques més utilitzades que està permetent una gran expansió en el coneixement de la biologia de la reproducció és l'ús d'espectrometria de masses en l'estudi del proteoma d'individus fèrtils. L'aplicació d'aquesta tècnica -òmica en l'estudi de pacients infèrtils també està permetent d'identificar mecanismes patogènics que podrien estar relacionats amb la infertilitat masculina, els quals podrien derivar en nous potencials biomarcadors d'alta utilitat en la pràctica clínica (Tomar *et al.*, 2012; Sharma *et al.*, 2013a; Amaral *et al.*, 2014b; Azpiazu *et al.*, 2014; Codina *et al.*, 2015; Gilany *et al.*, 2015; Bieniek *et al.*, 2016; Carrell *et al.*, 2016; Jodar *et al.*, 2017b; Saraswat *et al.*, 2017; Camargo *et al.*, 2018; Jodar *et al.*, 2020). El desenvolupament d'un panell de biomarcadors moleculars, a més d'ajudar a distingir l'origen de la infertilitat masculina, també podria ser una eina predictiva de l'èxit del tractament de la fertilitat, sent altament útil en la decisió del tractament de reproducció assistida a seguir (Jodar *et al.*, 2015, 2017b).

En els últims anys, hi ha un nombre creixent d'estudis de proteòmica centrats en l'avaluació del plasma seminal. L'anàlisi comparativa del proteoma del plasma seminal de diferents pacients infèrtils permet avaluar la funció de l'epidídim i de les glàndules sexuals accessòries, i descobrir l'impacte que les seves secrecions tenen per a la fisiologia de

l'espermatozoide i, fins i tot, en les primeres fases del desenvolupament embrionari (Wang *et al.*, 2009; Milardi *et al.*, 2013; Sharma *et al.*, 2013a; Jodar *et al.*, 2017b; Saraswat *et al.*, 2017; Camargo *et al.*, 2018; Druart and de Graaf, 2018; Samanta *et al.*, 2018; Jodar, 2019; Schjenken and Robertson, 2020). A més, el fet que el plasma seminal sigui un fluid biològic de fàcil obtenció el situa en una posició estratègica i òptima per a la identificació de biomarcadors moleculars per a un diagnòstic no invasiu de les alteracions de l'aparell reproductor masculí (Drabovich *et al.*, 2014). Per tant, l'aplicació de la proteòmica en l'estudi del plasma seminal té una gran potencialitat per al descobriment d'alteracions en l'epidídim i en les glàndules sexuals accessòries que podrien resultar en infertilitat masculina, i obre la porta a la identificació de nous biomarcadors que permetin millorar el diagnòstic, la classificació o el tractament de la infertilitat (Milardi *et al.*, 2013; Gilany *et al.*, 2015; Bieniek *et al.*, 2016). Així mateix, la recerca sobre la participació de les VEs presents en el plasma seminal en la reproducció masculina i la funcionalitat espermàtica ha esdevingut primordial en aquests últims anys. La seva rellevància biològica i la possibilitat que alteracions en les VEs podrien estar relacionades amb certes alteracions de la infertilitat masculina ha fet que les VEs també comencin a ser estudiades per tècniques proteòmiques amb la finalitat d'identificar i desenvolupar nous biomarcadors per a la infertilitat masculina, així com per a desenvolupar noves teràpies per al tractament de la infertilitat masculina.

En aquesta tesi s'han aplicat tècniques proteòmiques d'alt rendiment per a estudiar el perfil proteòmic del plasma seminal de pacients amb diferents tipus d'infertilitat (treball 1 i 2) amb la finalitat d'identificar nous potencials biomarcadors que permetin una millor estratificació, diagnòstic i avaluació de la infertilitat masculina. A més, també s'han aplicat tècniques de microscòpia i moleculars, conjuntament amb anàlisis *in silico* i anàlisis de proteòmica, per tal d'explorar la comunicació intercel·lular entre el tracte reproductor masculí i els espermatozoides mitjançant VEs (treball 3), així com per determinar l'impacte de les VEs seminals en la funcionalitat espermàtica (treball 4).

4.1. Aplicació de tècniques proteòmiques en el plasma seminal de pacients amb diferents tipus d'infertilitat: a la recerca de nous biomarcadors de fertilitat i/o infertilitat

L'aplicació de la proteòmica en l'àmbit clínic ha esdevingut una nova eina molt útil per a la identificació de marcadors moleculars per a la patologia. En l'àmbit de la reproducció, l'estudi del proteoma del plasma seminal ha resultat ser molt atractiu per a l'avaluació de la fertilitat/infertilitat masculina (Milardi *et al.*, 2013).

L'actual classificació de la infertilitat masculina basada en el seminograma ha resultat ser força limitada i insuficient. Amb l'objectiu de clarificar els mecanismes patogènics que resulten en les alteracions espermàtiques, en el treball 1 d'aquesta tesi es va realitzar un

estudi del proteoma del plasma seminal imitant la classificació de la infertilitat basada en els paràmetres seminals. La definició del perfil proteòmic del plasma seminal de pacients categoritzats com a normozoospèrmics, astenozoospèrmics, oligozoospèrmics i azoospèrmics, i la seva comparació mitjançant l'aplicació d'anàlisis convencionals, va permetre la identificació de proteïnes desregulades (ex.: ANPEP) en pacients amb mobilitat espermàtica alterada, com ja s'havia descrit prèviament (Wang *et al.*, 2009), i també va permetre la identificació d'una disminució gradual en l'abundància d'algunes proteïnes en pacients amb una baixa o nul·la concentració d'espermatozoides. Aquest descens en els nivells d'algunes proteïnes es va confirmar per a les proteïnes CRISP1, NPC2, SPINT3 i ECM1, on l'abundància d'aquestes proteïnes es correlacionava negativament amb la concentració d'espermatozoides. Gràcies a l'ús de la base de dades *Human Protein Atlas* es va identificar com les proteïnes ECM1, SPINT3 i CRISP1 es trobaven principalment expressades en l'epidídim, i no en testicle, el que suggeria que les secrecions de l'epidídim podien estar regulades per la presència d'espermatozoides, un resultat que es troba en concordança amb observacions prèvies en l'espècie murina i bovina (Garrett *et al.*, 1990; Reyes-Moreno *et al.*, 2008).

No obstant això, la realització de l'anàlisi de correlació a nivell de pèptids, en lloc de proteïna, va demostrar que només la proteïna NPC2 mantenia la majoria dels seus pèptids correlacionats amb la concentració espermàtica, el que podria ser degut a la presència de diverses isoformes o modificacions posttraduccionals de proteïnes, tal com hem demostrat posteriorment (Jodar *et al.*, 2020), així com per l'alta presència de fragments proteolítics com a resultat de la liqüefacció del semen (Jodar *et al.*, 2017c; Camargo *et al.*, 2018). Per tal de superar aquesta limitació, en el mateix treball 1 es va desenvolupar una nova tècnica basada en l'anàlisi de parells de proteïnes estables (PPE), la qual s'havia prèviament aplicat per a l'estudi de RNA-seq (Lalancette *et al.*, 2009; Mao *et al.*, 2013). Amb l'ús d'aquesta nova estratègia s'aconsegueix reduir l'heterogeneïtat de les dades proteòmiques, ja que permet identificar parelles de proteïnes on la majoria dels seus pèptids estan correlacionats entre si en un conjunt de mostres. L'anàlisi PPE en els diferents grups de pacients infèrtils va mostrar que només els pacients normozoospèrmics presentaven un elevat número de PPE, mentre que el número de PPE disminuïa dràsticament en els grups de pacients amb els paràmetres seminals alterats. Mentre que la baixa presència de PPE en pacients azoospèrmics no va resultar sorprenent, ja que era un grup constituït per pacients azoospèrmics obstructius i no obstructius, sí que va sobtar per als pacients astenozoospèrmics i oligozoospèrmics. Aquesta troballa és especialment rellevant, ja que posa de manifest que l'existència de múltiples alteracions resulten en el mateix fenotip, i evidencia la necessitat de noves estratègies que permetin una millora en l'estratificació actual dels pacients infèrtils, on l'estudi dels perfils moleculars podria ser de gran utilitat. A més, l'aplicació de l'anàlisi PPE per a l'estudi d'alteracions de mostres individuals fa d'aquesta una tècnica molt interessant per a la identificació de biomarcadors relacionats amb la infertilitat masculina i obre la porta a la seva utilització

per a una medicina personalitzada, el que resultaria en una millora de les estratègies clíniques dels pacients infèrtils.

Una altra aproximació és l'aplicació de les tècniques proteòmiques per a la identificació de potencials biomarcadors que proporcionin una millora en les estratègies diagnòstiques i terapèutiques. Aquests biomarcadors són altament necessaris quan els resultats de les actuals eines diagnòstiques poden ser inconclusius i, a més, poden ajudar a la presa de decisions durant el seguiment clínic del tractament. Per exemple, l'hipogonadisme secundari es caracteritza per uns nivells baixos de testosterona en sang combinats amb uns nivells normals o baixos de FSH i LH en sang. Uns nivells de testosterona inferiors a 8 nmol/L determinen perfectament l'hipogonadisme, mentre que uns nivells de testosterona superiors a 12 nmol/L representen els nivells normals. Actualment està en debat el diagnòstic de l'hipogonadisme i l'adequació d'un tractament en aquesta franja incerta entre els 8 i els 12 nmol/L i, en aquest cas, la identificació de nous biomarcadors sobre l'acció dels andrògens podria ser valuós per orientar el diagnòstic, per prendre una decisió sobre la teràpia de reemplaçament de testosterona (TRT) i pel seguiment clínic del pacient.

Així doncs, en el treball 2 es va realitzar una anàlisi de proteòmica quantitativa del plasma seminal de pacients amb hipogonadisme hipogonadotròpic (HH), i es va avaluar l'efecte de la teràpia TRT en les secrecions de l'epidídim i de glàndules sexuals accessòries. En primer lloc, es van identificar un total d'11 proteïnes en abundància disminuïda en el plasma seminal de pacients amb HH, en comparació amb el plasma seminal d'individus fèrtils. Aquest conjunt de proteïnes identificades podria resultar en el desenvolupament d'un panell de biomarcadors relacionats amb l'acció de la testosterona en la funció secretora de l'epidídim i de les glàndules sexuals accessòries, ja que aquestes proteïnes disminuïdes representen la resposta *in vivo* d'aquests teixits al dèficit de testosterona. A més, la modulació de la secreció d'aquestes proteïnes per la manca de la testosterona podria resultar en la identificació de nous biomarcadors d'alta utilitat en el diagnòstic de l'hipogonadisme masculí, principalment en pacients que presenten els nivells de testosterona en la franja entre els 8 i els 12 nmol/L.

L'anàlisi i comparació dels proteomes del plasma seminal dels pacients amb HH, abans i després de TRT, va evidenciar la presència de 6 proteïnes (PIP, PPAP, TRLF, MUC6, SEMG1 i SEMG2) les quals incrementaven amb el tractament amb testosterona. Cinc d'aquestes sis proteïnes (PIP, PPAP, TRLF, SEMG1 i SEMG2) s'havien prèviament detectat en abundància reduïda en pacients HH en comparació amb individus fèrtils, i els seus nivells van incrementar després de TRT. Aquests resultats es troben en concordança amb resultats previs, on 3 de les 6 proteïnes identificades (PIP, PPAP i TRFL) ja s'havien descrit com a incrementades amb el tractament TRT en un estudi qualitatiu del plasma seminal en pacients amb HH (Milardi *et al.*, 2014). Per tant, aquest conjunt de proteïnes podria representar un panell de biomarcadors per al diagnòstic de l'hipogonadisme i per al

seguiment clínic del tractament. En definitiva, la identificació d'aquest conjunt de proteïnes implicades en l'acció dels andrògens podria representar la base per a clarificar com els andrògens actuen en la fisiologia de l'epidídim i de les glàndules sexuals accessòries i podrien resultar en biomarcadors d'utilitat clínica per al diagnòstic de pacients amb diferents graus d'hipogonadisme i per al seguiment clínic de la teràpia amb testosterona.

Les anàlisis de proteòmica quantitativa d'alt rendiment del plasma seminal realitzades en la present tesi doctoral se sumen a la gran quantitat d'estudis que han permès una millor comprensió de la participació de les secrecions de l'epidídim i de les glàndules sexuals accessòries en la fisiologia de la reproducció masculina (Batruch *et al.*, 2012; Milardi *et al.*, 2013; Gilany *et al.*, 2015; Jodar *et al.*, 2017b, 2017c; Camargo *et al.*, 2018), i han proporcionat nous coneixements sobre mecanismes patogènics resultants en infertilitat masculina. Addicionalment, l'aplicació d'una nova tècnica d'anàlisi basada en PPE, així com les troballes derivades d'aquests estudis de proteòmica, representen una veritable promesa per a la identificació de nous potencials biomarcadors no invasius que permetin una optimització de les estratègies clíniques per a l'avaluació i tractament de la infertilitat masculina, i ens apropa cap a una medicina personalitzada.

4.2. Participació i impacte de les vesícules extracel·lulars del plasma seminal en la maduració i funció espermàtica

Com s'ha pogut observar al llarg de la tesi, la importància que juga el plasma seminal per a la correcta reproducció i funcionalitat de l'espermatozoide ha estat àmpliament demostrada (Lindholmer, 1974; Primakoff and Myles, 2002; Milardi *et al.*, 2013; Drabovich *et al.*, 2014; Jodar *et al.*, 2017c; Saraswat *et al.*, 2017; Camargo *et al.*, 2018; Druart and de Graaf, 2018; Samanta *et al.*, 2018; Barrachina *et al.*, 2019; Schjenken and Robertson, 2020). No obstant això, no ens podem oblidar de l'alta població de VEs que es troben presents en el plasma seminal. Les VEs seminals són secretades per l'epidídim i per les glàndules sexuals accessòries i tenen una especial rellevància, ja que sembla que proporcionen a l'espermatozoide, una cèl·lula transcripcionalment i traduccionalment inerta, el seu contingut ric en proteïnes, RNAs i lípids. Aquesta fusió entre les VEs seminals i els espermatozoides sembla que té un rol fonamental per a la funcionalitat espermàtica, la maduració posttesticular de l'espermatozoide i l'adquisició del potencial de fertilització de l'espermatozoide (Aalberts *et al.*, 2014; Tannetta *et al.*, 2014; Sullivan, 2015; Foster *et al.*, 2016; Machtinger *et al.*, 2016; Jodar *et al.*, 2017c; Simon *et al.*, 2018; Murdica *et al.*, 2019b), rols que s'havien descrit pel plasma seminal.

Malgrat que hi ha evidències de la interacció entre VEs seminals i espermatozoides (Frenette *et al.*, 2002, 2010; Schwarz *et al.*, 2013; Păunescu *et al.*, 2014; Reilly *et al.*, 2016; Battistone *et al.*, 2019; Nixon *et al.*, 2019; Zhou *et al.*, 2019), i de la transferència de RNAs

als espermatozoides mitjançant VEs (Reilly *et al.*, 2016; Sharma *et al.*, 2016, 2018), la transferència de proteïnes per via de VEs seminals encara no està prou caracteritzada.

El treball 3 d'aquesta tesi es focalitza en la demostració de la participació de les VEs seminals en la transferència de proteïnes cap als espermatozoides. A causa de la gran heterogeneïtat de VEs seminals, ens vam focalitzar específicament en les secrecions de l'epidídim i en el seu contingut d'epididimosomes, ja que el pas de l'espermatozoide per l'epidídim suscita molt interès perquè és el lloc on es produeix principalment la maduració posttesticular de l'espermatozoide (Yanagimachi et al., 1985; Sullivan et al., 2007; Sullivan and Saez, 2013; Sullivan, 2015; Gervasi and Visconti, 2017). Primerament, amb l'ampliació d'una anàlisi *in silico* prèviament realitzada en el nostre grup (Castillo *et al.*, 2018), es van identificar un conjunt de proteïnes espermàtiques amb un potencial origen en l'epidídim conservat tant en l'espècie humana com en la murina. L'adquisició d'aquestes proteïnes per part de l'espermatozoide durant el seu trànsit per l'epidídim podria ser clau per a la maduració funcional que es duu a terme en els espermatozoides que han abandonat el testicle (Sullivan *et al.*, 2007; Breton *et al.*, 2016; Zhou *et al.*, 2018), ja que les proteïnes identificades tenen rols relacionats amb la maduració i funció espermàtica, fertilització, producció d'energia, protecció antioxidant i immunoregulació, entre d'altres.

El possible origen epididimari de 4 de les proteïnes espermàtiques (SLC27A2, EDDM3B, KRT19 i WFDC8) identificades en el treball 3 es va validar per microscòpia confocal d'alta resolució en humà i en ratolí. La validació de l'origen epididimari d'aquestes 4 proteïnes seleccionades a l'atzar fa pensar que les altres proteïnes espermàtiques predites en el nostre estudi *in silico* també provenen de l'epidídim. La identificació d'aquestes 4 proteïnes en epididimosomes que estaven en contacte amb espermatozoides va confirmar que les VEs secretades per l'epidídim contenen una càrrega específica de proteïnes epididimàries que poden ser transferides als espermatozoides. Per tant, sembla evident que els epididimosomes podrien estar modulant el proteoma de l'espermatozoide gràcies a l'aportació del seu contingut en proteïnes i, conseqüentment, podrien ser un dels responsables del procés de maduració posttesticular que té lloc a l'epidídim.

Una de les quatre proteïnes espermàtiques presents en els epididimosomes, concretament la proteïna SLC27A2, es va trobar exclusivament expressada en les cèl·lules clares de l'epidídim. Aquesta troballa va resultar ser sorprenent, ja que les cèl·lules clares de l'epidídim es caracteritzen per tenir una alta capacitat d'endocitosi (Hermo and Robaire, 2002; Zhou *et al.*, 2018; Breton *et al.*, 2019), i el present treball obre les portes a pensar que aquestes cèl·lules també podrien tenir un rol secretor i participar activament en la producció d'epididimosomes que podrien participar en la transformació funcional dels espermatozoides.

La comprensió del rol que juga l'epidídim, així com les glàndules sexuals accessòries, en la funció espermàtica i en la reproducció mitjançant la participació de les VEs seminals és crucial per a la millora de la comprensió de la biologia reproductiva, i podria permetre la

identificació de dianes moleculars extratesticulars que podrien tenir una implicació directa en la salut reproductiva masculina. Una de les aproximacions que recentment s'està duent a terme per tal d'elucidar l'impacte de les VEs seminals en la fertilitat masculina és l'aprofundiment en el coneixement de la seva composició molecular (Utleg *et al.*, 2003; Rejraji *et al.*, 2006; Frenette *et al.*, 2006, 2010; Thimon *et al.*, 2008; Poliakov *et al.*, 2009; Girouard *et al.*, 2011; Belleannée *et al.*, 2013a, 2013b; Brouwers *et al.*, 2013; Vojtech *et al.*, 2014; Abu-Halima *et al.*, 2016; Reilly *et al.*, 2016; Sharma *et al.*, 2016; Yang *et al.*, 2017; Barceló *et al.*, 2018, 2019; García-Rodríguez *et al.*, 2018; Lin *et al.*, 2019; Murdica *et al.*, 2019a; Nixon *et al.*, 2019; Kaddour *et al.*, 2020). Tanmateix, per tal de descobrir la rellevància biològica de les VEs seminals, els mètodes d'aïllament d'aquestes VEs haurien d'assegurar una bona purificació VEs seminals previ a la caracterització molecular del seu contingut.

Creiem que una bona metodologia per a l'aïllament de les VEs seminals hauria de permetre l'enriquiment en VEs seminals, i la reducció de la majoria dels contaminants, com serien les partícules lipídiques o els agregats proteics, entre d'altres (Colombo *et al.*, 2014; Lötvall *et al.*, 2014; Szatanek *et al.*, 2015; Konoshenko *et al.*, 2018; Brennan *et al.*, 2020). El fet que es coneguin marcadors específics de VEs presents en la superfície de la seva membrana, tals com el CD63, CD9 i CD81, fa que sigui possible aïllar específicament aquestes VEs per immunoafinitat (Clayton *et al.*, 2001; Koliha *et al.*, 2016; Li *et al.*, 2017b). Amb la finalitat d'identificar quina podria ser la metodologia òptima per a l'aïllament de VEs seminals, en el treball 4 es va dur a terme una comparació molecular i proteòmica de diferents estratègies per purificar VEs basades en metodologies estàndard de precipitació directa, de selecció de mida i densitat, i de selecció específica per immunoafinitat. L'aïllament de VEs seminals mitjançant ultracentrifugació en coixins de sacarosa al 25-30% seguit de l'ús de boles magnètiques unides a l'anticòs específic de VEs CD63 va resultar ser l'estratègia més adequada per a una correcta purificació de VEs provinents del semen. Les dades proteòmiques van mostrar que, a més d'aconseguir un enriquiment en VEs CD63+, també es va trobar un increment dels altres marcadors de VEs CD9 i CD81, el que dóna suport a l'ús d'aquesta tècnica per a l'aïllament de VEs seminals.

El fet que es va trobar un enriquiment en els processos biològics "glicòlisi" i "reconeixement oòcit-espermatozoide" en les VEs seminals CD63+ d'individus normozoospèrmics va corroborar que aquesta població específica de VEs seminals podria estar implicada en la mobilitat espermàtica i en l'adquisició del potencial de fertilització de l'espermatozoide, tal com s'havia prèviament descrit per a les VEs seminals en general (Sullivan and Saez, 2013; Aalberts *et al.*, 2014; Sullivan, 2016; Simon *et al.*, 2018; Jodar, 2019; Baskaran *et al.*, 2020). A més, la identificació de la majoria d'aquestes proteïnes relacionades amb la glicòlisi i amb la fertilització en VEs seminals CD63+ de pacients azoospèrmics per vasectomia emfatitzava la rellevància que tenen les secrecions de les glàndules sexuals accessòries en la funció espermàtica i la reproducció (Saez *et al.*, 2003; Burden *et al.*, 2006; Duncan and Thompson, 2007; Aalberts *et al.*, 2014; Saez and Sullivan,

2016; Sullivan and Belleannée, 2017; Zhou *et al.*, 2018). La participació de les proteïnes presents en les VEs seminals CD63+ en la mobilitat espermàtica és recolzada per l'observació que un terç de les proteïnes espermàtiques alterades en pacients astenozoospèrmics han estat identificades en les VEs seminals CD63+.

La participació de l'epidídim, la pròstata i les vesícules seminals en la secreció de VEs seminals CD63+ també es va demostrar, ja que s'han identificat proteïnes en el proteoma de les VEs CD63+ que només s'expressen en aquests teixits. L'expressió específica en l'epidídim d'algunes d'aquestes proteïnes de VEs seminals ja les havíem validat en els estudis previs que formen part d'aquesta tesi, com seria el cas de la proteïna ECM1 (Barrachina *et al.*, 2019), i les proteïnes EDDM3B i WFDC8 (Barrachina *et al.*, 2020). A més, la identificació de les proteïnes EDDM3B i WFDC8 mitjançant proteòmica en VEs seminals CD63+ consolida la nostra troballa prèvia, on es va descriure que els epididimosomes contenien aquestes proteïnes i les podien proporcionar als espermatozoides. La identificació d'algunes proteïnes de VEs seminals CD63+ amb un origen específic en les vesícules seminals va resultar ser molt interessant, ja que fins ara es creia que les VEs seminals provenien principalment de l'epidídim (epididimosomes) i de la pròstata (prostasomes) (Baskaran *et al.*, 2020), però aquesta troballa suggeria que les vesícules seminals també podrien contribuir en la secreció de VEs.

Un dels punts més rellevants d'aquest estudi recau en el fet que s'ha utilitzat una metodologia innovadora, basada en l'ús de boles magnètiques unides a l'anticòs específic de VEs CD63, que ha permès una millora en la purificació de VEs seminals i, conseqüentment, en la identificació del contingut proteic de les VEs CD63+ del semen. Som conscients que les proteïnes identificades no corresponen a la totalitat de les VEs seminals, ja que una de les limitacions d'aquesta tecnologia és que només permet aïllar VEs que continguin el marcador específic en la superfície de la seva membrana. No obstant això, proporcionen una visió real del contingut d'aquestes VEs específiques i, per tant, permet determinar la rellevància biològica de les VEs seminals CD63+. Futurs estudis utilitzant aquesta mateixa metodologia però amb altres marcadors de VEs, com el CD81 o el CD9, permetran caracteritzar amb major profunditat la composició molecular de l'heterogènia població de VEs presents en el semen. A més, el fet que aquesta tecnologia permet l'aïllament d'una determinada població de VEs obre la porta a l'ús de marcadors específics de glàndules sexuals accessòries que permetin descobrir quina és la seva participació en la funcionalitat de l'espermatozoide i en la fertilitat en general. Un cop determinada la contribució de les VEs seminals en condicions fisiològiques, seria també interessant avaluar com les alteracions en les glàndules sexuals accessòries modifiquen el contingut de les seves VEs, el que podria ser una font de biomarcadors per a l'avaluació del correcte funcionament d'aquestes glàndules. A més, també podria resultar en la identificació de biomarcadors útils per al diagnòstic i seguiment del càncer de pròstata, on s'ha demostrat la participació de VEs secretades per les cèl·lules prostàtiques canceroses

en la regulació del desenvolupament del càncer i la metàstasi (Shephard *et al.*, 2017; Vlaeminck-Guillem, 2018; Lorenc *et al.*, 2020).

En resum, els presents estudis realitzats en VEs seminals han contribuït a ampliar el coneixement de la participació de les VEs seminals en la funció espermàtica i, per tant, en la biologia reproductiva. L'aplicació de la proteòmica en l'estudi de VEs seminals prèviament aïllades per marcadors específics té una gran potencialitat per identificar mecanismes moleculars alterats resultants d'una disfunció de l'epidídim o de les glàndules sexuals accessòries que podrien estar afectant la correcta reproducció, i obre la porta a la identificació de nous biomarcadors que permetin identificar on es troba el possible origen de la infertilitat masculina. Addicionalment, a més de poder resultar en noves eines diagnòstiques i pronòstiques, l'habilitat de les VEs seminals de fusionar-se i proporcionar el seu contingut també podria derivar en el seu ús com a nova eina terapèutica per al tractament de casos específics d'infertilitat.

5 CONCLUSIONS

CONCLUSIONS

Les conclusions derivades dels resultats obtinguts en aquesta tesi doctoral són les següents:

1. S'ha desenvolupat una nova estratègia per a l'estudi de perfils proteòmics basada en l'anàlisi de parells de proteïnes estables (PPE) que permet identificar parelles de proteïnes on la majoria dels seus pèptids estan correlacionats entre si i, per tant, aconsegueix reduir l'heterogeneïtat de les dades proteòmiques.

2. L'aplicació de l'anàlisi PPE per a l'estudi del proteoma del plasma seminal de pacients amb alteracions en els paràmetres seminals ha demostrat que l'existència de múltiples alteracions proteòmiques resulten en el mateix fenotip, suggerint que l'estratificació actual de la infertilitat masculina basada en el seminograma resulta insuficient per a discriminar entre diverses potencials etiologies.

3. L'anàlisi PPE també ha permès la identificació de proteïnes o mecanismes alterats en mostres individuals, sent d'utilitat per a un diagnòstic personalitzat de la infertilitat masculina.

4. L'anàlisi de proteòmica quantitativa del plasma seminal de pacients amb hipogonadisme secundari ha permès la identificació de nous biomarcadors per al diagnòstic de l'hipogonadisme hipogonadotròpic, així com per al seguiment de la teràpia de reemplaçament de testosterona.

5. S'han identificat i parcialment validat un conjunt de 25 proteïnes espermàtiques que són transferides als espermatozoides durant el seu pas per l'epidídim, les quals, en estar evolutivament conservades, podrien ser especialment rellevants per a la maduració i funció espermàtica.

6. S'ha demostrat que els epididimosomes estan implicats en una transferència de proteïnes específiques entre l'epidídim i els espermatozoides i, per tant, podrien participar en el procés de maduració posttesticular de l'espermatozoide que té lloc a l'epidídim.

7. S'ha proporcionat nova evidència científica sobre el possible rol de les cèl·lules clares de l'epidídim en la secreció d'epididimosomes, demostrant així que les cèl·lules principals no són les úniques cèl·lules epididimàries responsables de secretar vesícules extracel·lulars (VEs).

8. S'ha descrit una nova metodologia per a l'aïllament de VEs seminals basada en la ultracentrifugació en coixins de sacarosa al 25-30% seguit d'una immunoselecció mitjançant l'ús de boles magnètiques unides a l'anticòs específic de VEs CD63 que suposa una millora en les tècniques de purificació de VEs seminals.

9. L'estudi de la composició de les VEs seminals CD63+ mitjançant proteòmica d'alt rendiment ha permès la caracterització d'una població de VEs seminals fins ara mai estudiada, i s'han identificat un total de 832 proteïnes en individus amb paràmetres seminals normals.

10. S'ha suggerit que les VEs seminals CD63+ secretades per l'epidídim i/o per les glàndules sexuals accessòries podrien participar en funcions tant importants com la mobilitat espermàtica o l'adquisició del potencial de fertilització per part de l'espermatozoide.

11. S'han identificat proteïnes de les VEs seminals CD63+ exclusives de l'epidídim, la pròstata i les vesícules seminals, de tal manera que s'ha corroborat la participació d'aquests òrgans en la producció de VEs seminals i el seu impacte en la funció espermàtica.

CONCLUSIONS

The conclusions derived from the results obtained in this doctoral thesis are the following:

1. *A new strategy for the analysis of proteomics data based on the establishment of stable-protein pairs has been developed, allowing to identify protein pairs where most of their peptides are correlated with each other and, therefore, contributing to reduce the heterogeneity of the proteomic data.*

2. *The use of stable-protein pairs analysis for the study of the seminal plasma proteome signatures in patients with alterations in seminal parameters has shown that the existence of multiple proteomic alterations is present in patients with the same phenotype. This in turn suggests that the current male infertility stratification based on a seminogram is insufficient to discriminate between various potential etiologies.*

3. *Stable-protein pairs analysis has also enabled the identification of altered proteins or pathogenic mechanisms in individual samples, therefore opening a window to its application in the personalized diagnosis of male infertility.*

4. *The quantitative proteomic analysis of the seminal plasma of patients with secondary hypogonadism has permitted the identification of novel biomarkers for the diagnosis of hypogonadotropic hypogonadism, and for the testosterone replacement therapy follow-up.*

5. *A subset of 25 sperm proteins transferred to sperm during their passage through the epididymis have been identified and partially validated. As they are evolutionarily conserved, these extra-testicularly acquired sperm proteins could be especially relevant for sperm maturation and function.*
6. *It has been demonstrated that epididymosomes are involved in a specific protein transfer from the epididymis to spermatozoa and, therefore, epididymosomes could participate in the sperm post-testicular maturation process that takes place in the epididymis.*
7. *New scientific evidence has been provided on the potential role of epididymal clear cells in the secretion of epididymosomes, thus demonstrating that principal cells are not the only epididymal cell type responsible for secreting extracellular vesicles (EVs).*
8. *A new methodology for the purification of seminal EVs based on ultracentrifugation in 25-30% sucrose cushions followed by immuno-isolation using magnetic beads coated with the CD63 antibody has been applied for the first time, representing an enhanced methodology to isolate seminal EVs.*
9. *The assessment of the protein content of CD63+ seminal EVs using high-throughput proteomics has allowed the characterization of a specific population of seminal EVs, identifying up to 832 EVs proteins from individuals with normal seminal parameters.*
10. *It has been suggested that CD63+ seminal EVs secreted by the epididymis and/or by the male accessory sex glands could have a fundamental role in sperm motility and the acquisition of the sperm fertilizing potential.*
11. *Proteins from CD63+ seminal EVs originating from the epididymis, prostate, and seminal vesicles have been identified, thereby evidencing the participation of these organs in the production of seminal EVs and their importance for sperm function.*

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7 ANNEXOS

Annex 1_

BARRACHINA F, SOLER-VENTURA A, OLIVA R, JODAR M (2018)

Chapter 2: Sperm Nucleoproteins (histones and protamines).

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Chapter 2
Sperm Nucleoproteins (Histones and Protamines)

Ferran Barrachina, Ada Soler-Ventura, Rafael Oliva, and Meritxell Jodar

2.1 Introduction

Diploid spermatogonial stem cells differentiate into haploid spermatozoa by an accurately controlled process termed spermatogenesis (Fig. 2.1). Spermatogenesis comprises three distinct phases: a mitotic proliferation phase, a meiotic phase, and the differentiation/maturation phase also known as spermiogenesis [1]. During this last phase, the round spermatids undergo significant nuclear, morphological, and cytoplasmic changes to end up becoming motile, haploid, and highly condensed spermatozoa.

One of the most remarkable features of spermatogenesis is the chromatin dynamics along the different phases (Fig. 2.1) [2–5]. Similarly to somatic cells, the DNA in differentiating spermatogonia is packaged by nucleosomes. Spermatogonia replicate by mitosis to ensure the maintenance of germinal stem cell population. However, certain spermatogonia will enter into meiosis to halve its chromosome content and give rise to haploid germ cells. In the prophase of the first meiotic division, the homologous chromosome recombination occurs. One prerequisite for the homologous recombination is the introduction of DNA double-strand breaks (DSB) and its subsequent repair. A high number of DSB are induced in meiotic cells, and only few of them will be resolved as chromosome crossovers, therefore

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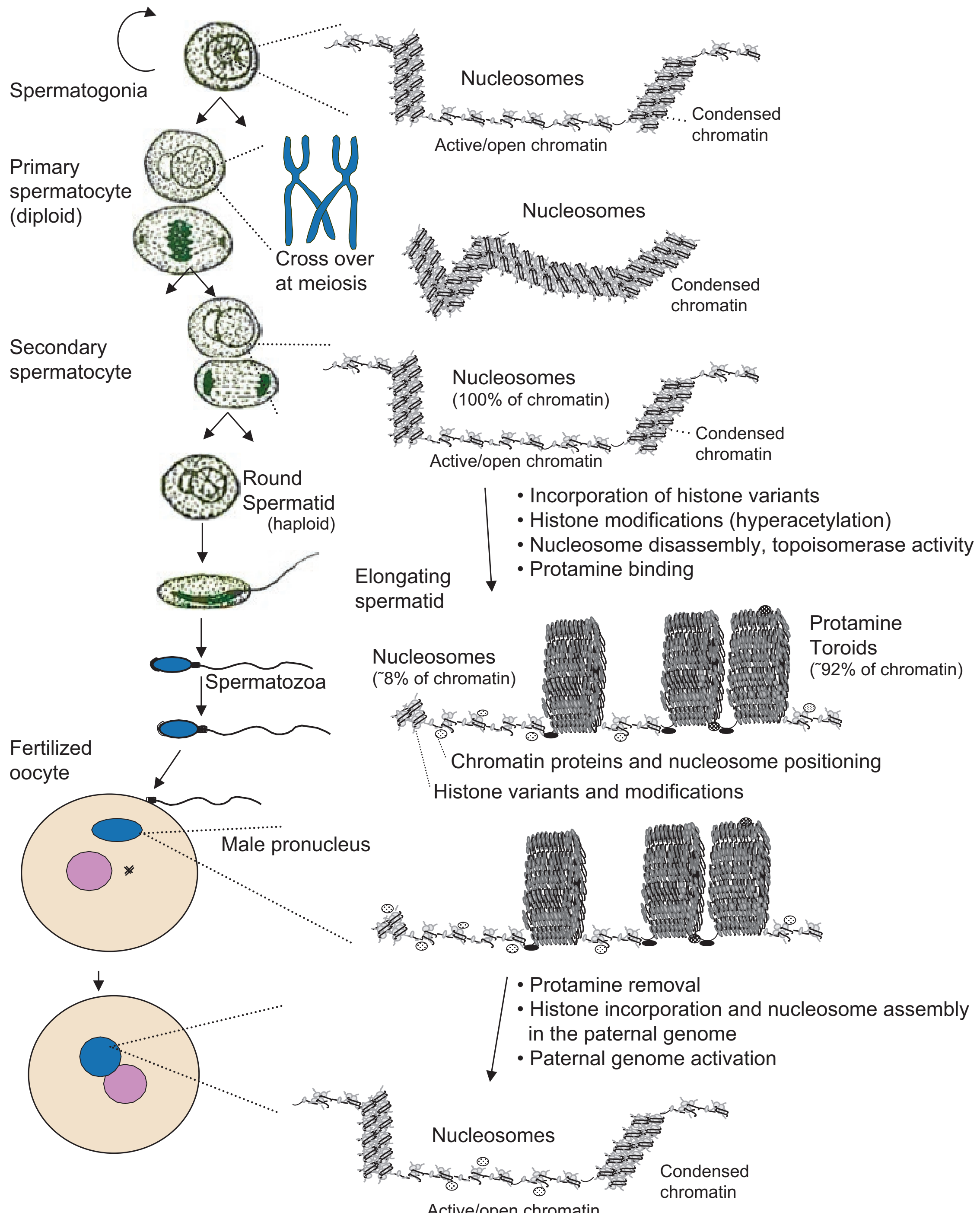

Fig. 2.1 Cellular and chromatin changes during spermatogenesis and fertilization. The main cellular changes (left) are represented together with the concomitant main chromatin changes (right). Spermatogonia replicate and differentiate into primary spermatocytes, which undergo crossing over at meiosis and genetic recombination, and give rise to the secondary spermatocytes after division. Secondary spermatocytes will then divide and give rise to the haploid round spermatids. The round spermatids possess a chromatin structure similar to that of the preceding cells and somatic cells formed by nucleosomes. However, a differentiation process called spermiogenesis is then initiated where the nucleosomal chromatin structure is disassembled and replaced by a highly compact nucleoprotamine complex. The disassembly of nucleosomes changes the superhelicity of the DNA and requires the action of topoisomerases. In the human sperm, about 92% of the chromatin DNA is condensed by protamines forming highly compact toroidal structures each packaging about 50 KB of DNA, and about 8% of the chromatin is formed by nucleosomes. The genes and repetitive sequences are specifically distributed in the nucleohistone and nucleoprotamine structure, and this peculiar chromatin structure is transferred to the oocyte at fertilization. After fertilization, the paternal chromatin must undergo the nucleoprotamine disassembly and the de novo assembly of nucleosomes before paternal gene expression starts

ensuring the genetic variability of the resulting germ cells [6]. In the meiotic and postmeiotic germ cells, the canonical histones are replaced sequentially, first by histone variants [7], subsequently, during spermiogenesis, by transition proteins (TNPs), and, finally, by protamines, following a precise and well-established timing (see Chap. 1) [2, 3, 5, 8]. This process results in a dramatic reorganization of the chromatin exchanging the nucleosomal histone-based structure in the diploid spermatogonia to a nuclear structure tightly packaged by protamines in the haploid spermatozoa, with a potential function in the sperm DNA protection [2, 3]. The multistep procedure of histone exchange requires the contribution of histone variants, as well as histone posttranslational modifications (PTMs); chromatin readers, for example, BRDT [9]; and the transient induction of DSB by topoisomerases to probably eliminate DNA supercoils formed during histone removal [10]. Of relevance, topoisomerases or topoisomerase activity also seems to be present in the final sperm chromatin and may be related to sperm DNA integrity (see Chap. 3) [11]. However, the underlying mechanisms of chromatin reorganization in developing spermatozoa are still poorly understood. Although most of the histones are replaced by protamines during spermatogenesis, the human sperm retains approximately 5–15% of its genome packaged by histones [12]. After fertilization, when the sperm nucleus enters into the oocyte cytoplasm, protamines are quickly replaced by maternal histones, although this process is also poorly understood [13]. However, it has been suggested that the sperm chromatin bound to histones could act as an epigenetic signature with a pivotal role during the activation of zygote genome in early embryogenesis, as well as on transgenerational epigenetic inheritance [14–16].

In this chapter, we highlight the most relevant proteins present in mature spermatozoa, the protamines, and histones, including their variants, their PTMs distribution in the sperm chromatin, and their potential correlation with male infertility.

2.2 Nucleoprotamine Complex in Sperm

Protamines are the most abundant sperm nuclear proteins in many species and in human are packing approximately the 85–95% of the paternal DNA [2, 3, 17–19]. Protamines are small basic proteins rich in positively charged arginine residues, allowing the formation of a highly condensed complex with the negatively charged paternal DNA. Additionally, protamines are rich in cysteine residues, which allow the formation of disulfide bonds and zinc bridges among intra- and inter-protamine molecules resulting in the compact toroidal nucleoprotamine complex [20, 21]. In mammals, two types of protamines have been described, the protamine 1 (P1) and the protamine 2 (P2) family. All mammal species harbor P1 in spermatozoa, but the P2 family, composed by the P2, P3, and P4 components, is solely expressed by some mammal species, such as humans and mice [18, 22]. Typically, the genes encoding protamines (*PRM1* and *PRM2*) are clustered together. In human, the protamine gene cluster is located in chromosome 16 together with the transition nuclear protein 2 (*TNP2*) gene [23]. Whereas P1 is synthesized as a mature form, P2 family is generated from the proteolysis of the protamine 2 precursor resulting in the

different components of P2 family (P2, P3, P4), which differ among them only by one to four amino acid residues on the N-terminal extension, being the P2 the most abundant [17, 18].

Although several hypotheses of the P1 and P2 family functions have been proposed [2, 3, 18], the most accepted protamine functions are:

(i) To tightly package the paternal genome in a more compact and hydrodynamic nucleus required for a proper sperm motility
(ii) To protect the paternal genome from exogenous or endogenous mutagens or nucleases potentially present in the male and/or female tracts
(iii) To compete with and remove transcriptional factors and other nuclear proteins from the spermatid chromatin, leaving the paternal genome in a "blank state" so that the paternal genome could be reprogrammed by the oocyte
(iv) To be involved in the imprinting of the paternal genome during spermatogenesis and to confer new epigenetic marks in certain areas of the sperm genome, leading to gene reactivation or repression in the first steps of early embryo development [3, 18]

2.2.1 Protamine Post-translational Modifications

In contrast to the well-known roles of histone PTMs, such as acetylation, methylation, and phosphorylation (see Sect. 3.2), relatively little is known about protamine PTMs. The most well-studied protamine PTM has been phosphorylation (Fig. 2.1, Table 2.1) [2, 3]. Protamines are quickly phosphorylated after their synthesis in elongated spermatids, as a requisite for the proper protamine binding to sperm DNA [17]. However, after the protamine-DNA binding, protamines are extensively dephosphorylated except in some residues whose phosphorylation can still be observed in the mature sperm (Fig. 2.2, Table 2.1) [24, 27–29]. Another type of protamine PTM is the differential processing of protamine 2 precursors. In fact, protamine 2 is synthesized as a long precursor protein which is then proteolytically processed to give rise to the mature P2, P3, and P4 components [30]. More recently, the use of mass spectrometry has allowed to identify additional PTMs in both protamines, suggesting the existence of a protamine code similar to the histone code [31, 32] that could be relevant for zygote epigenetic reprogramming [26, 33, 34]. In mature human sperm, the analysis of the extracted intact protamines by mass spectrometry has enabled to identify mono-, di-, and tri-phosphorylations, di-acetylations, and a mono-methylation for P1 [25]. Using the same strategy, only the intact P3 component could be identified from the P2 family with two potential PTMs (one acetylation and one methylation) [25]. However, further studies are required in humans including the amino acid sequencing by mass spectrometry in order to identify new protamine PTMs and localize the modified residues, as has been recently described in mouse (Fig. 2.2, Table 2.1) [26].

Table 2.1 Posttranslational modifications (PTMs) detected in human and mouse protamine amino acid sequences

Specie	Protamine	Amino acid residue	Post-translational modification	Methodology	Reference
Human	Protamine 1	S8	Phosphorylation	Electrospray mass spectrometry	Chirat et al. [24]
				Phosphoserine conversion and protein sequencing	Pirhonen et al. [29]
		S10	Phosphorylation	Electrospray mass spectrometry	Chirat et al. [24]
				Phosphoserine conversion and protein sequencing	Pirhonen et al. [29]
		S28	Phosphorylation	Phosphoserine conversion and protein sequencing	Pirhonen et al. [29]
		ND	Phosphorylation	Mass spectrometry	Castillo et al. [25]
		ND	Acetylation	Mass spectrometry	Castillo et al. [25]
	Protamine 2	S50	Phosphorylation	Phosphoserine conversion and protein sequencing	Pirhonen et al. [29]
		S58	Phosphorylation	Electrospray mass spectrometry	Chirat et al. [24]
				Phosphoserine conversion and protein sequencing	Pirhonen et al. [29]
		S72	Phosphorylation	Phosphoserine conversion and protein sequencing	Pirhonen et al. [29]
Mouse	Protamine 1	S8	Phosphorylation	Mass spectrometry	Brunner et al. [26]
		S42	Phosphorylation	Mass spectrometry	Brunner et al. [26]
		S42	Acetylation	Mass spectrometry	Brunner et al. [26]
		T44	Phosphorylation	Mass spectrometry	Brunner et al. [26]
		K49	Methylation	Mass spectrometry	Brunner et al. [26]
		K49	Acetylation	Mass spectrometry	Brunner et al. [26]
		N-terminal	Acetylation	Mass spectrometry	Brunner et al. [26]
	Protamine 2	S55	Phosphorylation	Mass spectrometry	Brunner et al. [26]
		S55	Acetylation	Mass spectrometry	Brunner et al. [26]
		K57	Acetylation	Mass spectrometry	Brunner et al. [26]
		K64	Acetylation	Mass spectrometry	Brunner et al. [26]

The table shows the PTMs identified in human and mouse protamine 1 or protamine 2 amino acid residues and the methodology performed

ND Not determined

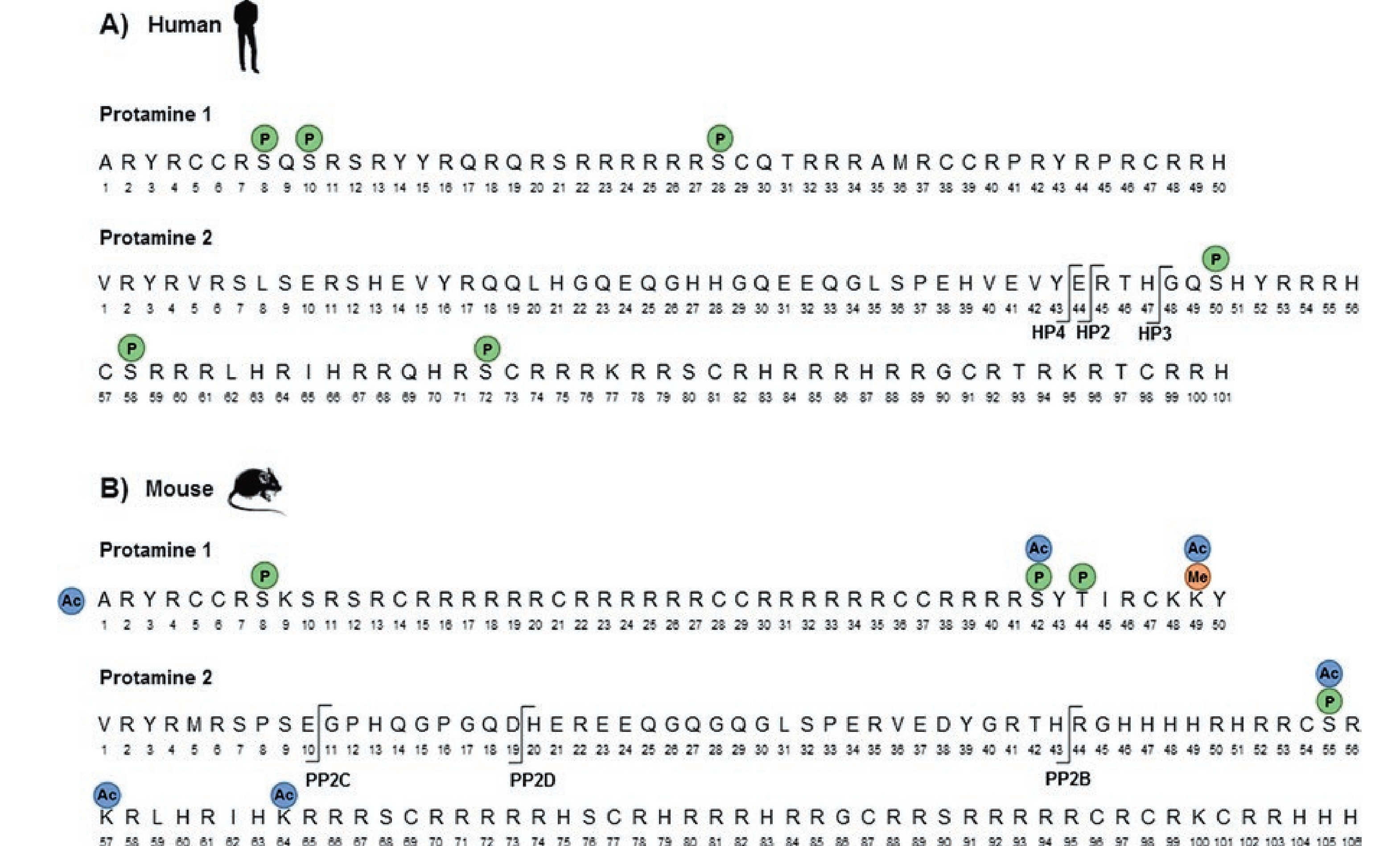

Fig. 2.2 Protamine amino acid sequences and posttranslational modifications (PTMs) detected in human and mouse spermatozoa. PTMs are indicated by Ac for acetylation, Me for methylation, and P for phosphorylation. Residues are numbered after the cleaved methionine. (**a**) Human protamine 1 and protamine 2 amino acid sequences with the described PTMs. The human HP2, HP3, and HP4 cleavage sites are indicated in the sequence. (**b**) Mouse protamine 1 and protamine 2 amino acid sequence with the described PTMs. The mouse PP2B, PP2C, and PP2D cleavage sites are indicated in the sequence

2.2.2 Protamine Alterations in Infertile Patients

The relative ratio of the abundance of the protamine 1 (P1) and the protamine 2 (P2) has been widely studied as a measure of sperm chromatin maturity and normality/abnormality [18]. A prospective study in the general population proposed the presence of a wide range for P1/P2 ratio that can oscillate between 0.5 and 1.5 [35]. However, from a reproductive view, several groups proposed a P1/P2 ratio around 1 (0.8–1.2) for fertile males [36, 37]. An altered P1/P2 ratio (below 0.8 or above 1.2) has been correlated among seminal parameter alterations, DNA damage, and low success rate of assisted reproduction techniques (Table 2.2). A recent meta-analysis comparing infertile and fertile patients, including data from nine different studies, has demonstrated a significantly increased P1/P2 ratio in subfertile patients [57].

P2 deregulation occurs more frequently than P1 deregulation, indicating that a P2 deregulation is normally responsible for the P1/P2 ratio alteration. Lower sperm count and sperm motility and/or abnormal sperm morphology have been correlated with abnormal P1/P2 ratio (Table 2.2) [38–45, 47, 48, 58, 59]. Furthermore, some studies have shown that the total absence of P2 or the incomplete processing of the P2 precursors reflected by a decreased pre-P2/P2 ratio is also linked to a lower sperm count, a lower sperm motility, and an abnormal sperm morphology (Table 2.2) [41, 44–46]. Additionally, an altered P1/P2 ratio or a decreased pre-P2/P2 ratio was also linked to an increased sperm DNA damage or to an augmented reactive oxygen species levels (Table 2.2) [41, 49–54, 58]. These studies suggest that an altered P1/P2 ratio results in a sperm DNA more accessible to nuclease activity and, therefore, DNA damage increases. A correct protamination, as a measure of a correct P1/P2 ratio, could be crucial for the DNA protection [49]. However, the meta-analysis performed by Ni et al. could not establish an association between an altered P1/P2 ratio and DNA damage [57]. Otherwise, several studies have also correlated an altered P1/P2 ratio with a low fertilization rate, a low implantation rate, a low embryo quality score, and a low pregnancy outcome using in vitro fertilization (IVF) with or without intracytoplasmic sperm injection (ICSI) treatments (Table 2.2) [40, 43, 45, 52, 55, 59]. Likewise, a decreased pre-P2/P2 ratio has been correlated to a low implantation rate and a poor pregnancy outcome [45], and the total absence of P2 has been correlated to a low sperm penetration ability in IVF [46]. Taken together, these results suggest that protamine deregulation could be involved in fertilization and early embryo development processes. Other parameters such as men's age and smoking have been proposed to alter the protamine P1/P2 ratio (Table 2.2) [38, 39, 54]. In addition, it has been reported that a mutation in the *PRM1* gene promoter (−191AA genotype) causes an increased P1/P2 ratio suggesting that genetic mutations could be the cause of a defective protamination [56]. All these studies suggest that a correct P1/P2 ratio is important for men's fertility and for proper embryo development.

Table 2.2 Altered P1/P2 ratio in infertile patients

Study	P1/P2 ratio	Outcome
Correlation with seminal parameters		
Simon et al. [38]	Altered P1/P2	Lower sperm count; lower semen volume
Hamad et al. [39]	Increased P1/P2	Lower sperm count; lower sperm vitality
Aoki et al. [40]	Altered P1/P2	Lower sperm count; lower sperm motility; abnormal sperm morphology
Torregrosa et al. [41]	Decreased pre-P2/P2	Lower sperm count; lower sperm motility; abnormal sperm morphology
Aoki et al. [58]	Altered P1/P2	Lower sperm count; lower sperm motility; abnormal sperm morphology
Mengual et al. [42]	Increased P1/P2	Lower sperm count
Khara et al. [43]	Altered P1/P2	Lower sperm count; lower sperm motility; abnormal sperm morphology
de Yebra et al. [44]	Altered P1/P2	Lower sperm count
	No P2	Lower sperm count; lower sperm motility; abnormal sperm morphology
De Mateo et al. [45]	Decreased P1/P2	Lower sperm motility
	Decreased pre-P2/P2	Lower sperm count; lower sperm motility
Aoki et al. [59]	Altered P1/P2	Lower sperm count; lower sperm motility; abnormal sperm morphology
	Decreased P1/P2	Abnormal sperm head morphology
Carrell and Liu [46]	No P2	Lower sperm motility; abnormal sperm morphology
Bach et al. [47]	Altered P1/P2	Altered seminal parameters
Lescoat et al. [48]	Altered P1/P2	Altered seminal parameters
Correlation with DNA damage		
Ribas-Maynou et al. [49]	Increased P1/P2	Increased DNA damage (SCD assay)
García-Peiró et al. [50]	Increased P1/P2	Increased DNA damage (SCD assay)
Castillo et al. [51]	Decreased P1/P2	Increased DNA damage (alkaline comet assay)
Simon et al. [52]	Increased P1/P2	Increased DNA damage (alkaline comet assay)
Aoki et al. [53]	Altered P1/P2	Increased DNA damage (TUNEL assay)
Torregrosa et al. [41]	Decreased pre-P2/P2	Increased DNA damage (TUNEL assay)
Aoki et al. [58]	Decreased P1/P2	Increased DNA damage (SCSA assay)
Hammadeh et al. [54]	Increased P1/P2	Increased reactive oxygen species (ELISA assay)
Correlation with assisted reproduction techniques		
Simon et al. [52]	Decreased P1/P2	Low fertilization rate (IVF)
De Mateo et al. [45]	Decreased P1/P2	Low fertilization rate (IVF); low implantation rate (IVF and/or ICSI); low pregnancy outcome (IVF and/or ICSI)
	Decreased pre-P2/P2	Low implantation rate (IVF and/or ICSI); low pregnancy outcome (IVF and/or ICSI)
Aoki et al. [40]	Altered P1/P2	Low fertilization rate (IVF)
	Decreased P1/P2	Low chemical-pregnancy and clinical-pregnancy rates (IVF and/or ICSI)

(continued)

Table 2.2 (continued)

Study	P1/P2 ratio	Outcome
Aoki et al. [59]	Decreased P1/P2	Low fertilization rate (IVF and ICSI)
Nasr-Esfahani et al. [55]	Increased P1/P2	Low fertilization rate (ICSI); low embryo quality score in day 3 (ICSI)
Khara et al. [43]	Altered P1/P2	Low fertilization rate (IVF)
Carrell and Liu [46]	No P2	Low sperm penetration ability (IVF)
Correlation with other parameters		
Simon et al. [38]	Altered P1/P2	Men's age
Hamad et al. [39]	Increased P1/P2	Smokers
Hammadeh et al. [54]	Increased P1/P2	Smokers
	Decreased P2	Smokers
Jodar et al. [56]	Increased P1/P2	Mutation in the PRM1 gene promoter (−191AA genotype)

Correlation of protamine P1/P2 ratio with seminal parameters, DNA damage, assisted reproduction techniques outcome, and other parameters
SCD sperm chromatin dispersion, *TUNEL* terminal deoxynucleotidyl transferase dUTP nick end labeling, *SCSA* sperm chromatin structure assay, *ELISA* enzyme-linked immunosorbent assay, *IVF* in vitro fertilization, *ICSI* intracytoplasmic sperm injection

2.3 Nucleohistone Complex in Sperm

As mentioned before, the human spermatozoon retains approximately a 5–15% of its chromatin packaged in nucleosomes [18]. The nucleosome structure in sperm seems to be similar to that from somatic cells and consists on 147 base pairs of DNA wrapped around an octameric histone core including two of each H2A, H2B, H3, and H4 histones [60, 61]. Adjacent nucleosomes are interconnected by a linker DNA that can be up to 80 bp long. Members of histone H1 family (linker histones) are situated at the site of DNA entry and exit from the core particle binding around 20 nt of linker DNA. Apart from acting as a linker, histone H1 plays an important role in the chromatin folding modulation. The final result is a constrained DNA that approximately achieves a fivefold compaction. Despite the high degree of compaction that nucleosomes confer, histone-packaged chromatin sperm is more open and dynamic than the protamine-packaged chromatin and could be modulated and regulated by the incorporation of histone variants [7, 62], histone PTMs [31], and nuclear factors that modulate the DNA and histone interactions [3, 63].

2.3.1 Histone Variants

During spermatogenesis, some canonical histones are replaced by histone variants, and a subset of those remains in the nucleus of mature spermatozoa. Several histone variants have been identified in mature sperm by mass spectrometry, including the histone H4, which is less diversified compared with the most diverse H2A and H2B histones (Table 2.3). Despite the fact that histone variants have only small changes

Table 2.3 Human sperm histone variants identified in mature sperm

Protein name	Gene name	Localization	♂KO effect on reproduction and embryogenesis	References
Histone H1 family				
Histone H1t	HIST1H1T	Testis	Normal phenotype	Lin et al. [64], Fantz et al. [65]
Testis-specific H1 histone (H1t2)	H1FNT	Testis	Oligozoospermia, asthenozoospermia, teratozoospermia, abnormal spermiogenesis, reduced male fertility, and impaired fertilization	Martianov et al. [66], Tanaka et al. [67]
Histone H1x	H1FX	All tissues	ND	–
Histone H1.2	HIST1H1C	All tissues	Normal phenotype	Fan et al. [68]
Histone H1.3	HIST1H1D	All tissues	Normal phenotype	
Histone H1.4	HIST1H1E	All tissues	Normal phenotype	
Histone H1.5	HIST1H1B	All tissues	ND	–
Histone H2 family				
Histone H2A type 1	HIST1H2AG	Testis	ND	–
Histone H2A type 1-A (TH2A)	HIST1H2AA	Testis	ND	–
Histone H2B type 1-A (TH2B)	HIST1H2BA	Testis	ND	–
Histone H2A-Bbd type 1	H2AFB1	Testis	ND	–
Histone H2A-Bbd type 2/3	H2AFB2	Testis	ND	–
Histone H2A type 1-B/E	HIST1H2AB	Enriched in testis	ND	–
Histone H2A type 1-H	HIST1H2AH	Enriched in testis	ND	–
Histone H2B type 1-B	HIST1H2BB	Enriched in testis	ND	–
Histone H2B type 1-J	HIST1H2BJ	Enriched in testis	ND	–
Core histone macro-H2A.1 (mH2A1)	H2AFY	All tissues	Normal phenotype	Changolkar et al. [69], Boulard et al. [70]
Histone H2A type 1-C	HIST1H2AC	All tissues	ND	–
Histone H2A type 2-A	HIST2H2AA3	All tissues	NP	–
Histone H2A type 2-C	HIST2H2AC	All tissues	ND	–
Histone H2A.V	H2AFV	All tissues	ND	–
Histone H2AX	H2AFX	All tissues	Seminiferous tubules reduced diameter, small testes, male meiosis arrest, and male infertility	Celeste et al. [71]

(continued)

Table 2.3 (continued)

Protein name	Gene name	Localization	♂KO effect on reproduction and embryogenesis	References
Histone H2A.Z	H2AFZ	All tissues	Not viable	Faast et al. [72]
Histone H2B type 1-C/E/F/G/I	HIST1H2BC	All tissues	ND	–
Histone H2B type 1-D	HIST1H2BD	All tissues	NP	–
Histone H2B type 1-H	HIST1H2BH	All tissues	ND	–
Histone H2B type 1-K	HIST1H2BK	All tissues	ND	–
Histone H2B type 1-L	HIST1H2BL	All tissues	ND	–
Histone H2B type 1-M	HIST1H2BM	All tissues	ND	–
Histone H2B type 1-N	HIST1H2BN	All tissues	ND	–
Histone H2B type 1-O	HIST1H2BO	All tissues	NP	–
Histone H2B type 2-E	HIST2H2BE	All tissues	KO not fertility related	Santoro et al. [73]
Histone H2B type 2-F	HIST2H2BF	All tissues	NP	–
Histone H2B type 3-B	HIST3H2BB	All tissues	ND	–
Histone H2B type F-S	H2BFS	All tissues	NP	–
Histone H2A type 1-D	HIST1H2AD	–	ND	–
Histone H2A type 2-B	HIST2H2AB	–	ND	–
Histone H3 family				
Histone H3.1	HIST1H3A	Testis	ND	–
Histone H3.1 t (H3t)	HIST3H3	Testis	NP	–
Histone H3.3C	H3F3C	Testis	NP	–
Histone H3.2	HIST2H3A	All tissues	ND	–
Histone H3.3	H3F3A	All tissues	Reduced male fertility	Tang et al. [74]
Histone H3-like centromeric protein A (CENP-A)	CENPA	All tissues	Not viable	Howman et al. [75], Kalitsis et al. [76]
Histone H4 family				
Histone H4	HIST1H4L	All tissues	ND	–
Histone H4	HIST1H4A	All tissues	ND	–
Histone H4-like protein type G	HIST1H4G	ND	NP	–

Integrative table of the human sperm histone families combining the protein/gene name, GTEx localization, and the knockout effect on reproduction/embryogenesis using Mouse Genome Informatics database

ND no data, *NP* not present in mouse

in their primary structure compared with the canonical histones, those little differences can lead to major changes in the nucleosome structure, stability, and function [62]. The destabilization of DNA-protein interaction by incorporation of histone variants during spermatogenesis allows the transition from the nucleohistone complex to the nucleoprotamine complex [7, 18, 62, 77].

Although there are histone variants widely expressed in all tissues, there are some testis-specific variants that are essentially expressed in spermatocytes [78]. Targeting the individual histone variants in mouse models (knockouts) has revealed which histone variants are crucial for male fertility and reproduction (Table 2.3). Unfortunately, there is a lack of information about a set of histone variants that are not present or have not been detected in mouse (NP) or the corresponding knockout model has not been generated yet (ND). In addition, it is not possible to assess the effect on male reproduction of some histone variants because the knockouts have resulted in embryonic lethality [72, 75, 76], pointing out the need to generate conditional knockout models to assess their importance in testes function (Table 2.3).

Knockouts of some histone variants display a normal phenotype without negative impact on fertility, for example, histone H1t, mH2A1, H2B type 2-E, H1.2, H1.3, and H1.4, suggesting that they are not essential for male fertility (Table 2.3) [64, 65, 68–70, 73]. However, it could be expected that different testis-specific histone variants should have a major importance for proper fertility. As observed in Table 2.3, the knockout models of some testis-specific histones or widely expressed histone variants seem to result in reproductive failure. This is the case of a testis-specific histone, the H1t2, and the widely expressed histones H2AX and H3.3. Each knockout of these three different histone variants displayed male infertility although due to different reasons. For example, H1t2 knockout displays an abnormal spermatogenesis, sperm defects, and impaired fertilization, because this histone is necessary for DNA condensation and nuclear modulation during the last steps of spermatogenesis [66, 67]. In contrast, the disruption of H2AX and H3.3 in mice results in male meiosis arrest, since H2AX is crucial for meiosis because it facilitates the repair of induced DSBs [62, 71] and H3.3 is essential for chromosome segregation that takes place during meiosis (Table 2.3) [74].

2.3.2 Histone Post-translational Modifications

The early events during the transition of histones to protamines throughout spermiogenesis involve the incorporation of histone variants and histone PTMs, which enable the chromatin remodeling and trigger the protamination. Both histones and histone variants are modified by different PTMs [79]. The most known histone PTMs are acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and ribosylation, among other forms [79]. The different combinatorial patterns of the huge number of histone PTMs create a complex histone code that contributes to chromatin organization and dynamics, as well as to gene expression [7, 60]. For example, the massive increase of histone acetylation is one of the first signs that the

protamine replacement during spermiogenesis will start [3, 18, 80–82]. Histone hyperacetylation relaxes the chromatin and decreases the affinity of the sperm histones to the DNA, allowing the removal and replacement of histones, firstly, by TNPs and, finally, by protamines [3, 18, 63, 83]. Actually, histone H4 hyperacetylation in elongating spermatids is a prerequisite for the histone-to-protamine replacement [84], and an aberrant H4 hyperacetylation pattern results in impaired spermatogenesis [3, 18, 81, 85]. Apart from histone acetylation, there are other histone modifications throughout spermatogenesis such as methylation, which could also be associated with nucleosome dismantlement and histone eviction. Histone methylation seems to modulate epigenetic signals necessary for spermatogenesis [14, 60]. This is the case of H3K4me, a methylation mark that is necessary to turn spermatogonia into spermatocytes [86, 87], and H3K9me and H3K27me, marks that regulate gene expression during spermatogenesis [88].

Although histone variants and histone PTMs allow chromatin remodeling and most of them are replaced by protamines during late spermatogenesis, some of the modified nucleosomes are not replaced and are retained in the mature sperm. More than 100 histone post-translational modifications have been identified in the remaining histones present in human sperm, including acetylation, methylation, phosphorylation, butyrylation, and crotonylation [31, 89, 90]. Surprisingly, some histone PTMs found in human mature sperm showed a high degree of conservation with mouse sperm, which further supports an evolutionary conserved role of histone PTMs [31]. Those modified paternal histones maintained in the sperm are inherited by the zygote, and they have been proposed to play a role in the epigenetic control of embryogenesis [34]. For example, alterations in the histone methylation (H3K4me2) pattern in mice sperm impair the development and survivability of the offspring, indicating the importance of the sperm epigenome in the health of the progeny [91].

2.3.3 Histone-Bound Sperm Chromatin

Many studies suggest that the 5–15% retained nucleosomes in mature sperm are not randomly distributed through the sperm genome but occupy specific loci [12, 14, 15, 19, 32, 89, 92, 93]. This is supported by recent sperm chromatin high-throughput genome-wide dissection studies indicating that there is a differential distribution of genes and repetitive sequences between nucleohistone and nucleoprotamine complexes.

The first studies using human sperm chromatin fractionation followed by microarrays or high-throughput sequencing concluded that mature sperm histones are associated with DNA enriched at gene regulatory regions and genes involved in developmental processes, including promoters of embryonic transcription factors and signaling pathway proteins, as well as miRNA clusters and imprinted genes (Table 2.4) [19, 89, 92]. In contrast, protamines seemed to be enriched at olfactory receptors genes and ZNF genes [92]. Interestingly, the use of sperm chromatin immunoprecipitation (ChIP) of specific histone PTMs followed either by microarray

Table 2.4 Sperm nucleosomal DNA distribution in healthy men

Study	Methodology	Main outcomes
Arpanahi et al. [92]	Sperm salt extraction and endonuclease digestion or micrococcal nuclease (MNase) digestion followed by a microarray-based genome-wide analysis. Additionally, after digestion, ChIP-chip for acH4 was also used	Endonuclease-sensitive DNA regions are enriched in gene regulatory regions including promoter sequences involved in the development and CTCF-recognized sequences
Hammoud et al. [89]	Sperm MNase digestion followed by either array analysis or high-throughput sequencing. Additionally, ChIP-chip and ChIP-seq for H3K9me3, H3K27me3, H3K4me2/me3, TH2B, and H2A.Z were performed	Sperm nucleosomes are enriched at loci of developmental importance including imprinted gene clusters, miRNA clusters, HOX gene clusters, and promoters of embryo developmental transcription and signaling factors. Histone modifications (H3K4me2/3 and H3K27me3) localize to particular developmental loci
Brykczynska et al. [32]	Sperm MNase digestion followed by mononucleosomal DNA isolation and ChIP for H3K4me2 and H3K27me3 combined with microarray analysis or high-throughput sequencing	Sperm nucleosomes are slightly enriched at TSS. H3K27me3 and H3K4me2 are retained at regulatory sequences in mature human spermatozoa and marks promoters of genes related with spermatogenesis and early embryonic development
Vavouri et al. [15]	Reanalysis of the data from Arpanahi et al. [92], Hammoud et al. [89], and Brykczynska et al. [32]	Nucleosome retention, which is determined by the base composition, occurs in both genic and nongenic regions of the genome. Nucleosomes at GC-rich sequences with high nucleosome affinity are retained at TSSs and at developmental regulatory genes, particularly TSSs of most housekeeping genes. Also, there is a link between nucleosome retention in sperm and DNA unmethylated regions in the early embryo
Samans et al. [93]	Sperm cell fractionation by micrococcal nuclease followed by DNA high-throughput sequencing of the nucleosomal fraction	Sperm chromatin nucleosomes are enriched in certain repetitive DNA elements, as centromere repeats and retrotransposons (LINE1 and SINEs), and the majority of nucleosomal binding sites are enriched in distal intergenic regions. Nucleosome depletion was observed within exons, the majority of promoters, 5′-UTRs, 3′-UTRs, TSS, and TTS. Function of paternally derived nucleosomes in postfertilization processes
Castillo et al. [19]	Sperm chromatin fractionation using salt extraction followed by restriction enzyme digestion or MNase digestion, followed by high-throughput sequencing and proteomic analyses (LC-MS/MS)	Nucleosomal and subnucleosomal DNA regions are highly enriched at gene promoters, CpG island promoters, and linked to genes involved in embryo development

TSS Transcription start site, *TTS* Transcription termination site

(ChIP-chip) or DNA sequencing (ChIP-seq) has revealed that H3K4me2 and H3K4me3 are enriched at developmental promoters expressed in the four- to eight-cell stage embryos, suggesting a potential epigenetic function of those modified sperm histones in early embryogenesis [89]. The specific study of sperm mononucleosomal DNA has shown slight differences, for example, H3K4me2 marks genes involved in spermatogenesis and cellular homeostasis, while H3K27me3 marks developmental regulators and HOX genes [32]. These differences could be attributed to different technical issues in the preparation of the human sperm mononucleosomal DNA in contrast to all nucleohistone complex [32]. In silico analysis from the studies mentioned above revealed that spermatozoal nucleosomes are retained at GC-rich loci and that nucleosome retention in the sperm cell is linked to demethylated DNA in the early embryo [15].

In contrast to the mentioned findings above, one study claimed that retained nucleosomes in sperm are enriched in certain repetitive DNA sequences, such as centromere repeats and retrotransposons (LINE1 and SINE), and the majority of nucleosomal binding sites were enriched at distal intergenic regions [93]. However, these contradictory observations are probably due to technical issues or differences in the computational methodology used [94].

As a summary, there is huge evidence suggesting the existence of a differential distribution between histone-packaged and protamine-packaged sperm chromatin, which is involved in a potential sperm epigenetic signature transferred into the oocyte. The sperm nucleosome enrichment at developmental regulatory genes and gene regulatory sequences suggest that it could regulate the gene expression in early embryogenesis when zygote genome activation occurs and indicate that sperm chromatin is much more complex than it was previously thought.

2.3.4 Histone Alterations and Male Infertility

In contrast to the vast number of studies assessing the potential correlation between protamines (P1/P2 ratio) and male infertility (see Sect. 2.2), very few studies have evaluated sperm histones in infertile patients. Early obervations already indicated that a large proportion of the sperm samples with an altered P1/P2 ratio also had increased levels of histones [3, 18, 44]. Focusing on specific histones, it has been described that γH2AX levels are higher in the sperm of infertile patients than in fertile men, and it has been correlated to an increased number of sperm DSBs [95]. It has also been reported that semen samples from infertile men have a significant higher H2B/(P1+P2) ratio than do fertile men, suggesting that an alteration of H2B/(P1+P2) ratio could reflect an abnormal chromatin structure that results in male infertility [96–98]. Moreover, it has also been found an increased H2B/(P1+P2) ratio in smokers [39], implying a negative effect between smoking cigarettes and male fertility. Finally, a correlation has been found between alterations of a testis-specific histone variant (TH2B) and male fertility, which indicates that TH2B is involved in sperm chromatin compaction and male pronucleus development [99].

Apart from the abovementioned alterations in histone content, the sperm of infertile men has also shown an altered histone localization pattern [100]. The study of these infertile men revealed a randomly distributed pattern of nucleosome retention in the sperm chromatin [100]. This alteration in nucleohistone-bound genome could be attributed to a disrupted chromatin remodeling machinery or due to an improper histone hyperacetylation signaling during the histone exchange by protamines [100]. On a different line of experiments, evidence for a substantial deregulation of histones has been detected in normozoospermic sperm cells from male infertile patients with failed assisted reproduction outcomes after ICSI [101]. Overall, these studies demonstrate the importance of an appropriate distribution of genes in the sperm chromatin structure. Therefore, the potential side effects in the embryo associated to an improper histone retention in the sperm are an aspect that deserves further investigation in the future.

2.4 Concluding Remarks

Protamines have been largely studied and correlated with male infertility, specifically by P1/P2 ratio measurement. Similarly, alterations of specific histones have also been associated with sperm defects. Recent studies support the idea that the distribution of the nucleohistone and nucleoprotamine complexes in the sperm chromatin is not random. The intracytoplasmic sperm injection (ICSI) of mouse round spermatids, that did not complete the histone replacement yet, into mature oocytes, derived in embryos with aberrant patterns of gene expression, thereby suggesting that the paternal chromatin structure is important for the first steps of early embryo development [5]. The complexity of sperm chromatin highlights the need to perform further studies in sperm nucleoproteins content and distribution, including the assessment of their variants and PTMs, in order to clarify the significance of the sperm chromatin in male infertility and early embryo development as well as to shed light into the possible effects across generations. Furthermore, it will be particularly interesting to determine the specific role of the hundreds of chromatin-associated proteins present in the normal sperm chromatin, in addition to histones and protamines, as derived from recent high-throughput proteomic studies [102–104].

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Annex 2_

BARRACHINA F, ANASTASIADI D, JODAR M, CASTILLO J, ESTANYOL JM,
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Identification of a complex population of chromatin-associated proteins in the European sea bass (Dicentrarchus labrax) sperm.

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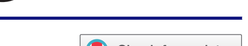

RESEARCH ARTICLE

Identification of a complex population of chromatin-associated proteins in the European sea bass (*Dicentrarchus labrax*) sperm

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ABSTRACT

A very common conception about the function of the spermatozoon is that its unique role is to transmit the paternal genome to the next generation. Most of the sperm genome is known to be condensed in many species by protamines, which are small and extremely positively charged proteins (50–70% arginine) with the functions of streamlining the sperm cell and protecting its DNA. However, more recently, it has been shown in mammals that 2–10% of its mature sperm chromatin is also associated to a complex population of histones and chromatin-associated proteins differentially distributed in the genome. These proteins are transferred to the oocyte upon fertilization and may be involved in the epigenetic marking of the paternal genome. However, little information is so far available on the additional potential sperm chromatin proteins present in other protamine-containing non-mammalian vertebrates detected through high-throughput mass spectrometry. Thus, we started the present work with the goal of characterizing the mature sperm proteome of the European sea bass, with a particular focus on the sperm chromatin, chosen as a representative of non-mammalian vertebrate protamine-containing species. Proteins were isolated by acidic extraction from purified sperm cells and from purified sperm nuclei, digested with trypsin, and subsequently the peptides were separated using liquid chromatography and identified through tandem mass spectrometry. A total of 296 proteins were identified. Of interest, the presence of 94 histones and other chromatin-associated proteins was detected, in addition to the protamines. These results provide phylogenetically strategic information, indicating that the coexistence of histones, additional chromatin proteins, and protamines in sperm is not exclusive of mammals, but is also present in other protamine-containing vertebrates. Thus, it indicates that the epigenetic marking of the sperm chromatin, first demonstrated in mammals, could be more fundamental and conserved than previously thought.

Abbreviations: AU-PAGE: acetic acid–urea polyacrylamide gel electrophoresis; CPC: chromosomal passenger complex; DTT: dithiothreitol; EGA: embryonic genome activation; FDR: false discovery rate; GO: Gene Ontology; IAA: iodoacetamide; LC: liquid chromatography; LC-MS/MS: liquid chromatography coupled to tandem mass spectrometry; MS: mass spectrometry; MS/MS: tandem mass spectrometry; MW: molecular weight; PAGE: polyacrylamide gel electrophoresis; PBS: phosphate buffered saline; SDS: sodium dodecyl sulfate; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCA: trichloroacetic acid.

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Introduction

The spermatozoa are highly specialized cells usually with a markedly condensed nucleus, almost no cytoplasm, and a very long flagellum. In all mammals, the majority of the sperm chromatin is condensed by protamines forming compact toroidal structures (Oliva and Dixon 1991; Oliva 1995; Balhorn 2007; Oliva and Castillo 2011a, 2011b; Barrachina et al. 2018). This sperm DNA condensation by protamines also occurs in a substantial proportion of vertebrates (Miescher 1874; Kossel 1928; Felix 1960; Bloch 1969; Iatrou et al. 1978; Oliva and Dixon 1991; Saperas et al. 1993, 1994; Zilli et al. 2004, 2005; Kurtz et al. 2009; Li et al. 2010; Wu et al. 2011; Nynca et al. 2014; Dietrich et al. 2016). Furthermore, protamines or protamine-like basic proteins are also found in sperm from many invertebrate species (Subirana 1983; Eirín-López et al. 2006; Martínez-Soler et al. 2007; Dorus et al.

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2008; Elnfati et al. 2015; Gärtner et al. 2015; Kimura and Loppin 2016; Rivera-Casas et al. 2017), as well as from algae and other eukaryotes (Kasinsky et al. 2014). It has been proposed that this sperm chromatin condensation by protamines may serve (1) to reduce the size of the sperm cell nuclei, allowing the spermatozoon to adopt a hydrodynamic shape so that it can swim faster and (2) to protect the paternal genome during its journey and delivery to the oocyte to form the zygote and final embryo (Oliva and Dixon 1991; Oliva 2006; Balhorn 2007; Björndahl and Kvist 2011). In fact, there are a substantial number of studies strongly supporting these functions of condensation and protection of the paternal genome (Balhorn et al. 1988; Ausió 1999; Castillo et al. 2011; Kasinsky et al. 2011; Simon et al. 2011; Ausió et al. 2014; Zatecka et al. 2014; Lüke et al. 2016). In addition, the deregulation of protamines in mammalian spermatozoa has been associated to male infertility (Balhorn et al. 1988; de Yebra et al. 1993, 1998; Bench et al. 1998; Aoki et al. 2006; Oliva 2006; Gázquez et al. 2008; de Mateo et al. 2009; Jodar et al. 2011; Azpiazu et al. 2014; Jodar and Oliva 2014).

However, while it is clear that the main function of the sperm cell is to transmit the paternal genome to the next generation, more recently additional important functions are starting to be uncovered. For instance, in mammals (such as human, mouse, and bull), it has been demonstrated that the mature sperm cell contributes to the embryo with around 2–8% of its DNA organized by histones and nucleosomes (Gatewood et al. 1987, 1990; Zalensky et al. 2002; Nazarov et al. 2008; Hammoud et al. 2009; Castillo et al. 2014a, 2014b, 2015; Sillaste et al. 2017), and that there is a differential distribution of the genes in the protamine-condensed versus the histone-complexed sperm chromatin domains (Arpanahi et al. 2009; Hammoud et al. 2009; Samans et al. 2014; Royo et al. 2016). Similarly, the differential poising of the sperm chromatin is also present in vertebrate species which do not express protamines, but that organize its full complement of the sperm DNA with histones and histone variants, containing histone modifications, and other chromatin marks (Wu et al. 2011). Furthermore, the presence of relevant chromatin-associated proteins in sperm has also been described in invertebrates such as *Drosophila melanogaster* (Dorus et al. 2008; Elnfati et al. 2015; Kimura and Loppin 2016; Pimenta-Marques et al. 2016).

In addition, the recent application of high-throughput protein identification strategies based on mass spectrometry (MS) have greatly expanded the sensitivity to dissect the molecular composition of the sperm cell (de Mateo et al. 2007; Oliva et al. 2008; Rousseaux and Khochbin 2012; Carrell et al. 2016; Jodar et al. 2017a, 2017b). The outcomes of these approaches demonstrate that the mammalian sperm contains hundreds of chromatin-associated proteins in addition to histones and protamines (de Mateo et al. 2011; Chocu et al. 2012; Baker et al. 2013; Amaral et al. 2014; Azpiazu et al. 2014; Castillo et al. 2014b, 2015; Codina et al. 2015; Vandenbrouck et al. 2016). Therefore, the sperm chromatin epigenetic information provided to the embryo includes DNA methylation, histone post-translational modifications, other chromatin-associated proteins, a unique chromatin structure, and chromosome territories in the nucleus (Zalensky et al. 1995; Zalenskaya et al. 2000; Gawecka et al. 2015; Pantano et al. 2015; Skinner et al. 2018). In zebrafish (*Danio rerio*), it has been shown that the DNA methylation patterns are transmitted to the early embryo from the sperm, and not from the oocyte (Jiang et al. 2013), and that the maternal genome undergoes reprogramming to match the paternal DNA methylation patterns (Potok et al. 2013). Thus, there is a compelling amount of evidence pointing out that the mature sperm cell also contributes with a complex chromatin structure and epigenetic information to the next generation (Nanassy et al. 2010; Chocu et al. 2012; Dacheux et al. 2012; Castillo et al. 2018; Ioannou and Tempest 2018).

However, so far, very few proteomic studies based on MS have been performed on the sperm chromatin of other non-mammalian protamine-containing vertebrates, such as the majority of teleost fish and birds (Zilli et al. 2005; Nynca et al. 2014; Labas et al. 2015). This information is highly needed and strategic from a phylogenetic and functional perspective, in order to determine whether, as in mammals, their sperm nuclei contain other chromatin-associated proteins in addition to the protamines. So far, conventional protein analyses have been performed on the sperm chromatin of many vertebrate species (Felix 1960; Iatrou et al. 1978; Oliva and Dixon 1991; Saperas et al. 1993, 1994; Zilli et al. 2005; Kurtz et al. 2009). In some cases, there is evidence indicating the presence of a minor proportion of proteins with an electrophoretic migration profile similar to that of histones, coexisting with a major proportion of protamines in the mature sperm cells (Saperas et al. 1993, 1994; Zilli et al. 2005; Kurtz et al. 2009). Furthermore, proteomic studies on the seminal proteins from different fish species and from other vertebrates and invertebrates are now available (Dorus et al. 2008, 2012; Awe and Renkawitz-Pohl 2010; Oliva 2012; Elnfati et al. 2015; Gärtner et al. 2015; Kimura and Loppin 2016). However, to our knowledge, only two studies have been performed so far to describe the proteomic composition of protamines-expressing teleost fish sperm. The first one was performed on the European sea bass (*Dicentrarchus labrax*) and was based on a two-dimensional electrophoretic separation of the proteins followed by MALDI-TOF identification (Zilli et al. 2005), while the

more recent one was performed on the rainbow trout (*Oncorhynchus myskiss*) through conventional polyacrylamide gel electrophoresis (PAGE) prefractionation combined with liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) protein identification (Nynca et al. 2014). However, these two studies were not focused on the sperm chromatin composition. Thus, in the present work, we chose the European sea bass as a representative model of non-mammalian vertebrate protamine-containing species, which is also one of the richest fish species in terms of available genomic resources (Louro et al. 2014; Tine et al. 2014), to characterize the mature sperm proteome using current high-throughput proteomic approaches based on LC-MS/MS and with a particular focus on the sperm chromatin.

Results

Characterization through polyacrylamide gel electrophoresis (PAGE) of the sperm proteins extracted from isolated sperm and nuclei

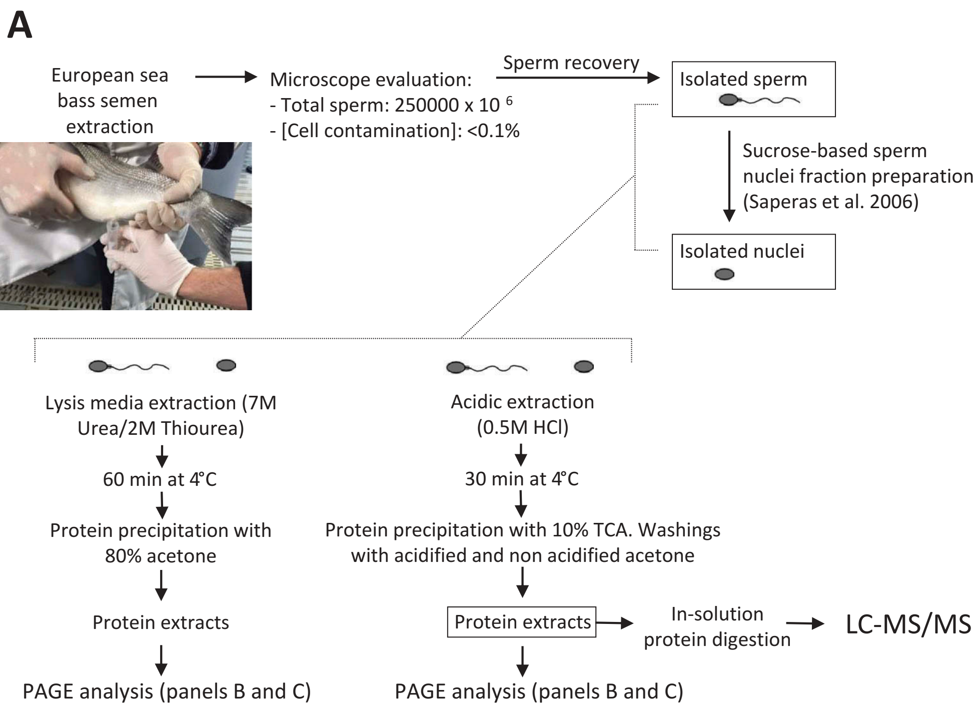
The electrophoretic patterns of the proteins obtained from isolated sperm cells and from purified sperm nuclei, extracted using two different procedures (7 M Urea/2 M Thiourea lysis media vs. 0.5 M HCl extraction; see Figure 1A for methodological details), are shown in Figure 1B (Sodium dodecyl sulfate PAGE; SDS-PAGE) and Figure 1C (Acetic acid-urea PAGE; AU-PAGE). The separation of the proteins according to molecular weight by SDS-PAGE detects the presence of bands with an equivalent mobility to the linker and core histones, as well as of additional proteins with higher molecular weight (Figure 1B). It is well known that the SDS-PAGE system does not allow the separation of protamines since they are insoluble in the presence of sodium dodecyl sulfate (SDS) because of their extremely positively charged nature (Soler-Ventura et al. 2018). In contrast, the AU-PAGE system is able to separate proteins according to both charge and size resulting in the efficient separation of the protamines from other basic proteins (Figure 1C). It should be pointed out that most of the extracted nuclear proteins by acidic extraction corresponded to protamines, as it is visualized by the fast mobility intense band at the bottom of the gel (lanes 5 and 7 in Figure 1C). In addition, different bands with similar electrophoretic migration pattern to that of histones were detected (lanes 5 and 7 in Figure 1C).

When comparing the amount of proteins recovered with the two different extraction media (0.5 M HCl vs. 7 M Urea/2 M Thiourea), it turns out that the acidic extraction (0.5 M HCl) was the only method that allowed the extraction of protamines and that resulted in a higher yield of proteins with a histone-like electrophoretic mobility (Figure 1B and 1C). For these reasons, the acidic extraction procedure was selected to proceed to the MS protein identification. At the same time, the combination of acidic extraction and AU-PAGE was used to estimate the relative amount of extracted basic proteins corresponding to protamines and proteins with histone-like electrophoretic mobility. The acidic extraction resulted in the detection of a Coomassie blue staining intensity of 45.5% for the protamines and 12.7% for the proteins with mobility similar to histones, in reference to the total lane intensity from purified sperm cells (Figure 1C). In the case of the purified sperm cell nuclei, the acidic extraction resulted in the detection of a Coomassie blue staining intensity, in reference to the total lane intensity, of 59.5% for the protamines and 16.9% for the proteins with mobility similar to histones (Figure 1C).

Mass spectrometry identification of the sperm proteins isolated by acidic extraction from purified spermatozoa and sperm nuclei

The overall summary of the workflow and the number of proteins identified through LC-MS/MS are shown in Figure 2, and the complete list of identified proteins is reported in Suppl. Table 1 (proteome from purified whole spermatozoa and proteome from purified sperm nuclei). A total of 232 proteins were identified from the European sea bass isolated whole sperm and 157 from the isolated sperm nuclei (Figure 2; Suppl. Table 1). From those, 139 proteins were detected only in the whole sperm, 64 were detected exclusively in the isolated sperm nuclei, and 93 proteins were identified both in the whole sperm cells and nuclei protein extracts (Figure 2B). The 139 proteins detected only in the whole sperm proteome correspond mostly to proteins present in sperm structures such as the tail, the intermediate piece, and the cytoplasmic membrane, which are lost during the process of sperm nuclei purification. The 64 proteins detected only in the proteome of the isolated sperm nuclei correspond most likely to low abundant proteins below the MS detection threshold in the whole sperm proteome determination.

Furthermore, Gene Ontology (GO) enrichment analyses of the identified proteins were conducted in this study, and the results are shown in Suppl. Table 2. The enriched GO-terms in the whole sperm cell proteome were related to metabolic, cellular, and chromatin pathways, whereas the chromatin and cellular structure annotations were detected as enriched in the protein set extracted from the isolated nuclei (Suppl. Table 2).



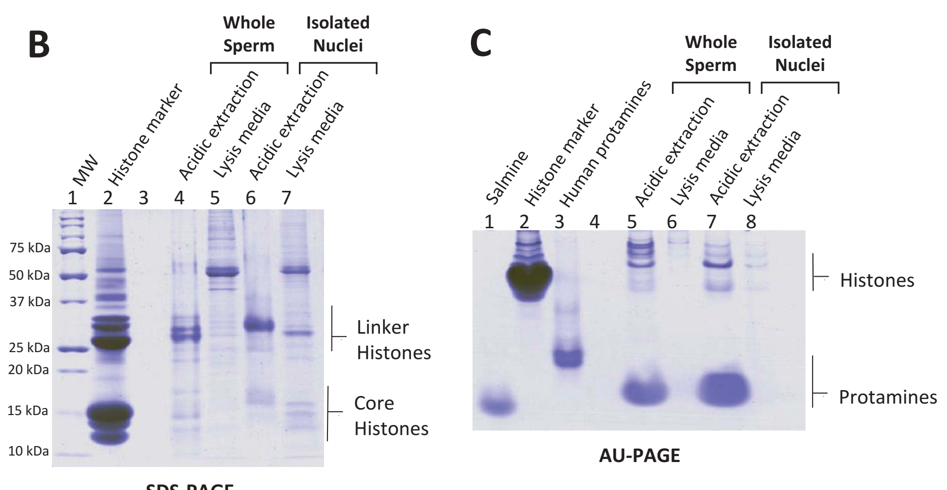
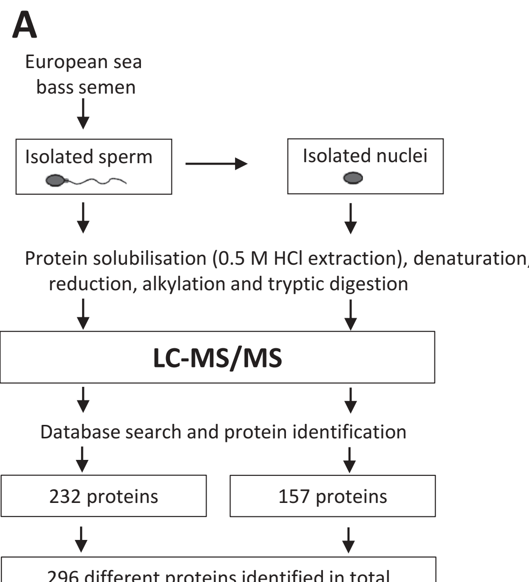


Figure 1. Outline of the purification of European sea bass whole sperm and nuclei, protein extraction, and analyses by electrophoresis and LC-MS/MS. (A) Sperm collection from European sea bass (*Dicentrarchus labrax*), quality control checks, and basic steps for sperm isolation, nuclei purification, and protein extraction (see material and methods section for details). (B) Polyacrylamide gel electrophoresis in SDS gels (SDS-PAGE) stained with Coomassie blue of the proteins extracted from whole purified European sea bass sperm cells and from isolated sperm cell nuclei. Bands were detected according to the expected molecular weight-based migration in SDS-PAGE for linker and core histones. It is important to note that this type of gel does not allow protamines separation, since their extremely positively charged nature causes protamines precipitation in the presence of SDS and, therefore, they do not enter the gel (Soler-Ventura et al. 2018). Lane 1: molecular weight (MW) marker, Lane 2: chicken erythrocyte histone marker, Lanes 4 and 5: proteins extracted from whole sperm using acidic extraction (0.5 M HCl) or lysis media (7 M Urea/2 M Thiourea), respectively, Lanes 6 and 7: proteins extracted from isolated sperm nuclei using acidic extraction (0.5 M HCl) or lysis media (7 M Urea-2 M Thiourea), respectively. (C) Polyacrylamide gel electrophoresis in acetic acid-urea gels (AU-PAGE) stained with Coomassie blue of the proteins extracted from whole European sea bass purified sperm cells and from isolated European sea bass sperm cell nuclei. This type of acidic gel and procedure allow the detection of both the predominant protamines with a fast migration at the bottom of the gel and proteins with a histone-like migration. The predominant protamine fast migrating bands are seen at the bottom of the gel, corresponding to salmine (Lane 1; protamine from salmon sperm) and to the protamine from European sea bass sperm and nuclei (Lanes 5 and 7, respectively). The identification of this major high mobility band in Lanes 5 and 7 as protamine is based on the very good concordance of the results obtained here and those obtained in previous articles (Oliva and Dixon 1991; Saperas et al. 1993; Saperas et al. 2006; Soler-Ventura et al. 2018). Also, a histone marker (Lane 2; slightly overloaded) isolated from chicken erythrocytes and a human protamines standard (Lane 3) have been included. Note that the bands with a histone-like migration are seen in Lanes 5 and 7, which represent a Coomassie blue stain intensity of 12.7% and 16.7%, respectively.



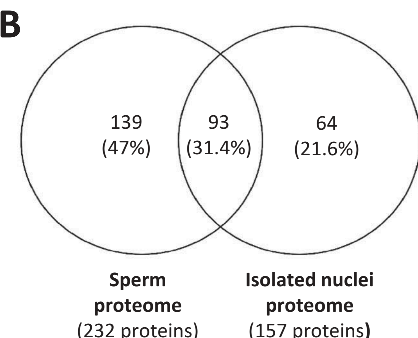


Figure 2. Overall schematic proteomic strategy used for the identification of proteins from sperm cells and the sperm nuclei-enriched fraction of the European sea bass (*Dicentrarchus labrax*). (A) The soluble proteins from both the isolated sperm cells free of seminal fluid and the isolated nuclei fraction were extracted (acidic extraction; 0.5 M HCl), reduced, alkylated, and digested with trypsin. The peptides were subsequently separated by liquid chromatography (LC) and identified through tandem mass spectrometry (MS/MS). (B) Overview of the protein identification for the European sea bass whole sperm and isolated nuclei fraction. A total of 296 proteins were identified in the overall sperm European sea bass proteome. Of those, 64 (21.6%) were exclusively present in the isolated nuclei fraction, 139 (47%) were just found in the whole sperm, and 93 (31.4%) were identified in both the isolated nuclei and whole sperm protein extracts.

Interestingly, up to 94 chromatin-related proteins were identified, including histone and histone variants, histone modifiers, centrosomal proteins, and zinc fingers and transcription factors among other chromatin-related proteins, which are reported herein according to the gene name, as well as classified into different categories (Table 1). Out of these 94 chromatin proteins, 9 correspond to histones and histone variants, which are further detailed in Tables 1 and 2. It should be noted that the European sea bass protamine was not detected through LC-MS/MS since, due to its high content of arginines (~62% of the amino acid sequence), the peptides obtained after trypsinization are too small to be detected by the shotgun protein identification strategy used in this study. However, its identification is clear and unambiguous from the result shown in Figure 1C and its concordance with those obtained in previous articles (Oliva and Dixon 1991; Saperas et al. 1993; Saperas et al. 2006; Soler-Ventura et al. 2018).

Table 1. List of chromatin-related proteins identified in the European sea bass (*D. labrax*) sperm (*n* = 94 proteins). Only proteins containing at least one unique peptide are reported.

Protein category	Gene names
Histones and histone variants	H1F0, HIST1H1A, H2AFV, H2AFY, H2AFY2, HIST1H2BC, HIST3H3, H3F3B, CENPA
Histone modifiers	1433B, HSP90BA, DPY30, GSG2, ATAD2, HUWE1, SKP1, YWHAE1
Zinc fingers and transcription factors	ZNF813, DZIP1, NCOA6, SUB1, CNBP, HMGB1A, HMGB2, C1QBP, PFD1, JUPA, PSMA6A, PRDX3, MLF1, HSP70, AKNA
Centrosomal proteins	CEP135, CEP57L1, CEP63, RANBP1, CETN3, POC5, SSNA1, TCP1, ALMS1, CCDC61, CCDC77, CCT4, CCT5, CCT8, CETN1, MZT2B, NDK7, POC1B, RPGR, RTTN, NM23, DYL1, DYNLL2, RILPL1, B9D1, DYNLRB1, EFHC1
Other chromatin-related proteins	PSMD14, PSMD1, PSMD2, PSMD7, HINT1, RAD23A, UBA1, NSFL1C, PDCD5, PRDX5, TXN, HSBP1, BADB, SPATA18, SEPT6, SEPT7, SEPT7 (isoform 2), DNAL1, PFDN5, MYCBP, ALYREF, DDB1, TSNAX, COMD3, PTGES3A, RCC1, BANF1, SSBP1, NUP153, RAN1, VCP, USP14, HSPA8, MIF, MRPL12

Table 2. List of histones and histone variants identified in the European sea bass (*D. labrax*) sperm proteome. The different protein names and gene names of the identified histones in *D. labrax* are shown. At the right column, the corresponding ortholog in *H. sapiens* is indicated.

Histone type	Protein name (*D. labrax*)	Gene name (*D. labrax*)	Ortholog (*H. sapiens*)
H1	H1 histone	H1F0	Histone H1.0
	Histone H1.1	HIST1H1A	Histone H1.1
H2a	H2a histone family member v variant 1	H2AFV	Histone H2A.V
	Core histone macro-like isoform x2	H2AFY	Core histone macro-H2A.1
	Core histone macro-H2A	H2AFY2	Core histone macro-H2A.2
H2b	Histone H2b 1 2-like	HIST1H2BC	Histone H2B type 1-C/E/F/G/I
H3	H3 histone family 3b variant 2	H3F3B	Histone H3.3
	Histone H3.1t	HIST3H3	Histone H3.1t
	Histone H3-like centromeric protein a	CENPA	Histone H3-like centromeric protein A

Table 3. Conservation of European sea bass chromatin-related sperm proteins within vertebrates. Number of chromatin-related proteins identified in the European sea bass sperm (*Dicentrarchus labrax*), and divided by categories, whose orthologs have been found in the sperm from rainbow trout (*Oncorhynchus mykiss*) (*n* = 206, whole sperm proteome), mouse (*Mus musculus*) (*n* = 2888, whole sperm proteome), and human (*Homo sapiens*) (*n* = 6871, whole sperm proteome). The detailed list of the 94 chromatin-related sperm proteins in *D. labrax* and its comparison with the other species is shown in the Supplementary Table 3.

Number of Chromatin-related sperm proteins in *D. labrax* (*n* = 94)		Number of orthologs found in other species		
		O. mykiss	*M. musculus*	*H. sapiens*
Histones and histone variants	*n* = 9	3	6	6
Histone modifiers	*n* = 8	3	7	8
Zinc fingers and transcription factors	*n* = 15	2	7	10
Centrosomal proteins	*n* = 27	4	17	23
Other chromatin-related proteins	*n* = 35	6	31	33

Conservation within different protamine-containing vertebrate species of the chromatin-related sperm proteins identified in the European sea bass (D. labrax)

The presence of the orthologs corresponding to the 94 European sea bass sperm chromatin-related proteins identified in this study was assessed in the sperm proteomes from rainbow trout (*Oncorhynchus mykiss*), mouse (*Mus musculus*), and human (*Homo sapiens*), as representatives of additional protamine-containing vertebrates species (Table 3, Suppl. Table 3). Of note, 80 of these 94 proteins were also identified in the human sperm (Table 3), which is the most studied male germ cell by proteomic approaches. In contrast, only 18 and 68 orthologs were found in the rainbow trout and the mouse sperm, respectively (Table 3). However, it is important to take into account that no specific methods for the enrichment of chromatin-associated proteins were applied in these species, which may reduce the number of positive matches. Therefore, sperm chromatin proteins seem to be highly conserved among protamine-containing vertebrates, which suggest their potential critical role in sperm function. Interestingly, while the groups that showed more variability were the histone and histone variants, and the zinc fingers and transcription factors, the groups of histone modifiers, centrosomal proteins, and other chromatin-related proteins are much more conserved.

Discussion

In this study, we report the identification of a total of 296 different proteins in the European sea bass (*D. labrax*) mature sperm cell and discuss their potential function with a specific focus on the chromatin-associated proteins.

Classically, in vertebrate species replacing histones by protamines, the sperm chromatin structure has been considered to be constituted by the entire DNA complement condensed by protamines (Bloch 1969; Oliva and Dixon 1991; Balhorn 2007). This idea may have been originated in part by the results obtained after the stringent chromatin purification methods applied to isolate and sequence the protamines, which removed many chromatin-associated proteins, and, therefore, resulted in the exclusive detection of protamines by electrophoresis (Ando et al. 1973; Balhorn et al. 1977, 1987; McKay et al. 1985, 1986; Ammer et al. 1986; Bélaïche et al. 1987; Bellvé et al. 1988; Sautière et al. 1988; Martinage et al. 1990; Arkhis et al. 1991; Chauvière et al. 1992; Alimi et al. 1993; de Yebra and Oliva 1993; Saperas et al. 1993). However, it has been clearly demonstrated that the mammalian mature sperm cell still contains around 2–8% of the DNA packaged by histones and forming nucleosomes (Gatewood et al. 1987, 1990; Zalensky et al. 2002; Hammoud et al. 2009; Castillo et al. 2014a; Sillaste et al. 2017). This proportion of nucleohistone is of relevance since the existence of a non-randomly differential distribution of the genes among the protamine- and the histone-condensed sperm chromatin domains has been proposed in mammals, raising the potential for transmission of epigenetic information to the oocyte (Gatewood et al. 1987; Zalensky et al. 1995; Zalenskaya et al. 2000; Wykes and Krawetz 2003; Martínez-Heredia et al. 2006; Oliva et al. 2009; de Mateo et al. 2011; Jodar et al. 2011, 2012; Oliva and Ballescà 2012; Jodar et al. 2017a; Castillo et al. 2018). But little information is currently available on the additional potential sperm chromatin proteins present in other protamine-containing non-mammalian vertebrates, which can be identified by the current sensitive and high-throughput MS protein identification strategies. This information is needed from a strategic phylogenetical perspective to clarify whether the coexistence of histones, protamines, and additional chromatin-associated proteins in the mature sperm is exclusive of mammalian vertebrates, or, in contrast, it is also present in other protamine-containing vertebrate species. For that reason, we have included the modern teleost fish European sea bass (*D. labrax*) in this proteomic study, as a representative of protamine-containing non-mammalian vertebrate species.

Interestingly, our results demonstrate the presence of 94 chromatin-associated proteins in the mature European sea bass sperm (Table 1). However, in order to deeply

study the putative role of these proteins during fertilization and beyond, it is important to exclude any possibility of contamination by somatic cells in the recovered sperm. To this extent, no round or somatic cells were detected after counting 4000 sperm cells (thus, with a potential contamination <0.1%). In addition, it is important to remark that some of the proteins detected within the European sea bass sperm proteome (such as the HIST3H3-H3.1t) are known to be exclusively expressed during the germinal cell line (Witt et al. 1996; Churikov et al. 2004). Also, the Coomassie blue staining intensities of the proteins extracted from purified sperm nuclei and separated by AU-PAGE, in reference to the total lane intensity, were 59.5% for the protamines and 16.9% for those proteins with histone-like electrophoretic migration (Figure 1C), which supports that the majority of the proteins identified by LC-MS/MS correspond to sperm proteins.

Among the 94 chromatin-associated proteins found in the European sea bass sperm, 9 of them correspond to histones and histone variants. Specifically, the proteomic analyses underwent in this study resulted in the identification of two histone H1 variants, three histone H2A variants, one histone H2B variant, and three histone H3 variants (Table 2). These findings make interesting to further predict the putative impact of the European sea bass sperm histone variants, not only on the sperm chromatin packaging, but also as potential regulators of epigenetic information.

The two histone H1-type proteins identified in this study correspond to the H1F0 (also known as H1.0) and the HIST1H1A (also known as H1.1). Remarkably, those histone H1 variants have never been described in the well-characterized human sperm proteome, whereas the core histone variants identified in European sea bass are conserved between both species (Suppl. Table 3). These results may reflect the variability of linker histones' sequences between different species, which is well known to be much higher than that of the extremely well-conserved core histones (Hergeth and Schneider 2015).

Regarding the conserved core of histone variants, three H2A variants were identified in the European sea bass sperm. One of them, the H2AFV (ortholog of the mammalian H2A.Z) is known to be highly enriched in the transcriptional start sites of active genes (Bao and Bedford 2016), as well as to be associated to global DNA methylation reprogramming during early vertebrate embryonic development (Madakashira et al. 2017), suggestive of its crucial role during early embryogenesis. The potential crucial role of H2AFV/H2A.Z in sperm function is supported not only by the conservation of this variant across the different species assessed, i.e., the rainbow trout, the mouse, and the human (Suppl. Table 3), but also by its presence in zebrafish, which does not contain protamine in the sperm (Wu et al. 2011). In contrast, of the remaining two H2A variants identified in the mature European sea bass sperm, the H2AFY (ortholog of the mammalian macro-H2A.1) and the H2AFY2 (ortholog of the mammalian macro-H2A.2), the macro-H2A.1 (H2AFY) is known to be associated to transcriptionally inert chromatin, being essential to recruit remodeling complexes required for heterochromatinization during mouse meiosis, as well as to participate in the transcriptional repression of X- and Y-linked genes (Hoyer-Fender et al. 2004; Baumann et al. 2011; Bao and Bedford 2016). In contrast, only one H2B variant has been found in the European sea bass sperm in the current study, the histone H2b 1 2-like (ortholog of the mammalian HIST1H2BC). This variant was found to be fivefold times increased in mouse round spermatids, suggesting that it might have an impact on the histone variant exchanges during spermiogenesis (Li et al. 2014). However, the HIST1H2BC promoter was found during a genome-wide search for H4K12ac in human sperm, which could also imply a potential regulatory role after fertilization (Paradowska et al. 2012). Concerning the European sea bass sperm histone H3 variants detected, the H3F3B (H3.3 in mammals) is known to constitute the predominant form of histone H3 in non-dividing cells as the spermatozoa. More interesting is the fact that H3F3B is deposited at sites of nucleosomal displacement throughout transcribed genes, suggesting that it represents an epigenetic imprint of transcriptionally active chromatin (Elsaesser et al. 2010). Although the essential role of this variant during spermatogenesis is already known, since male mice containing H3F3B heterozygotic mutations are sterile due to an arrest at the round spermatids stage, the retention of H3F3B in mature sperm might also mark potential transcriptionally active chromatin sites at the time of the embryonic genome activation (EGA) (Tang et al. 2015). Furthermore, the European sea bass sperm also contains Histone H3-like centromeric protein A (CENPA), which is specifically found in centromeric nucleosomes (Shelby et al. 1997). Interestingly, the CENPA protein is not displaced during the histone-to-protamine transition and, therefore, it behaves as an inherited element conserved in the two daughter chromatids during S phase. Thus, the inheritability of CENPA may have an important role in fertilization (Kimmins and Sassone-Corsi 2005; Miller et al. 2010). In fact, the presence of CENPA subtly modifies the nucleosome structure and, therefore, it may serve as an epigenetic mark that propagates the centromere identity through replication and cell division. This histone variant is also known to be deposited on newly duplicated centromeres, being required for the recruitment of other proteins to centromeres and kinetochores, thus

suggesting that its presence in mature spermatozoa could play a role in the paternal genome during early embryogenesis (Govin et al. 2004).

A histone that has not been detected in the European sea bass sperm is histone H4. This may indicate either its absence or its presence at a lower abundance as compared to other histones and, therefore, under of the detection range of the technique. In any case, it may suggest the presence of a very peculiar chromatin structure in the European sea bass sperm cell, possibly involving non-canonical nucleosomes and/or nucleosomal-like particles with a special histone stochiometry. In fact, a structure more sensitive to MN digestion, detected as a discrete 60 bp DNA fragment, and specifically associated with two novel histone H2A variants was reported in mice spermatogenic cells (Govin et al. 2007). Also, we have previously reported the presence of subnucleosomal particles in human sperm chromatin, which were associated to 40–80 bp fragments and highly enriched at promoters of genes and CpG islands (Castillo et al. 2014a).

Apart from the above indicated histone variants, it is also very interesting to highlight the large variety of other chromatin-related proteins that have been found in the European sea bass sperm proteome and its conservation between the assessed species (Tables 1 and 3). It is worth to point out a few of these additional chromatin proteins present in the mature European sea bass sperm because of their potential involvement in epigenetic regulation. For instance, a remarkable proportion of histone modifiers, zinc fingers and transcriptional factors, and other chromatin-related proteins have been identified. This is the case of the E3 Ubiquitin-Protein Ligase HUWE1 (HUWE1), which has been shown to play an important role in mouse embryo development, and its deregulation in human sperm seems to be associated with poor embryo development (Chen et al. 2016). Also, the European sea bass sperm contains the DPY30 homolog isoform 1 (DPY30), a component of the histone methyltransferase complex which is involved in the trimethylation and acetylation of histone H3 (Wang et al. 2009; Xu et al. 2009; Jiang et al. 2011). Of interest, the serine/threonine-protein kinase HASPIN (GSG2) is also present in European sea bass sperm (Table 1) and is known to promote the activation of components of the chromosomal passenger complex (CPC) at centromeres to ensure proper chromatid cohesion (Sato et al. 2011). Also remarkable is the identification of the nuclear receptor coactivator 6-like (NCOA6), since it is known to be an important developmental regulator. Of interest, the promoter of NCOA6 has been found to be depleted in H4K12ac binding sites in the human spermatozoa of subfertile patients, thus suggesting a potential altered transfer of epigenetic marks to the oocyte (Vieweg et al. 2015). Similarly, the Translin-associated protein X (TSNAX) could be related to the regulation of the well-known presence of RNAs and non-coding RNAs in sperm and its deregulation in infertile males (Jodar et al. 2012, 2013, 2015; Pantano et al. 2015; Gomez-Escobar et al. 2016).

The European sea bass sperm also contains a subset of centrosomal proteins (Table 1). These proteins are highly relevant since the sperm is specifically providing the initial centriole to the oocyte in most species, which builds the first centrosome that is essential for early development (Pimenta-Marques et al. 2016). Furthermore, it is known that alterations in centrosomal proteins cause male infertility and, in some cases, severe developmental defects. For instance, the European sea bass sperm contains the centrosomal protein of 63 kDa (CEP63), whose whole mutations are known to cause male infertility, in addition to microcephaly and dwarfism (Marjanović et al. 2015); the Centrin-1 protein (CETN1), whose germ line deletion in mice has been reported to cause male infertility (Avasthi et al. 2013); and the centrosomal protein of 135 kDa (CEP135), whose homozygous mutation has been associated with multiple sperm flagella morphological abnormalities in infertile men, as well as pregnancy failure following embryo transfer (Sha et al. 2017). Finally, the Alstrom syndrome protein 1 (ALMS1) has also been found in the European sea bass sperm, whose mutations in the corresponding human gene are responsible for Alstrom syndrome, a disorder in which key metabolic and endocrinological features are disturbed, resulting in childhood-onset obesity, metabolic syndrome, diabetes, and infertility (Arsov et al. 2006). Thus, the many different centrosomal proteins detected in the present work together with the multiple lines of evidence linking their anomalies with male infertility also suggest a relevant function for the European sea bass sperm proteins.

Of note, an additional group of nuclear proteins have been detected in this study, which, although not being associated to chromatin, is interesting to highlight. This is the case of the proteosomal proteins, since the proteosomal regulation is essential not only for the correct development of spermatogenesis, but also for the early events occurring at fertilization, which includes the process of sperm mitophagy, where potentially damaged paternal mitochondrial DNA is eliminated from the early embryo (Song et al. 2016; Sutovsky 2018).

Finally, it is also worth pointing that the European sea bass sperm contains proteins which are related to oxidative stress and redox signaling during development, such

as the Thioredoxin-dependent peroxide mitochondrial precursor (PRDX3), or the Peroxiredoxin 5 protein (PRDX5), which has been reported to have a role in sperm function and male fertility (Buckman et al. 2013; Tirmarche et al. 2016; Ryu et al. 2017).

In conclusion, the present work contributes to support the idea that the presence of histones and chromatin-associated proteins in the mature sperm cell nuclei of vertebrate protamine-containing species is more conserved among vertebrates than previously thought. Since the potential differential distribution of the genes within the histone- and protamine-condensed regions might raise the possibility of transmission of epigenetic information to the offspring (Gatewood et al. 1987; Zalensky et al. 2002; Arpanahi et al. 2009; Hammoud et al. 2009; Samans et al. 2014; Castillo et al. 2014a; Royo et al. 2016; Sillaste et al. 2017), the present description of the European sea bass sperm proteome, as well as the demonstration of the important proportion of chromatin-related proteins, apart from protamines, open up the possibility to further study their distribution and function in the European sea bass genome, epigenome, and development. Also, in future studies, it will be interesting to determine the relative proportion, degree of conservation in the sperm of independent individuals, and the specific genomic sequences associated to the chromatin-associated proteins reported here.

Materials and methods

Animal care and semen sample collection

The European sea bass (*D. labrax*, Family Moronidae) used in this study was maintained at the Institute of Marine Sciences, Barcelona, Spain. The fish was treated according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS Nu 123, 01/01/91). The experimental protocol was approved by the Spanish National Research Council (CSIC) Ethics Committee within the project AGL2016-78710-R. The semen sample was obtained from a 5-year-old European sea bass with a body weight of 1.7 kg. The sea bass was anesthetized with 2-phenoxyethanol (0.2 ml/l). The semen sample (5.4 ml) was collected by abdominal massage into a sterile tube, avoiding contamination by blood, urine, or feces. A semen analysis was performed in order to check sperm concentration (4.7 x 10^{10} spermatozoa/ml) with a Makler counting chamber (Sefi-Medical Instruments ltd., Haifa, Israel). Furthermore, potential round cell contamination was monitored by phase contrast microscopy (Olympus CX41, Olympus, Tokyo, Japan), and no round cells were detected after counting over 4000 mature spermatozoa (<0.1%). Then, the semen sample was centrifuged thrice with phosphate buffered saline (PBS) at 800×g for 5 min at 4°C, and the obtained pellet (containing spermatozoa free of seminal plasma) was stored at 4°C until the next step.

Sperm nuclei fraction preparation

The methodology followed to obtain a nuclear fraction from sperm cells, which is based in a sucrose solution, was performed as previously described by Saperas et al. (2006), with some modifications. Briefly, the purified spermatozoa was suspended in 0.25 M sucrose, 5 mM $CaCl_2$, 10 mM Tris-HCl (pH 7.0), and 10 mM benzamidine chloride, homogenized in ice using a Dounce homogenizer (Wheaton, Millville, NJ, USA), and centrifuged at 2000×g for 5 min at 4°C. Then, the pellet was collected, homogenized, and centrifuged up to three times in the same buffer with additional 0.1% Triton X-100. Subsequently, the pellet was homogenized in 20 mM EDTA, 10 mM Tris-HCl (pH 7.0), and centrifuged at 2000×g for 5 min at 4°C. The final pellet, which is enriched in sperm nuclei, was homogenized for the last time in 10 mM Tris-HCl (pH 7.0). The resulting sperm nuclei fraction was checked in an Olympus CX41 Microscope.

Protein solubilization

Protein extracts were obtained from the purified nuclei and total sperm by using two different protein extraction procedures: (i) In order to perform an acidic extraction, 0.5 M HCl was added to the pelleted purified nuclei or total sperm for 30 min at 4°C, with vortexing every 5 min. After a centrifugation at 23700×g for 10 min at 4°C, the supernatants (soluble proteins) were collected and the proteins present in the HCl extracts were precipitated by adding trichloroacetic acid (TCA) to a final concentration of 10% (v/v) for 30 min at 4°C. The protein precipitate was collected by centrifugation at 23700×g for 10 min at 4°C, and the pellet was rinsed once with acidified cold acetone (acetone/0.0185% HCl (37%), v/v), and twice with cold 100% acetone (Saperas et al. 2006). The protein pellet was finally either dried using a speed-vacuum for MS/MS analysis or resuspended in sample loading buffer for gel electrophoresis; and (ii) A lysis buffer containing 7 M Urea, 2 M Thiourea, 1% CHAPS, 1% n-octyl-glucopyranoside, and 18 mM dithiothreitol (DTT) (Amaral et al. 2013; de Mateo et al. 2013; Bogle et al. 2017) was added to the pelleted nuclei and total sperm, and incubated under shaking for 60 min at 4°C. After centrifugation at 16000×g for 10min at 4°C, the supernatant (soluble

proteins) was kept and the proteins were precipitated overnight adding 100% cold acetone to a final concentration of 80% (v/v). The protein precipitate was collected by centrifugation at 17530×g for 10 min at 4°C, and the protein pellet was resuspended in sample loading buffer for gel electrophoresis (Martínez-Heredia et al. 2006; de Mateo et al. 2013).

Electrophoresis and protein separation

Proteins extracted from the whole sperm or purified nuclei were separated using two different one-dimensional gel electrophoresis methodologies, as previously described elsewhere (de Yebra and Oliva 1993; Mengual et al. 2003; Torregrosa et al. 2006; Castillo et al. 2014a; Soler-Ventura et al. 2018): (i) For SDS-PAGE, dried proteins were solubilized in a 1× SDS sample buffer (2.2% (w/v) SDS, 5% (v/v) glycerol, 60 mM Tris-HCl pH 6.8, and 0.1 M DTT) and separated through SDS-PAGE (3.9% acrylamide stacking gel and 12% acrylamide resolving gel) at 20 mA (Amaral et al. 2013); (ii) For AU-PAGE, dried proteins were resuspended in a 1× acid sample buffer (5.5 M urea, 20% (v/v) b-mercaptoethanol, and 5% (v/v) acetic acid) and separated in acetic acid–urea polyacrylamide gels (2.5 M Urea, 0.9 M acetic acid, 15% acrylamide, 0.5% TEMED, 0.5% PSA) at 150 V (Torregrosa et al. 2006; Castillo et al. 2011; Soler-Ventura et al. 2018). Afterwards, both gels were fixed with 50% methanol and 10% acetic acid, and stained with Comassie blue (EZBlue Gel Staining Reagent, Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer's instructions, and visualized using a GS-800 Calibrated Densitometer Scanner (Bio-Rad, Hercules, CA, USA). For histone and protamine evaluation, the protein extracts from purified nuclei and whole sperm obtained after acidic extraction (0.5 M HCl) in the AU-PAGE were quantified using the Quantity One software (BioRad, Hercules, CA, USA). In both cases, the optical densities of the bands corresponding to histones, protamines, and the total amount of proteins were quantified, and the percentages of histones (histones/total amount of proteins × 100) and protamines (protamines/total amount of proteins × 100) were calculated.

Analysis by LC (reverse phase) – MS/MS

The dried protein extracts, after acidic extraction (0.5 M HCl), from whole sperm and purified sperm nuclei were dissolved in 0.1% SDS. Disulphide bridges were reduced by incubation with 10 mM DTT for 30 min at 60°C, and cysteines were alkylated by incubation with 55 mM iodoacetamide (IAA) for 30 min at room temperature in the dark. Samples were then precipitated with 10% TCA, treated for 15 min at 4°C, and centrifuged at 16100×g for 15 min at 4°C. Finally, 1 ml of cold 100% acetone was added and then centrifuged at 16100×g for 15 min at 4°C. After discarding the supernatant, the protein-containing pellets were enzymatically digested for peptide-based analyses. First of all, the pellets were reconstituted in 2 µl of 8 M Urea and brought up to 10 µl in 25 mM Tris HCl buffer, pH 8. After that, proteins were digested into peptides with 1 µg trypsin (Promega, Madison, WI, USA) in 25 mM Tris-HCl buffer, pH 8.0 at 37°C overnight. Enzymatic digests were stopped by adding 1% formic acid. Reversed phase nano liquid chromatography–tandem mass spectrometry (nano LC-MS/MS) setup comprised a nano-LC Ultra 2D Eksigent coupled to an LTQ Velos-orbitrap mass spectrometer (Thermo scientific, San Jose, CA, USA). Peptides were injected onto a trap column C18 (L 0.5 cm, 300 µm ID, 5 µm, 100 Å; Thermo Fisher Scientific, San Jose, CA, USA) and, for chromatography purposes, a gradient was applied using an analytical column (L 15 cm, 75 µm ID, 3 µm, 100 Å; Thermo scientific, San Jose, CA, USA). Buffer System consists of buffer A (97% H_2O-3% ACN, 0.1% Formic acid) and buffer B (97% ACN-3% H_2O, 0.1% Formic acid). For the peptide mixture, the following gradient was applied: from 0–5 min 0% of B to 5% of B, from 5–120 min 5% of B to 40% of B, from 160–165 min 40% of B to 100% of B, at a flow rate of 500 nl/min, and from 165–180 min 100% of B at a flow rate of 600 nl/min to avoid carry over. MS/MS analyses were performed using an LTQ-Orbitrap Velos (Thermo Fisher Scientific, Waltham, MA, USA) with a nanoelectrospray ion source. The LTQ-Orbitrap Velos settings included one 30000 resolution scan for precursor ions followed by MS2 scans of the 20 most intense precursor ions in positive ion mode. MS/MS data acquisition was completed using Xcalibur 2.1 (Thermo Fisher Scientific, Waltham, MA, USA). For peptides identification, activation type of CID at 35% of normalized collision was applied.

Mass spectrometry data interpretation

Identified proteins were analyzed using PROTEOME DISCOVERER 1.4 (Thermo Fisher Scientific, Waltham, MA, USA). For database searching, processed data were submitted to the in-house dicLab1_scaffold.fasta (26654 proteins obtained from the European sea bass Genome Browser Gateway; http://seabass.mpipz.mpg.de/, dicLab1 v1.0c, 2012; Tine et al. 2014) using SEQUEST HT version 28.0 (Thermo Fisher Scientific, Waltham, MA, USA). Searches were performed using the following parameters: two maximum miss cleavages for trypsin, carbamidomethylation (+57.021 Da) as a static modification, methionine oxidation (+15.995 Da) as a dynamic modification, 20 ppm precursor mass tolerance, and 0.8 Da

fragment mass tolerance. A minimum of 1 peptide per protein (at least one unique peptide) and a false discovery rate (FDR) of 1% using Percolator were the criteria used for protein identification. In addition, all of the proteins were treated as dissociated or 'ungrouped' from their respective families, allowing the detection of the different isoforms of the same protein.

Proteomic analysis

Proteins identified in the *D. labrax* overall sperm proteome and isolated nuclei proteome were classified according to the Gene Ontology as GO-Slim biological process, GO-Slim molecular function, and GO-Slim cellular component annotations from the UniProt Knowledgebase (UniProtKB/Swiss-Prot) Web site (http://www.uniprot.org/). The gene-enrichment analysis significance was calculated by the bioinformatic tool Gene Ontology Consortium (http://www.geneontology.org/; Ashburner et al. 2000; Gene Ontology Consortium 2017) based on PANTHER v13.1 database (Release date 2018-02-03), displaying only results with p value <0.05 after FDR correction (Suppl. Table 2). The enriched GO-terms in *D. labrax* were based on the orthologs *Homo sapiens* gene annotations (n = 272 orthologs in *H. sapiens* out of the 296 proteins identified in *D. labrax*) extracted from the Uniprot database (http://www.uniprot.org/).

To further identify a subset of chromatin-related proteins in the *D. labrax* sperm proteome, we have selected proteins having at least one GO biological process, molecular function, and cellular component annotation, or a protein function related to chromatin. Basically, we have selected those proteins in the overall *D. labrax* proteome (n = 296) containing at least one of the following key words: *histone, chromatin, chromosome, nucleosome, centrosome, zinc fingers, transcription factors,* and *DNA.* Additionally, the little subset of proteins that did not have any ortholog in *Homo sapiens* was manually reviewed at the Uniprot database (http://www.uniprot.org/) not to exclude any protein with chromatin function. The complete list of 94 chromatin-related proteins is shown in Table 1.

Moreover, with the aim to evaluate the conservation of the chromatin-related sperm proteins among vertebrates, the 94 sperm proteins of *D. labrax* identified as chromatin-related (Table 1) were selected and compared with the rainbow trout (*Oncorhynchus mykiss*), mouse (*Mus musculus*), and human (*Homo sapiens*) sperm proteomes. These vertebrate's sperm proteome lists were obtained from published proteomic reports: *O. mykiss* (Nynca et al. 2014), *M. musculus* (Baker et al. 2008; Asano et al. 2010; Dorus et al. 2010; Chauvin et al. 2012; Guyonnet et al. 2012; Castillo et al. 2014b), and *H. sapiens* (Castillo et al. 2018). The gene names and ortholog gene names were extracted from the Uniprot database (http://www.uniprot.org/). The conservative comparison of the *D. labrax* chromatin-related sperm proteins within other species is shown in Table 3 and, more detailed, in Suppl. Table 3.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Notes on contributors

Each author has contributed to the research process. Conceived and designed the experiments: FB, RO. Performed the experiments: FB, JME, FP, RO. Analyzed and interpreted the data: FB, JC, MJ, JME, RO. Drafted the manuscript and the figures: FB, RO. Critically revised, and completed/corrected the manuscript, and approved the final version: All authors (FB, DA, MJ, JC, JME, FP, RO).

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Annex 3_

ARATO I*, GRANDE G*, BARRACHINA F*, BELLUCCI C, LILLI C, JODAR M, AGLIETTI MC, MANCINI F, VINCENZONI F, PONTECORVI A, CALAFIORE R, OLIVA R, LUCA G, MANCUSO F AND MILARDI D (2020)

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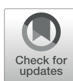

"*In vitro*" Effect of Different Follicle—Stimulating Hormone Preparations on Sertoli Cells: Toward a Personalized Treatment for Male Infertility

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Follicle-stimulating hormone (FSH), a major regulator of spermatogenesis, has a crucial function in the development and function of the testis and it is extensively given as a fertility treatment to stimulate spermatogenesis. We analyzed the effects of different FSH preparations (α-follitropin, β-follitropin, and urofollitropin) in combination with testosterone on porcine pre-pubertal Sertoli cells. To study the effect of the different FSH treatments in the Sertoli cell function we performed Real Time PCR analysis of AMH, inhibin B, and FSH-r, an ELISA assay for AMH and inhibin B, and a high-throughput comparative proteomic analysis. We verified that all three preparations induced a reduction of AMH in terms of mRNA and secreted proteins, and an increase of inhibin B in terms of mRNA in all the FSH formulations, while solely α-follitropin produced an increase of secreted inhibin B in the culture medium. Comparative proteomic analysis of the three FSH preparations identified 46 proteins, 11 up-regulated and 2 down-regulated. Surprisingly, the combination of testosterone with β-follitropin specifically induced an up-regulation of eight specific secreted proteins. Our study, showing that the three different FSH preparations induce different effects, could offer the opportunity to shed light inside new applications to a personalized reproductive medicine.

Keywords: Sertoli cells, alpha follitropin, beta follitropin, urofollitropin, proteomic analysis

INTRODUCTION

Follicle-stimulating hormone (FSH), a glycoprotein hormone secreted by the anterior pituitary gland, plays a key function in the treatment of human infertility. In infertile women it is widely prescribed to stimulate follicular development, meanwhile, in males, it is used alone or in association with Human chorionic gonadotropin (hCG) to trigger off and maintain spermatogenesis both in hypogonadotropic hypogonadism (1), and in oligozoospermic subjects with normogonadotropic hypogonadism (2).

FSH comprises two subunits, α and β, which are both glycosylated and contain four N-linked carbohydrates. The different content in sialic acid at the C-terminal determines a family of glycoforms that explain the structural and functional heterogeneity of the different FSH formulations (3).

The preparations of FSH available in the market are derived by either recombinant DNA technology (rFSH such as α- and β-follitropin) or post-menopausal urines (urofollitropin). α- and β-follitropins are synthesized by the same recombinant technology, producing identical dimeric α-FSH and β-FSH subunits, but with differences in the further glycosylation and in the procedures of purification. In contrast, urofollitropin consists of FSH with a minimal LH activity, and it has low specific activity (∼100–150 IU FSH/mg protein). The low specific activity of this preparation could be explained by the fact that more than 95% of the protein content correspond to non-specific co-purified urinary proteins (1).

Regarding the efficacy of the different FSH preparations in the female, many contradictory results have been published in the last 2 decades. The meta-analyses regarding the clinical efficacy of different FSH preparations demonstrated no significant differences in clinical or ongoing pregnancy and in the live-birth rate, in the miscarriage rate, or for the incidence of multiple pregnancy rate or ovarian hyperstimulation syndrome (OHSS) between rFSH and urofollitropin (4–8).

Up to now, in the male, no data exist regarding the efficacy of the treatment in relation to the FSH-therapy used. However, a meta-analysis reported a significant positive effect of the treatment with FSH both on sperm parameters and on pregnancy rate in oligozoospermic patients with normal FSH levels (9). Unfortunately, the studies included in the meta-analyses have an extremely heterogeneity in the selection criteria of the patients, in primary and secondary end-points, in the doses of FSH treatment and in time of treatment (10).

Sertoli cell (SC) is one of the principal actors in spermatogenesis as it provides nourishment, and structural and functional support to germ cells. Moreover, it protects germ cells by the blood-testis barrier (BTB) and by the production of immunomodulatory factors (11). In testis, FSH controls the function of SC through FSH receptors (FSH-r), which are only present in SC. In particular, FSH plays a pivotal role in the early stages of spermatogenesis, while testosterone has a major role in spermiogenesis (12).

In the prepubertal testis, SC is the most representative cell population. However, during this stage, there is a low activity of the hypothalamic–pituitary–gonadal axis reflected by the high levels of Anti-Müllerian Hormone (AMH) and inhibin B in serum (13). In contrast, during puberty, testosterone induces SC maturation and inhibits AMH production (13).

The aim of this research was to assess the effects of the different FSH formulation in an "*in vitro*" model of porcine pre-pubertal SC, in order to evaluate the SC responsiveness to pharmacological treatment of different FSH preparations, never assessed until now.

MATERIALS AND METHODS

SC Culture, Characterization, and Stimulation

Pure porcine pre-pubertal SC were isolated and characterized according to previously reported methods (14–16).

Purified SC cultures, as previously stated (14–16), were treated for 48 h as follows, and stimulation was performed according to the previously described protocol (17):

- Stimulated with testosterone (0.2 μg/ml; SIT, Pavia, Italy) (control group);
- Stimulated with α-follitropin (α-FSH) 100 nM and testosterone (0.2 μg/ml);
- Stimulated with β-follitropin (β-FSH) 100 nM and testosterone (0.2 μg/ml);
- Stimulated with Urofollitropin (u-FSH) 100 nM and testosterone (0.2 μg/ml).

We used testosterone in addition to any of the FSH formulation to mimic a physiological condition in testis, considering that both FSH and testosterone are essential for the adequate spermatogenesis (12).

Quantitative, Real-Time PCR

Analyses for AMH, inhibin B, and FSH receptor (FSH-r) were performed as previously described (17) employing the primers listed in **Table 1**. Total RNA was extracted from SC monolayers obtained in the experimental groups using Trizol reagent (Sigma-Aldrich, Milan, Italy), and quantified by reading the optical density at 260 nm. In detail, 2.5 μg of total RNA was subjected to reverse transcription (RT, Thermo Scientific, Waltham, MA, USA) to a final volume of 20 μl. We performed the qPCR with the use of 25 ng of the cDNA obtained by RT and a SYBR Green

TABLE 1 | Primer sequences for PCR analyses.

Gene	Forward sequences (5′-3′)	Reverse sequences (5′-3′)
AMH	GCGAACTTAGCGTGGACCTG	CTTGGCAGTTGTTGGCTTGATATG
Inhibin B	CCGTGTGGAAGGATGAGG	TGGCTGGAGTGACTGGAT
FSH-r	TGAGTATAGCAGCCACAGATGACC	TTTCACAGTCGCCCTCTTTCCC
β-actin	ATGGTGGGTATGGGTCAGAA	CTTCTCCATGTCGTCCCAGT

Master Mix (Stratagene, Amsterdam, The Netherlands). This procedure was performed in an Mx3000P cycler (Stratagene), using FAM for detection and ROX as the reference dye. We normalized the mRNA level of each sample against β-actin mRNA and expressed it as fold changes vs. the levels in the control group.

Culture Media Isolation

Aliquots of the culture media (CM) of SC were collected after 48 h of stimulation, centrifuged at 1,500 g for 10 min, and the supernatant was saved at −20°C for proteomic analysis and for an ELISA assay for Inhibin B and AMH secretion performed as previously described (18).

Proteomic Analysis of the SC Secretome

The SC CM for each sample was thawed and centrifuged at 3,000 g for 20 min at 4°C. The resulting supernatants were filtered (0.45 µm pore size) to remove cell debris and other impurities if any. Afterward, to perform protein solubilization, sodium dodecyl sulfate (SDS) and phenylmethylsulfonyl fluoride (PMSF) were added to a final concentration of 2% (w/v) and 1 mM, respectively. After 30 min, all samples were centrifuged at 4°C at 16,000 g for 10 min, and the supernatants (soluble proteins) were kept, and proteins were precipitated overnight in 80% cold acetone (v/v) at −20°C. After centrifugation at 17,530 g for 15 min at 4°C, the protein precipitates were collected and resuspended in 1% SDS, 1 mM PMSF in phosphate-buffered saline (PBS) to perform protein quantification using the BCA protein Assay Kit (Pierce^{TM} BCA protein Assay Kit, Thermo Fisher Scientific, Rockford, IL), according to manufacturer's recommendations.

The TMT labeling was performed as described previously (19, 20) and according to the manufacturer's instructions. Briefly, 50 µg of proteins from each sample were transferred to a new tube and adjusted to a final volume of 50 µl with 100 mM triethylammonium bicarbonate (TEAB) to obtain a 1 µg/µl concentration. Proteins were reduced in 9.5 mM tris (2-carboxyethyl) phosphine (TCEP), alkylated with 17 mM iodoacetamide (IAA), and precipitated by adding six volumes of 100% cold acetone. Then, samples were centrifuged at 17,500 g, and the acetone-precipitated protein pellets (containing 50 µg of proteins) were resuspended in 50 µl of 100 mM TEAB. Trypsin was added at a 1:22 protease-to-protein ratio and incubated overnight at 37°C. Prior to peptide labeling, an aliquot from each sample was taken and combined at equal amounts to form the internal control. After, 35 µg of peptides from each sample (including the internal control) were labeled with TMT isobaric tags (TMT 10-plex Mass Tag Labeling; Thermo Fisher Scientific, Rockford, IL). The technical reproducibility and analytical reliability of the approach were assessed by performing duplicate analyses on the internal control. Then, 15 µl of the TMT label reagents, previously resuspended in acetonitrile anhydrous (ACN), were added to the corresponding sample, followed by 1 h incubation at RT. Afterward, the reaction was stopped by adding 5% hydroxylamine. The TMT-labeled samples were combined at equal amounts constituting one multiplex pool, which was dried in a vacuum centrifuge and resuspended in 50 µl of 0.5% trifluoroacetic acid in 5% ACN. After, labeled peptides were cleaned up via reversed-phase C18 spin columns (Pierce C18 Spin Columns, Thermo Fisher Scientific, Rockford, IL), according to manufacturer's instructions. Then, the peptides were reconstituted in 0.1% formic acid (FA) to be processed by LC-MS/MS.

Our MS data was collected using a nano-LC Ultra 2D Eksigent (AB Sciex, Brugg, Switzerland) attached to an LTQ-Orbitrap Velos (Thermo scientific, San Jose, CA). Peptides were injected onto a C18 trap column (L 2 cm, 100 µm ID, 5 µm, 120 Å; NanoSeparations, Nieuwkoop, the Netherlands) and chromatographic analyses were performed using an analytical column (L 15 cm, 75 µm ID, 3 µm, 100 Å; Thermo scientific, San Jose, CA). The buffers used for the analysis were buffer A (97% H_2O-3% ACN, 0.1% FA) and buffer B (3% H_2O-97% ACN, 0.1% FA). A peptide mixture was loaded onto the analytical column with the following gradient: time 0–5 min, 0% of B; 5–180 min, 0–32.5% of B; 180–185 min, 32.5–100% of B at a flow rate of 400 ml/min; and 185–200 min, 100% of B at a flow rate of 400 nl/min to avoid carry-over. MS/MS analyses were performed using an LTQ-Orbitrap Velos (Thermo Fisher Scientific, Waltham, MA) with a nanoelectrospray ion source. The LTQ-Orbitrap Velos settings included one 30,000 resolution at 400 m/z MS1 scan for precursor ions followed by MS2 scans of the 15 most intense precursor ions, at 30,000 resolution at 400 m/z, in positive ion mode. The lock mass option was enabled, and mass calibration was performed on polysiloxane (m/z 445.12003). MS/MS data acquisition was completed using Xcalibur 2.1 (Thermo Fisher Scientific, Waltham, MA). MS2 experiments were performed using higher-energy collision dissociation (HCD) with a normalized collision energy of 42%.

LC-MS/MS data was analyzed using Proteome Discoverer 1.4.1.14 (Thermo Fisher Scientific, Waltham, MA, USA) based on SEQUEST HT cluster as search engine (University of Washington, licensed to Thermo Electron Corp., San Jose, CA) against UniProtKB/Swiss-Prot database with Sus scrofa (released September 2018; 3,339 sequences). Searches were run applying the following parameters: two maximum missed cleavage sites for trypsin, TMT-labeled lysine (+229.163 Da) and methionine oxidation (+15.995 Da) as dynamic modifications, cysteine carbamidomethylation (+57.021 Da) as a static modification, 20 ppm precursor mass tolerance, 0.6 Da fragment mass tolerance. Percolator was used for protein identification, applying the following identification criteria: at least one unique peptide per protein and a FDR of 1%. The reporter ion intensities were corrected according to the isotopic purities provided by the manufacturer.

For protein quantification purposes, only unique peptides were used, and protein ratios (i.e., TMT-127/TMT-126) were normalized to protein median. The cut-off for up-regulated proteins was $\geq$1.500, and for down-regulated proteins was $\leq$ 0.667 as previously reported (21).

Statistical Analysis

Values reported in the figures are the mean ± S.D. of three independent experiments, each one performed in triplicate. Statistical analysis was performed by the paired Student's *t*-test

using SigmaStat 4.0 software (Systat Software Inc., CA, USA). All tests were performed in triplicate, and statistically significance was assigned for $p < 0.05$.

RESULTS

Purification and Characterization of SC

The isolated SC culture was 95% pure as indicated by immunostaining for AMH (**Figure 1a**) with an extremely low percentage of non-SC cells (<5%) characterized by immunostaining for insulin-like 3-positive (Leydig) cells (INSL-3) (**Figure 1b**), alpha-smooth muscle actin positive (peritubular myoid) cells (ASMA) (**Figure 1c**) and protein gene product 9.5-positive (gonocytes and spermatogonial) cells (PGP9.5) (**Figure 1d**).

Inhibin B, AMH, and FSH-r Gene Expression in SC

AMH gene expression in SC was significantly down-regulated by treatment with α-, β-follitropin, and urofollitropin in combination with testosterone treatment compared with testosterone alone (**Figure 2A**, $p < 0.001$).

In contrast, inhibin B expression was significantly increased after treatment with α-, β-follitropin, and urofollitropin, each other combined with testosterone treatment compared with testosterone alone (**Figure 2A**, $p < 0.001$).

Moreover, we found a statistically significant reduction of FSH-r upon all three FSH preparations plus testosterone compared with testosterone alone (**Figure 2A**, $p < 0.001$).

Inhibin B and AMH Secretion Assay

The secretion of AMH was significantly down-regulated by α-, β-follitropin, and urofollitropin plus testosterone treatments compared with testosterone alone, consistent with the results of gene expression showed above (**Figure 2B**, $p < 0.001$).

Meanwhile, inhibin B was significantly increased in culture medium only after exposure to α-follitropin plus testosterone (**Figure 2B**, $p < 0.001$), while no changes were observed after β-follitropin plus testosterone stimulation. Interestingly, we observed a significant reduction of inhibin B after the stimulation with urofollitropin plus testosterone compared with testosterone alone (**Figure 2B**, $p < 0.05$).

Secretomic Protein Profiling

In order to evaluate the differences induced by the different FSH preparations on SC secretomic profiles, we performed a comparative proteomic analysis of the SC culture media in the groups treated with testosterone associated with α-, β-follitropin, and urofollitropin, and we compared it with the group of the testosterone treatment alone.

The proteomic analysis resulted in the identification of 46 TMT-labeled proteins in all the SC culture media proteomes (**Table 2**). Of those, 13 proteins were detected in a significantly altered abundance (**Table 3**). Specifically, 11 proteins were observed as up-regulated (**Table 3**; cut-off ≥ 1.500) and 2 proteins as down-regulated (**Table 3**; cut-off ≤ 0.667) by the different FSH preparations. All the different FSH preparations showed a down-regulation of the secreted SPARC protein

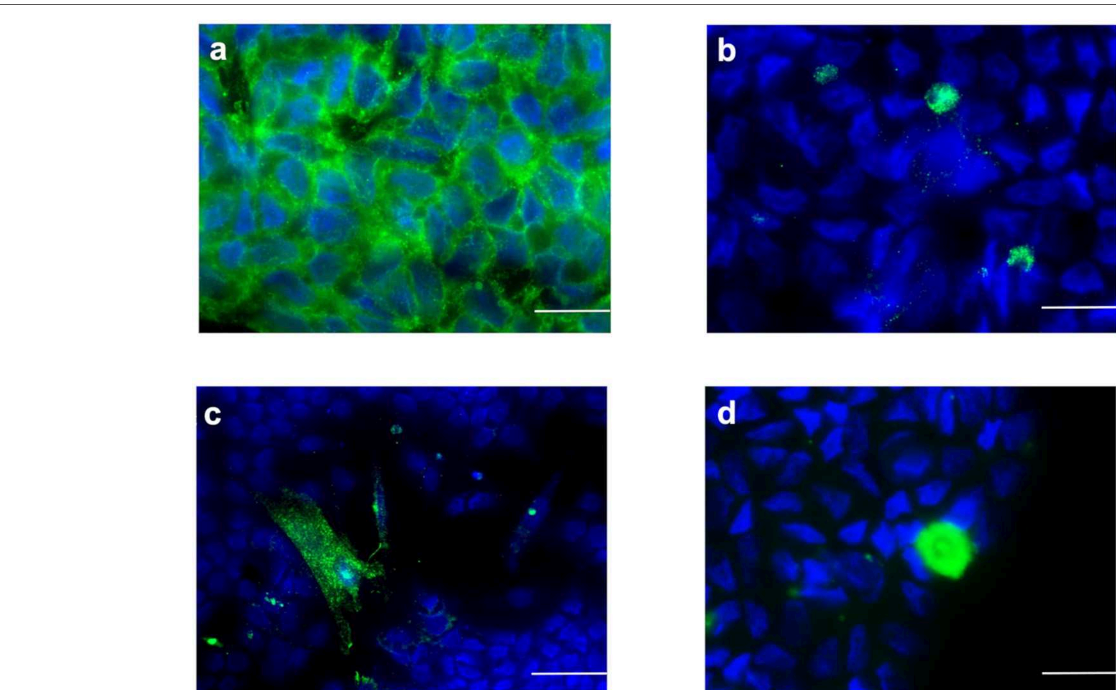

FIGURE 1 | Sertoli cells characterization by immunofluorescence staining (green color). Sertoli cell monolayers were characterized by the expression of **(a)** AMH, **(b)** INSL3, **(c)** ASMA, and **(d)** (PGP9.5). Nuclei are labeled with DAPI in blue color. Bars = 20 µm.

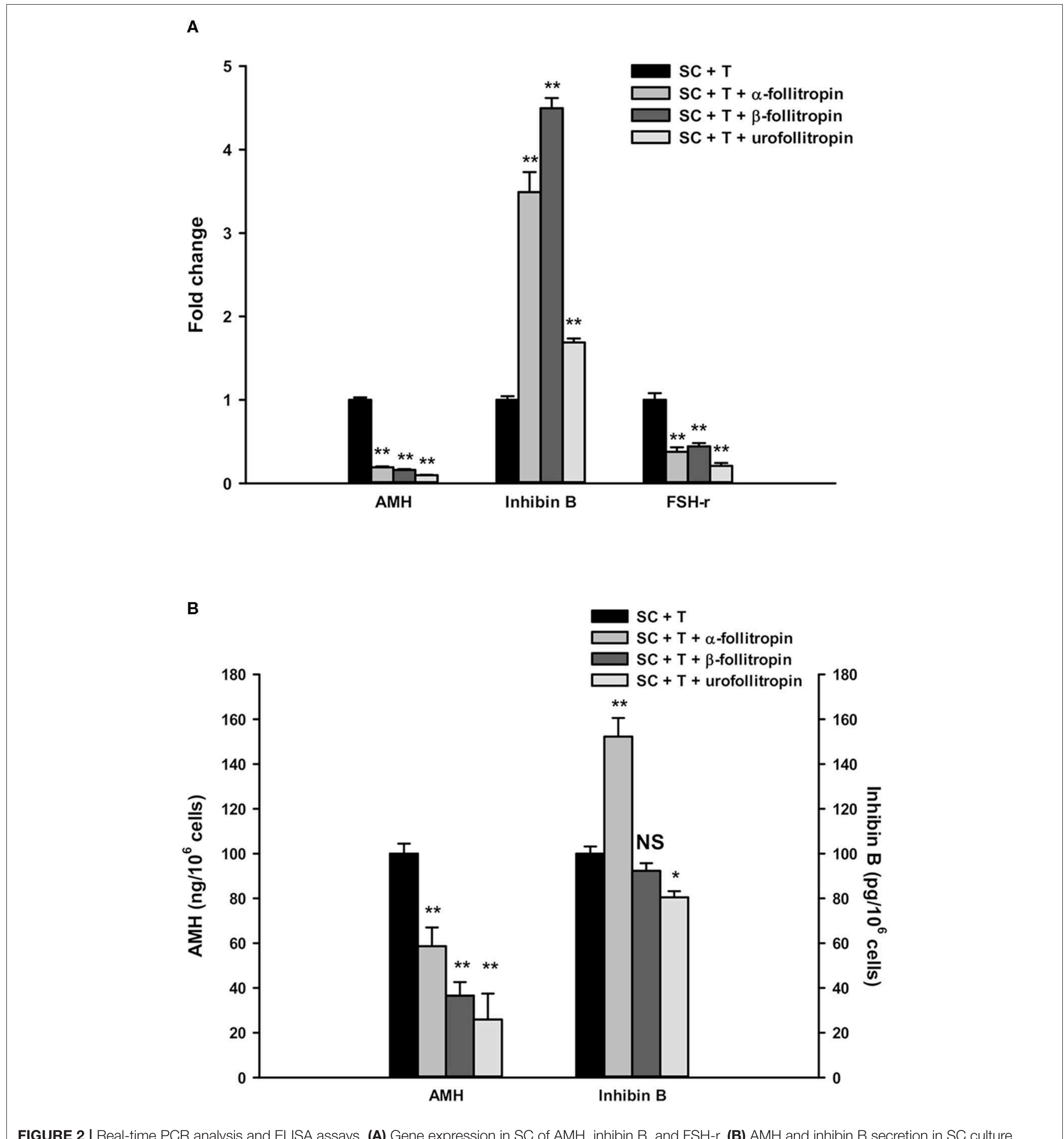

FIGURE 2 | Real-time PCR analysis and ELISA assays. **(A)** Gene expression in SC of AMH, inhibin B, and FSH-r. **(B)** AMH and inhibin B secretion in SC culture medium. Data represent the mean ± S.D. (*$p < 0.05$, **$p < 0.001$) of three independent experiments performed in triplicate.

and an up-regulation of 3 proteins (PLAT, INHBA, and TPI1, **Table 3**).

Interestingly, an up-regulation of 8 additional secreted proteins (ACTB, UBC, PRDX2, PPIA, SPP1, HBB, MIF, and S100A6) and a down-regulation of Histone H4 were specifically observed after the use of β-FSH with T, as reported in **Table 3**.

DISCUSSION

In the present work, we have focused on the "*in vitro*" effect of different FSH preparations on pre-pubertal porcine SC culture, evaluating the modulation of specific markers determined through different approaches. All the FSH preparations assessed

TABLE 2 | List of the 46 TMT-labeled proteins identified in the Sertoli cell culture.

Accession	Gene name	Description	Coverage	# Proteins	# Peptides	# Unique Peptides	MW (kDa)
P08835	ALB	Serum albumin	14.00	1	12	12	69.6
Q29549	CLU	Clusterin	40.36	1	14	14	51.7
P01025	C3	Complement C3	16.68	1	21	21	186.7
P04087	INHA	Inhibin alpha chain	31.04	1	8	8	39.2
Q6QAQ1	ACTB	Actin, cytoplasmic 1	22.40	1	6	2	41.7
P20305	GSN	Gelsolin (Fragment)	11.79	1	7	7	84.7
Q9GKQ6	BGN	Biglycan (Fragments)	31.99	1	7	7	30.4
P00761	N/A	Trypsin	9.96	1	2	2	24.4
P18648	APOA1	Apolipoprotein A-I	32.83	1	7	7	30.3
P02543	VIM	Vimentin	8.37	1	4	4	53.6
P04404	CHGA	Chromogranin-A (Fragment)	16.59	1	5	5	49.3
P20112	SPARC	SPARC	24.67	1	6	6	34.2
P68137	ACTA1	Actin, alpha skeletal muscle	15.65	1	5	1	42.0
P0CG68	UBC	Polyubiquitin-C	32.83	2	2	2	60.0
Q29095	PTGDS	Prostaglandin-H2 D-isomerase	16.40	1	3	3	20.6
Q8SQ23	PLAT	Tissue-type plasminogen activator	8.54	1	3	3	63.6
Q1KYT0	ENO3	Beta-enolase	8.06	1	2	2	47.1
P80031	GSTP1	Glutathione S-transferase P	7.73	1	1	1	23.5
P35624	TIMP1	Metalloproteinase inhibitor 1	9.18	1	1	1	23.1
P62802	N/A	Histone H4	11.65	1	1	1	11.4
A5A8V7	HSPA1L	Heat shock 70 kDa protein 1-like	6.08	1	2	2	70.3
P10668	CFL1	Cofilin-1	18.67	2	2	2	18.5
Q9XSD9	DCN	Decorin	5.28	1	2	2	39.9
Q49I35	LGALS1	Galectin-1	5.93	1	1	1	14.7
P52552	PRDX2	Peroxiredoxin-2 (Fragment)	7.09	1	1	1	14.2
O97763	NPC2	NPC intracellular cholesterol transporter 2	16.78	1	2	2	16.3
P79295	AMH	Muellerian-inhibiting factor	7.65	1	3	3	61.5
P62936	PPIA	Peptidyl-prolyl cis-trans isomerase A	5.49	1	1	1	17.9
Q29243	DAG1	Dystroglycan	1.82	1	1	1	95.4
P14287	SPP1	Osteopontin	5.28	1	1	1	33.6
P00690	AMY2	Pancreatic alpha-amylase	2.74	1	1	1	57.0
P43368	CAPN3	Calpain-3	1.83	1	1	1	94.5
P00172	CYB5A	Cytochrome b5	6.72	1	1	1	15.3
P29412	EEF1B	Elongation factor 1-beta	6.70	1	1	1	24.6
P02067	HBB	Hemoglobin subunit beta	6.12	1	1	1	16.2
O02705	HSP90AA1	Heat shock protein HSP 90-alpha	1.91	1	1	1	84.7
P03970	INHBA	Inhibin beta A chain	7.08	1	2	2	47.4
P01315	INS	Insulin	19.44	1	1	1	11.7
P80928	MIF	Macrophage migration inhibitory factor	7.83	1	1	1	12.4
Q2EN75	S100A6	Protein S100-A6	8.89	1	1	1	10.1
P03974	VCP	Transitional endoplasmic reticulum ATPase	2.11	1	1	1	89.2
Q29371	TPI1	Triosephosphate isomerase	6.05	1	1	1	26.7
P42639	TPM1	Tropomyosin alpha-1 chain	4.58	1	1	1	32.7
P09571	TF	Serotransferrin	2.01	1	1	1	76.9
P50390	TTR	Transthyretin	4.67	1	1	1	16.1
P04185	PLAU	Urokinase-type plasminogen activator	3.85	1	1	1	49.1

N/A, Not applicable.

in the current study, α-, β-follitropin, and urofollitropin, induced a significant and similar response in terms of down-regulation of both AMH gene expression and AMH secretion, up-regulation of inhibin B gene expression and down-regulation of the expression of FSH-r gene expression. AMH is a glycoprotein dimeric hormone, a member of the transforming growth factor β (TGF-β)

TABLE 3 | List of the up- and down-regulated proteins in Sertoli cell medium after stimulation with the different FSH preparations plus testosterone, compared with testosterone treatment alone.

Accession	Gene name	Description	αFSH+T/T	βFSH+T/T	uFSH+T/T
P03970	INHBA	Inhibin beta A chain	**1.804**	**1.835**	**3.084**
Q8SQ23	PLAT	Tissue-type plasminogen activator	**1.881**	**2.533**	**1.857**
Q29371	TPI1	Triosephosphate isomerase	**1.786**	**2.132**	**1.562**
Q6QAQ1	ACTB	Actin, cytoplasmic 1	1.377	**1.618**	1.147
P0CG68	UBC	Polyubiquitin-C	1.185	**1.500**	1.357
P52552	PRDX2	Peroxiredoxin-2 (Fragment)	1.459	**1.634**	1.077
P62936	PPIA	Peptidyl-prolyl cis-trans isomerase A	1.358	**1.980**	1.076
P14287	SPP1	Osteopontin	1.016	**2.134**	1.191
P02067	HBB	Hemoglobin subunit beta	1.270	**1.814**	1.336
P80928	MIF	Macrophage migration inhibitory factor	1.412	**1.513**	1.233
Q2EN75	S100A6	Protein S100-A6	1.302	**1.564**	1.100
P20112	SPARC	SPARC	**0.589**	**0.474**	**0.485**
P62802	N/A	Histone H4	1.275	**0.630**	1.049

N/A, Not applicable.
The values in bold indicate the cut-off ≥1.500 for up-regulated proteins, and ≤ 0.667 for down-regulated proteins.

family, that plays a pivotal function in fetal sex differentiation, being involved in the regression of the Müllerian ducts (22). In the male, SC secretes high amounts of AMH from fetal life until the onset of puberty. AMH is exclusively secreted by SC and, for this reason, it is widely considered an important marker of the testicular function during the pre-pubertal life (22). We observed that testosterone alone and in combination with the three FSH preparations induced a significant down-regulation in AMH mRNA expression and secretion. As expected, we also demonstrated a statistically significant reduction in FSH-r expression independently of the FSH preparation. These data are in accordance with literature reporting how the interaction of the hormone with its receptor leads to the down-regulation of FSH-r mRNA expression by a cAMP-dependent post-transcriptional mechanism (23).

Another specific and important marker of SC functionality is inhibin B, which provides for a negative feedback on FSH secretion. In particular, serum inhibin B concentration is high during early postnatal life, and then gradually is reduced to a detectable plateau-level until its increase at the beginning of puberty (12). The assay of inhibin B is used in clinical practice to evaluate the presence and function of SC during childhood. Additionally, in adult life, the inhibin B levels depend on the presence of germ cells thus reflecting the efficiency of spermatogenesis (24). Our results demonstrated that all three FSH preparations plus testosterone significantly induced an up-regulation in the levels of inhibin B mRNA, confirming the role of FSH in inducing the transcription of inhibin B gene.

Our SC secretomic analysis uncovered a similar response after stimulation of SC with the different FSH preparations. On the one hand, we demonstrated the reduction levels of secreted SPARC protein after stimulation of SC with the different FSH preparations. SPARC, also known as osteonectin or BM-40, is a multifunctional protein that can modulate cell shape, proliferation, differentiation, and migration (25). SPARC has been found to interact with structural matrix proteins and may act to mediate their interactions with cells (24–28). In addition, SPARC can regulate the activity of several signaling molecules, either by direct interaction or by interfering with their signaling pathways (29–31). In our scenario, it is known that SPARC is produced by Leydig and Sertoli cells (32, 33), and that it is internalized in Sertoli, Leydig, and germ cells (34), playing a paracrine regulatory role during fetal testis development. Furthermore, the expression of SPARC in SC bearing late-stage elongate spermatids might suggest a role in the spermiation of elongated spermatids (33). Future studies will comprehensively define the function of SPARC in Sertoli-germ cell interaction and spermiogenesis. Here we provide for the first time information about the down-regulation of SPARC secretion by FSH.

On the other hand, the proteomic analysis also showed that tissue-type plasminogen activator (PLAT), triosephosphate isomerase (TPI1), and inhibin beta A chain (INHBA) proteins were up-regulated by the stimulation with α-, β-follitropin, and urofollitropin in presence of normal androgen milieu. Specifically, the inhibin beta A chain was observed as upregulated in proteomics by all the FSH preparations, and the higher increase was observed for urofollitropin. Despite this evidence, only α-follitropin stimulation induced a significative increase in the inhibin B levels in the medium, as documented by ELISA. INHBA is a subunit of both activin B and inhibin B (24). In the testis, it has been postulated that activin B acts as an autocrine and paracrine regulator of spermatogenesis (35, 36), modulating the proliferation in the testis of germ cells and SC (37). We might speculate that β-follitropin and, especially, urofollitropin, induce the activation of the INHB gene and the production of INHB, but do not increase the levels of INHB in the medium since they might increase the levels of activin B instead of inhibin B. Further studies are so needed to understand how the different FSH preparations modify the inhibin B and activin B balance.

The remaining two up-regulated proteins have been previously associated with SC. For example, previous studies demonstrated low levels of PLAT activity in cultured SC under basal conditions, whereas FSH stimulation induces PLAT (38, 39). Our results through a quantitative *in vitro* secretomic approach support the increased secretion of PLAT after FSH treatment independently of the FSH preparation. Interestingly, the plasminogen activator system acts in the process of spermiation (40), the detachment of residual bodies from the mature spermatids (41), and the residual body phagocytosis by SC (42). Triosephosphate isomerase has moreover been previously reported to be expressed in SC (43). An increased TPI1 expression in SC may influence the early activities of spermatogenesis, such as mitosis or initiation of meiosis, by spermatogonia or pre-leptotene spermatocytes, respectively (43).

Surprisingly, the combination of β-follitropin with testosterone revealed specific effects in the SC function besides the aforementioned similar effect of all tested FSH treatments. Specifically, the levels of eight additional proteins were up-regulated, which were: Actin (ACTB), Polyubiquitin-C (UBC), Peroxiredoxin-2 (PRDX2), Peptidyl-prolyl cis-trans isomerase A (PPIA), Osteopontin (SPP1), Hemoglobin subunit beta (HBB), Macrophage migration inhibitory factor (MIF), and Protein S100-A6 (S100A6); and just one protein, Histone H4, was down-regulated.

It is important to underline that some of these proteins might have a pivotal role both in the germ cell migration and in the cell-to-cell contact at the blood-testis barrier (BTB). For example, actin filament bundles have been described in specific Sertoli cell regions that are adjoining to tight junctions and to the sites of adhesion to spermatogenic cells (44). During spermatogenesis, these actin bundles undergo organizational changes, which might play a role in changing the interrelationship between SC and germ cells by facilitating the movement of spermatogenic cells (44). Similarly, SPP1, synthesized by SC and germ cells, is involved in cell adhesion and migration (45), and MIF, produced by SC under basal conditions, induces the migration of spermatogonial cells (46). Also, we found up-regulation of PPIA, a protein highly expressed in SC that has been recognized as a crucial factor in BTB integrity and maintenance (47), as well as S100A6, a protein that promotes cell migration and influences cell junction of SC (48) that could be implied in the spermatogenesis and modulation of BTB.

Interestingly, β-follitropin also increased PRDX2, an antioxidant protein preferentially expressed in SC that might play a role in removing or regulating the intracellular levels of peroxides produced during metabolism (49, 50). Additional up-regulated proteins were HBB, a metalloprotein that acts as a scavenger balancing the level of carbon monoxide (CO) in testis (51), and UBC, which is required for normal spermatogenesis development (52).

In conclusion, α-, β-follitropin, and urofollitropin induced a similar response as expressed by the down-regulation of AMH gene expression and AMH secretion, and an up-regulation and down-regulation of inhibin B and FSH-r gene expression, respectively, thus exerting an interesting effect in inducing maturation of SC from a pre-pubertal to an adult phenotype. This data confirms that the FSH in presence of an androgenic milieu regulates the proliferation and functional maturation of Sertoli cell type (53). Moreover, all three FSH preparations induced a down-regulation of a spermiation related protein, the SPARC, supporting the role of FSH in the regulation of spermatogenesis. Nevertheless, there are some specific effects of each FSH preparation, which need consideration before their prescription to infertile males.

For instance, only α-follitropin, in association with testosterone, induced the secretion in the media of inhibin B. Since inhibin B secreted by Sertoli cells could serve as negative feedback control on the hypothalamic-pituitary system to decrease FSH release (54), we might suppose that α-follitropin could have an inhibitory effect on the hypothalamic/pituitary axis *in vivo*. If these preliminary data obtained in our *in vitro* model would be confirmed by further *in vitro* and *in vivo* studies, we might conclude that male secondary hypogonadism would represent the best indication for this treatment since it induces a good response in terms of Sertoli activation, and the increase in Inhibin B secretion does not have any clinical relevance. In contrast, the increase of Inhibin B secretion as a response to α-follitropin treatment might represent a problem for normogonadotropic infertile patients since it might inhibit physiological pituitary FSH secretion.

In the case of urofollitropin, it induces a similar secretomic profile but also an increase in the release of INHBA without increasing the levels of secreted inhibin B, suggestive of an increasing release of activin B. This panel of action might be useful for treating oligozoospermic patients with normal FSH secretion. Therefore, we might speculate that—if these preliminary data would be confirmed—urofollitropin might represent the best treatment option for patients with normogonadotropic hypogonadism, since it seems to induce an increase in the secreted proteins, including the A chain of inhibin B and, consequentially, activin B, without increasing the levels of inhibin B, which might interfere with pituitary FSH secretion.

Finally, β-FSH stimulation exhibits additional effect up-regulating specific proteins mainly related to spermatogenic cell migration and BTB maintenance. In this context, the stimulation of SC with β-follitropin exerts his effect in the modulation of additional proteins implicated in the last stage of spermiation and in the related antioxidant activity. Further studies are needed to confirm that β-follitropin treatment is the best treatment option for patients with a spermatogenic arrest at the spermatid stage, as suggested by these preliminary *in vitro* data.

This molecular and proteomic approach demonstrated how some molecular effects seem to have a specific signature depending on each FSH preparation. The different molecular responses could help to choose which of the different FSH preparations could be used for infertility treatment according to the different present physio-pathological conditions. We can argue that α-follitropin can find a specific clinical use in hypospermatogenesis due to hypogonadotropic stimulus or in inducing spermatogenesis in puberty; β-follitropin could be specifically indicated to improve spermiation or in case of spermatidic arrest; and, finally, urofollitropin could be useful

in idiopathic infertility in normogonadotropic patients. In conclusion, we performed for the first time a comparative study on the effects of different preparations of FSH on *in vitro* porcine pre-pubertal SC model. In the landscape of a personalized medicine, this study opens a window on the different use of the FSH formulations in relation to various clinical therapeutic targets.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by Italian Approved Animal Welfare Assurance (A-3143-01).

AUTHOR CONTRIBUTIONS

All authors had critically revised and approved the final version of the manuscript. IA, GG, and FB designed and drafted the manuscript. The experimental procedures and data analysis were performed by GG, FB, CB, CL, MJ, MA, FV, and FManci. AP, RC, and RO gave experimental guidance. GL, FMancu, and DM revised the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a past co-authorship with the authors IA, CL, GL, FM, and RC.

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Annex 4_

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Chapter 18: The use of sperm proteomics in the assisted reproduction laboratory.

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18

The Use of Sperm Proteomics in the Assisted Reproduction Laboratory

Meritxell Jodar, Ferran Barrachina, and Rafael Oliva

The Scientific and Biological Bases of Proteomics

About 84% of general population is able to conceive after 12 months of unprotected and regular intercourse.[1] However, 9% of women aged 20–44 years are unable to achieve a live birth during this period and often seek reproductive care.[2] Today, approximately 2% of the children born in Europe are conceived through assisted reproductive technology (ART).[3] Identifying the causes of infertility is crucial to establish the appropriate clinical treatment and minimize the risk of failure. Fertility evaluation for men is currently limited to examining semen parameters, which are able to reveal gross deficiencies in sperm count, motility, or morphology.[4] However, the etiology of male factor infertility remains largely idiopathic. Nowadays, fertility treatments offer a good rate of success, and approximately three out of four women will get pregnant as a result of ART. However, couples often have to face several attempts of ART cycles, which becomes a costly and time-consuming process. In Europe, this represents 134,422 live births from the 536,886 ART cycles including in vitro ART (in vitro fertilization [IVF] with or without intracytoplasmic sperm injection [ICSI]) and intrauterine insemination (IUI) reported in 2010 from women of all age groups.[3] This is a rather sobering statistic reflecting a per-ART-cycle failure rate of ~75% that is substantially higher than that of the naturally conceiving population.[3] Although the utility of some genetic tests (cytogenetic alterations and DAZ deletion) to predict unsuccessful results for ART cycles is unquestionable,[5,6] the series of semen parameters widely used in reproductive clinics to assess the male fertility are of little correlative value with respect to pregnancy outcome.[7] This observation emphasizes the need to develop alternative strategies for more accurate assessments.[8] High-throughput technologies such as proteomics provide the expression levels of all proteins of one functional state in a biological dynamic system. The application of proteomics to the study of the spermatozoa has progressed at a fast rate over the past 20 years (Figure 18.1).[9] The results obtained are shedding new light on the different issues of the sperm biology including generation, maturation, and metabolism of the mature sperm cell capable of fertilizing the oocyte.[10] Two main processes occur during spermatogenesis: (1) the replacement of histones by protamines and (2) the expulsion of majority cytoplasm during last steps of spermatogenesis, resulting in the blockage of nuclear transcription and translation in the mature sperm cells.[11,12] Then, the proteomic studies on the transcriptionally and translationally inert sperm cells, which are unable to generate new nuclear proteins, represent the final static picture of spermatogenesis. Results derived from comparative sperm proteomics between fertile and infertile males may provide insights into pathogenic mechanisms of male infertility. To date, sperm proteomics has only been applied in the research laboratory; however, the information derived from proteomics-based studies is likely to be useful in the development of fertility biomarkers. Therefore, proteomics holds promise of utility in clinical diagnostic testing of sperm for infertility, which is currently largely limited to the analysis of seminal parameters (sperm concentration, motility, and morphology).

Proteomic Techniques

The study of sperm proteins started more than a century ago with the isolation and identification by Friedrich Miescher in 1874 of a proteinaceous basic component from the sperm cell that he called "protamine" and that he found was coupled to what he called "nuclein" or what we know as DNA.[13] However, it was not until about 100 years later that the protein sequencing, separation, and detection methods were developed allowing the generalized study of the proteins (Figure 18.1).[14–16] Nevertheless, with these methods the proteins still had to be studied one at a time. The possibility to study the entire or a substantial proportion of the sperm proteome started much more recently, around 1995, with the application of mass spectrometry to the study of proteins (Figure 18.1).

The basic steps in most proteomic analysis at present are (1) protein or peptide extraction from the biological sample, (2) reducing the complexity of the protein or peptide extract, and (3) application of mass spectrometry and database comparisons to identify the different proteins or peptides (Figure 18.2).[17] The first step as applied to the sperm cell can be accomplished either by extracting the entire sperm or fluid proteome as well as by targeting specific cell compartments such as membrane systems, nucleus, tail or organelles, or fluid components.[18] The second step or reduction of the complexity of the initial protein or peptide extract can be accomplished using one-dimensional polyacrylamide gel electrophoresis (1D-PAGE) or 2D-PAGE (Figure 18.2). However, a more recent and high-throughput approach is to convert the initial protein extract into peptides on digestion with a protease and subsequently to fractionate the peptides using peptide isoelectric focusing (IEF) or monodimensional liquid chromatography (1D-LC) or 2D-LC (Figure 18.2).

The final step in a proteomic analysis is accomplished through mass spectrometry peptide and protein identification. Initial proteomic methods were developed that involved matrix-assisted laser desorption ionization—time of flight (MALDI-TOF), which relies on the accurate determination of peptide masses and comparison to peptide mass databases in search for identities. In a MALDI-TOF analysis, the proteins are typically excised from the gel, digested with trypsin, and the ratio of mass to charge of the resulting peptides determined. These peptide masses provide an accurate "peptide mass fingerprint" for

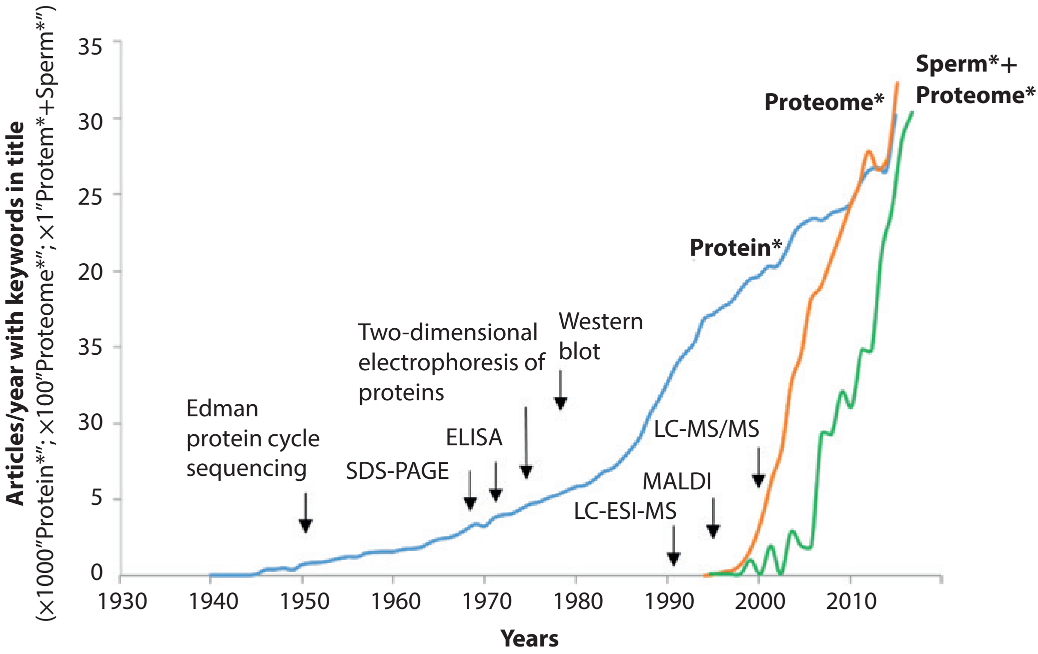

FIGURE 18.1 Pubmed publications where the keywords "protein*" or "proteome*" appear in the title. The asterisk "*" in "protein*" or "proteome*" indicates a wildcard. The year of the description of key methods to study proteins is indicated with arrows. It can be observed that proteomics is a relatively recent field as it started in 1990 with the application of mass spectrometry to study proteins.

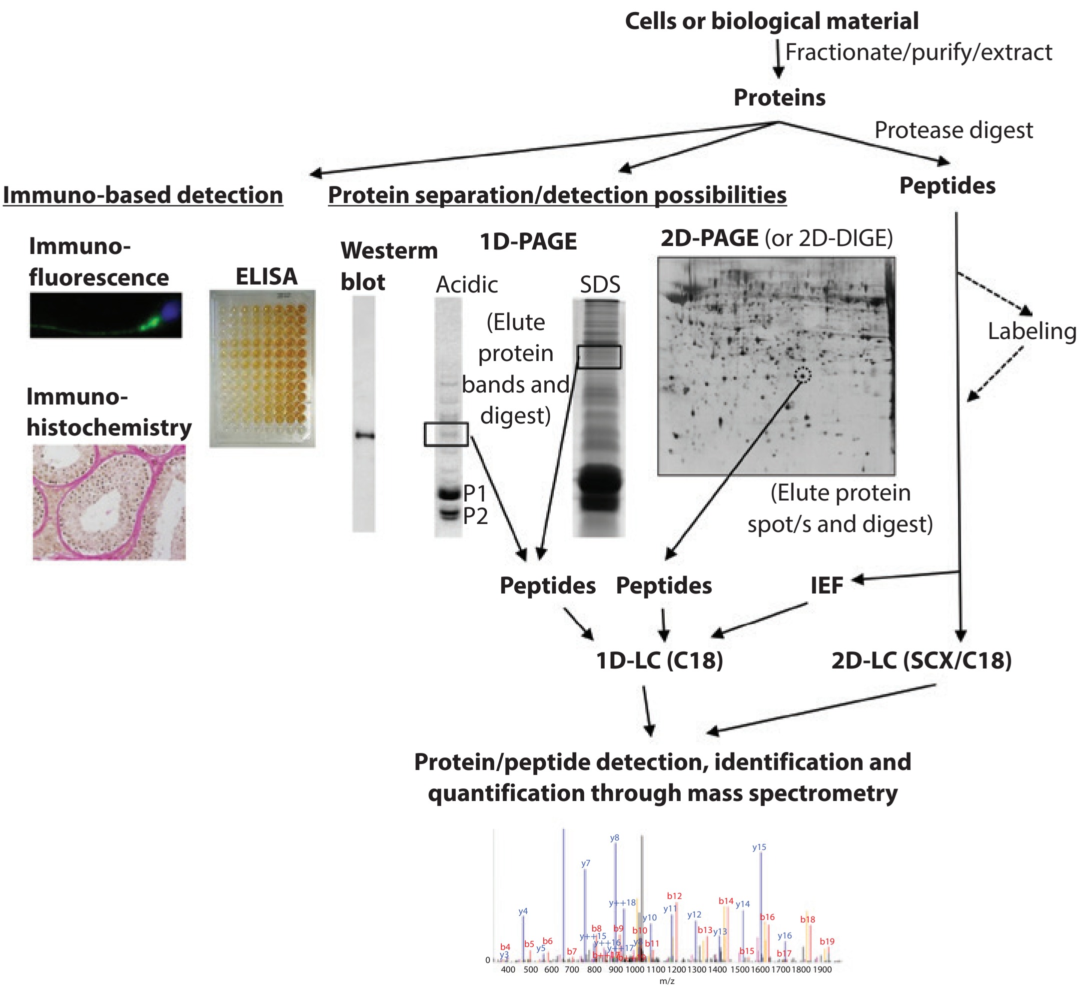

FIGURE 18.2 Many protein analysis options are currently available. Usually sperm cells or biological material must be processed, purified, or fractionated before proceeding to extract the proteins or their targeted detection. Cells or tissue sections can be directly used using immunohistochemistry or immunocytochemistry (left). Alternatively, proteins can be separated by gel electrophoresis (center) and the desired proteins eluted and digested into peptides. A current very high-throughput approach involves the digestion of the original protein mixture by proteases (usually trypsin) to convert it to peptides (right). The final stage is to separate the peptides through liquid chromatography and to proceed to identification using mass spectrometry.

the protein and are then compared against sets of masses from databases of in silico predicted peptides derived from the genome. If several of the experimentally determined peptide masses matched with the theoretical peptide matches derived from the proteins in the databases, then it is considered that the protein has been identified.[19] However, currently higher-throughput approaches based on tandem mass spectrometry (MS/MS) are being applied that also provide the opportunity for de novo peptide sequencing and posttranslational modifications detection (Figure 18.2).[17]

For protein quantification different possibilities are also available. Initial methods developed were based on enzyme-linked immunosorbent assay (ELISA)[14] or western blot[16] (Figure 18.2). These methods are extremely robust and useful but applicable only to study specific target proteins and cannot be applied to study many proteins simultaneously or even substantial proportions of the entire proteome. High-throughput approaches are currently available to quantify simultaneously many proteins in the proteome. Initial proteome quantification methods were based on measuring the protein intensities of proteins separated on 2D gels and identifying the corresponding protein spots.[20,21] However, current high-throughput quantification techniques rely on peptide quantification rather than protein quantification. Peptides can be quantified by spectral counting[22] or after their in vivo or in vitro labeling with tandem mass tags (Figure 18.2).[23,24]

Scientific Evidence

Currently, the analysis of the whole sperm proteome and subcellular proteome composition such as that corresponding to the sperm head,[25–27] tail,[26,28] and membranes[29,30] has resulted in the identification, with high confidence, of 6238 different proteins in the entire spermatozoa (Figure 18.3a).[10,18] Differential proteomics studies involving sperm cells from different subtypes of infertile patients

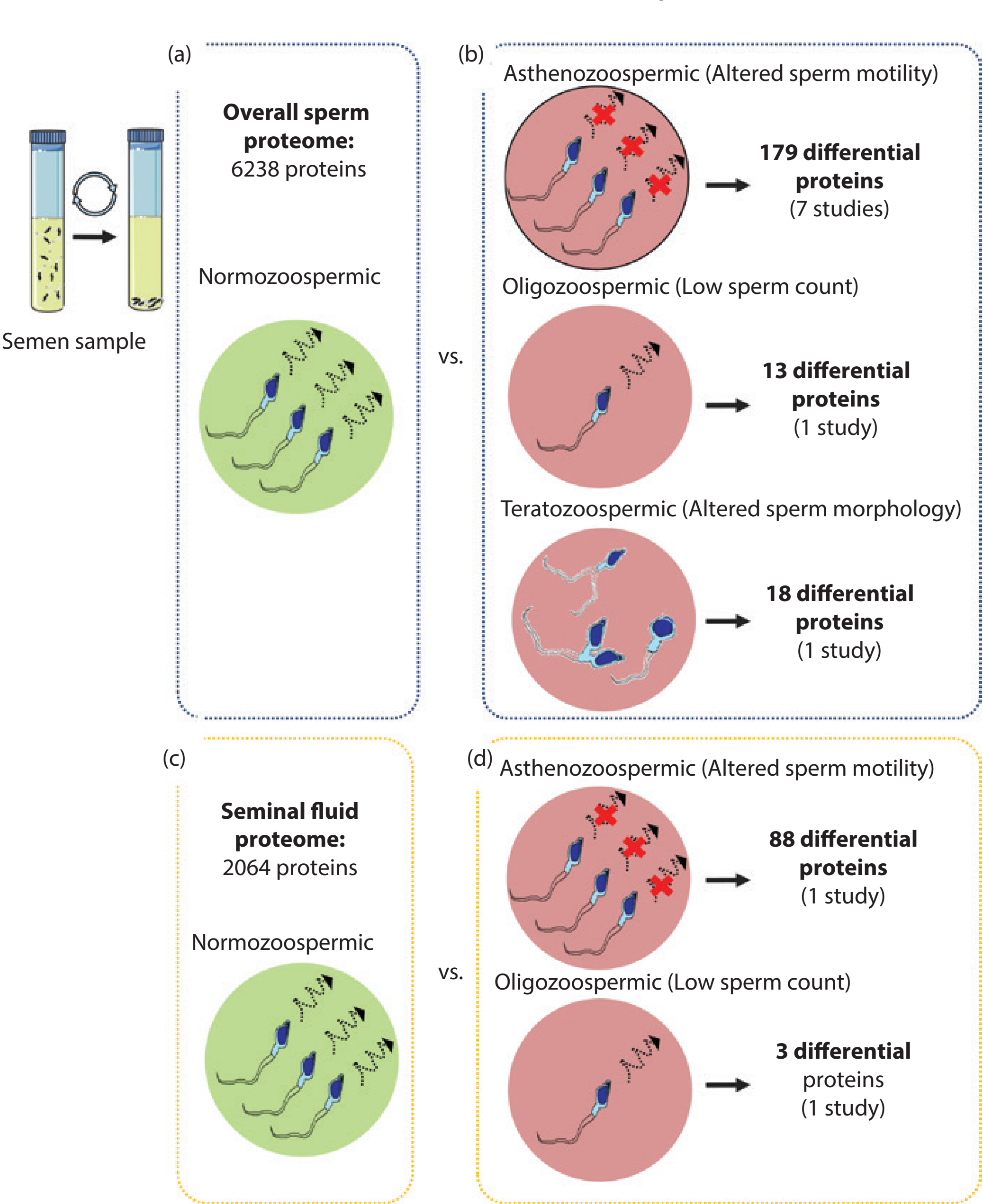

FIGURE 18.3 Descriptive and comparative semen proteomic studies according to seminal parameters. (a) A total of 6238 proteins have been identified in the sperm proteome of human normozoospermic spermatozoa. (b) Comparative sperm proteome studies between normozoospermic and different subtypes of male infertility according to their seminal parameters have revealed 179, 13, and 18 differential proteins for asthenozoospermic, oligozoospermic, and teratozoospermic patients, respectively. (c) A total of 2064 proteins have been identified in the seminal fluid proteome of human normozoospermic individuals. (d) Comparative seminal fluid proteome studies between normozoospermic and different subtypes of male infertility according to their seminal parameters have revealed 88 and 3 differential proteins for asthenozoospermic and oligozoospermic patients, respectively.

according to the seminal parameters has revealed functionally important proteins that could help to understand the various pathogenic mechanisms implicated (Figure 18.3b).[20,24,31–37] The most commonly studied sperm phenotype has been asthenozoospermia (altered sperm motility) comprising seven different studies and detecting a total of 179 differentially expressed proteins.[20,24,31–33,35,37] Most pathological mechanisms probably affecting sperm motility are based on the differential proteins detected and disturbances in the generation of energy required for sperm motility (those mainly involved in the citric acid cycle) and in the regulation of apoptosis. Spermatozoa only account for 5% of the ejaculate, whereas the remaining 95% corresponds to secretions from different accessory sex glands. A total of 2064 proteins have been identified in seminal fluid revealing that, contrary to being a simple medium to carry the spermatozoa through the female reproductive tract, the seminal fluid seems to be crucial for the regulation of semen coagulation and liquefaction, sperm motility, and fertilization[10,38] (Figure 18.3c). Comparative analysis of seminal fluid proteome from different infertile patients enables the assessment of the accessory sex glands function[39,40] and also their impact on sperm physiology.[41,42] A single study assessing differential proteins in the seminal fluid proteome from asthenozoospermic patients suggests the disturbance of processes associated with the energy production by glycolysis.[41] The glycolysis process seems to be related to the microvesicles contained in seminal fluid released mainly by prostate[43] with a known ability to produce extracellular adenosine triphosphate (ATP) from carbohydrates[44] and probably acting as an auxiliary tool to provide energy for sperm motility.[45] Proteomic approaches have emerged as an invaluable tool to understand the sperm physiology and pathogenic mechanisms associated with male infertility, and also comparing the abundance of thousands of proteins simultaneously in different subtypes of infertile patients might facilitate the identification of fertility biomarkers useful for the clinics or for the design of new fertility therapies or male contraceptive targeting.

Potential Clinical Use

Comparative semen proteomic studies from various functional states have produced a large number of candidate fertility biomarkers. A reliable biomarker should be accessible using noninvasive protocols, inexpensive to quantify, with a detection method that is sensitive and specific as well as highly reproducible among clinical laboratories. Although the extreme value of high-throughput proteomics as a biomarker discovery tool has been proven, some limitations hamper its routine use in the clinics.[46] First of all, sperm proteomics biomarker discovery experiments have shown so far a relatively low concordance between different laboratories. This is exemplified by the detection of only 17 out of the 179 differentially expressed proteins in at least two of the seven comparative studies in asthenozoospermia (Figure 18.3b). Interestingly, heat shock-related 70-kDa protein 2 (HSPA2) was found differentially expressed in four of the seven studies assessing protein changes in asthenozoospermia, suggesting that HSPA2 might be a good biomarker for altered sperm motility. The causes of the lack of detection of the same proteins between studies may be due to the following reasons: differences in sample collection, handling, and storage; different proteomic technologies applied; proportion of the proteome targeted; and the biological intra- and interindividual variance. Thus, because of the wide variety of conditions and approaches, the results obtained so far must be interpreted as being complementary rather than indicating genuine lack of concordance or reproducibility. Another limitation of the use of proteomics in the clinics is its prohibitive associated cost, including the requirement of skilled professionals and very expensive equipment. Thus, despite the latest advances in mass spectrometry technology, semen proteomics has only been used as a research and biomarker discovery tool so far. However, once the clinical value for fertility/infertility for some of the candidate biomarkers detected by proteomics is validated, it will be possible to develop cheaper and more feasible tests such as those based on protein microarrays, mass spectrometry selective reaction monitoring (SRM), or ELISA multiplexed to routinely test these specific biomarkers in the reproductive clinics. The results derived from the study of the semen proteome might provide an enhancement in some reproductive clinical applications as it is presented below.

Improvement in the Reproductive Counseling

Fertility treatment options can range from basic advice from the doctor to the most sophisticated therapies such as in vitro fertilization (IVF). The less invasive treatments include time intercourse (TIC), which simply identifies the days of the menstrual cycle when the woman could become pregnant, and IUI, which is based on the injection of sperm inside a woman's uterus, thus increasing the number of sperm that reach and could fertilize the oocyte. In comparison, IVF combines a sperm and an oocyte outside of the body in a laboratory dish with or without the help of ICSI, and only one or two fertilized eggs that start to develop to embryo are transferred into the woman's uterus. Reproductive counseling of infertile couples initiates with an extensive physical and molecular evaluation of the female and a basic physical analysis and seminal parameters evaluation in males.[47] Each couple receives advice about the appropriate fertility treatment based on evidence-based information about the success rate of different treatment options depending largely on the cause of infertility and the associated costs. Furthermore, the good practice in the reproductive clinic should also ensure that patients are not exposed to unnecessary invasive technologies or ineffective treatments. For example, when a known severe male or female infertility factor is identified (e.g., an ovulatory or tubular disorder in females or the diagnosis of azoospermia or severe oligoasthenozoospermia in males), the patients are advised to consider in vitro ART as the first treatment option. In contrast, reproductive treatments with minimal intervention are the first treatments that should be suggested for infertile couples with unexplained infertility or with mild to moderate female or male factor,[48] therefore reducing the clinical exposure of the women to intense treatments such as ovary hyperstimulation and egg collection. Although in vitro ART has a high success rate for couples with severely compromised semen parameters (around 40%), the success of TIC or IUI in infertile patients without severe alteration of seminal parameters is unpredictable. Only a single study attempted to identify potential protein biomarkers able to predict pregnancy outcome

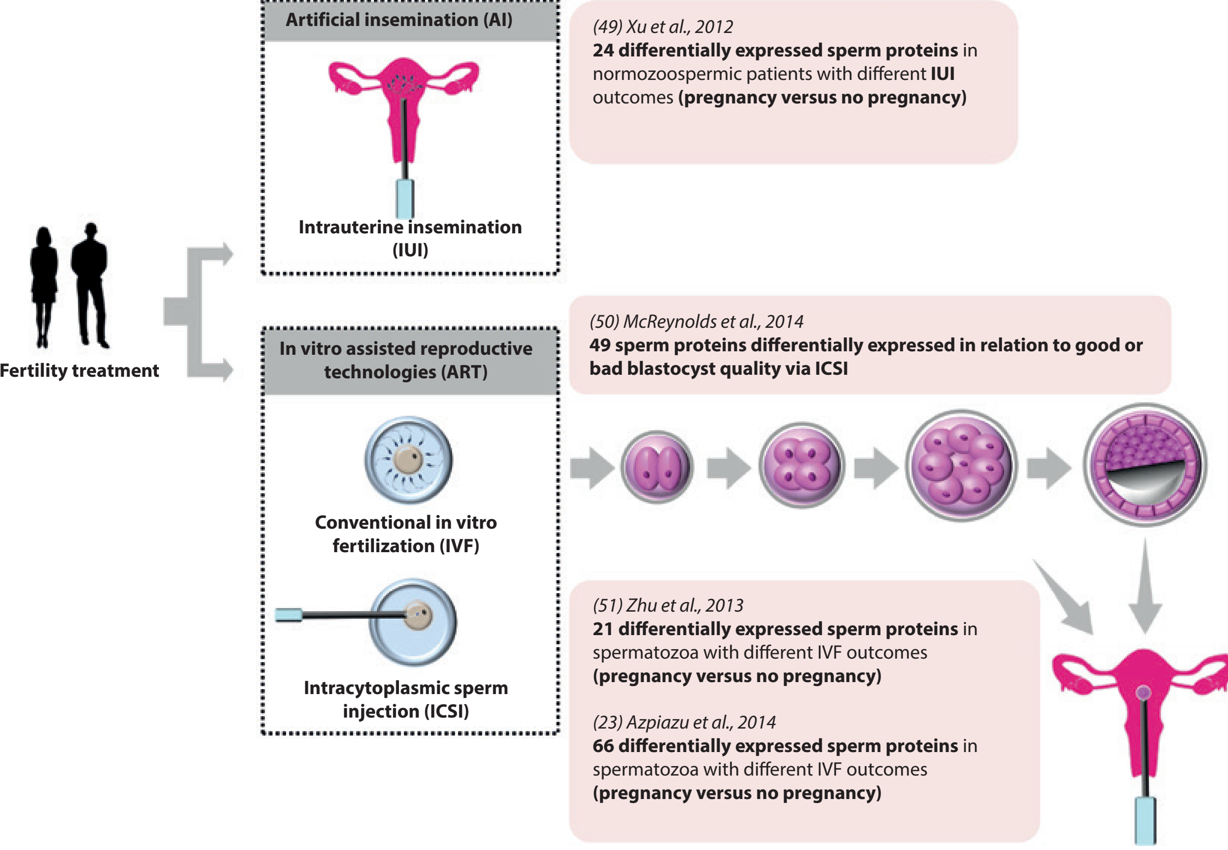

FIGURE 18.4 Comparative sperm proteomics related to assisted reproductive technology (ART). Comparative proteomic studies related to different ART outcomes (intrauterine insemination [IUI], in vitro fertilization [IVF], intracytoplasmic sperm injection [ICSI]) have detected several differentially expressed sperm proteins potentially able to predict success (pregnancy or blastocyst quality) from different fertility treatments.

by IUI (Figure 18.4).[49] A total of 24 differentially expressed proteins involved in sperm capacitation, acrosomal reaction, and sperm oocyte communication were identified in patients with normal semen parameters but unable to achieve a live birth by IUI.[49] Additionally, to enhance the outcome by in vitro ART, three different studies assessed the proteomic changes in sperm in relation to blastocyst quality development[50] and final pregnancy outcome[23,51] after in vitro ART. A total of 136 differential proteins were detected by the three different studies but only seven proteins were commonly affected in at least two of the three studies (Semenogelin-1, Semenogelin-2, Clusterin, Peroxiredoxin-5 mitochondrial, 5-oxoprolinase, Cysteine-rich secretory protein 2, and uncharacterized protein C17orf74). The low reproducibility between the few studies assessing the proteomic changes associated to the in vitro ART success indicates that we are in the infancy of the proteomics era in reproductive clinics. Infertility is a complex disease with underlying multiple causes. Therefore, an unmet need is the development of a panel of molecular biomarkers able to discern the male factor infertility and, thereby, be predictive of the different fertility treatment success reducing emotional and economical burn of the couples facing reproductive care.

Improvement in the Counseling of Azoospermic Patients Facing Invasive Procedures

Azoospermia, defined as the complete lack of spermatozoa in the ejaculate, is a severe disorder affecting nearly 5%–20% of infertile men.[22,52,53] Azoospermia is mainly classified as obstructive azoospermia (OA) or as nonobstructive azoospermia (NOA).[54] The majority of the patients with OA exhibit normal spermatogenesis and spermatozoa could be recovered for in vitro ART purposes by testicular sperm extraction (TESE).[55] In contrast, this invasive procedure (TESE) is not recommended for patients with NOA presenting a complete lack of spermatogenesis as, for example, for patients diagnosed as Sertoli cell-only syndrome (SCOS).[22] However, if NOA patients presented hypospermatogenesis or maturation arrest, there is the possibility to retrieve live spermatozoa from testis biopsy fragments, although with a low efficiency.

Currently, the main diagnostic method to discern OA from NOA and its different subtypes (hypospermatogenesis, maturation arrest, and SCOS) is the testicular biopsy.[56] There is a particular interest to explore whether some specific protein biomarkers in semen could be predictive for the presence of sperm in testis. The identification of such potential spermatogenic predictive biomarkers could let to the development of tests to avoid that patients without possibilities to recover spermatozoa (e.g., patients diagnosed as SCOS) underwent invasive and painful procedures such as testicular biopsy. Additionally, the diagnosis of NOA by testicular biopsy is not very accurate because it does not reflect the histology of the whole testis. Even if the general spermatogenesis within seminiferous tubules is not progressing in NOA patients, occasionally in some tubules sperm cells could be detected.

Proteomics has revealed several differential expressed proteins in the seminal plasma of men with different subtypes of azoospermia compared with individuals with normal spermatogenesis.[22,54,55,57–59] Recently, using MS/MS followed with a selected reaction monitoring (SRM), two protein biomarker candidates (epididymis-expressed protein ECM1 and the testis-expressed protein TEX101) were proposed for differential diagnosis of azoospermia (Figure 18.5).[60] The authors suggest that these two proteins are capable of differentiating OA from NOA as well as the different NOA subtypes. The high level of ECM1 expression in epididymis enables discerning patients with OA showing lower ECM1 expression than individuals without obstructive disorders including individuals with normal spermatogenesis or NOA patients.[60] Additionally, if the germ-specific protein TEX101 is almost absent in the seminal plasma, this is suggestive of absent spermatogenesis (SCOS) or vas deferens obstruction (OA and postvasectomy patients). In contrast, TEX101 is detected in higher levels in patients presenting maturation arrest or hypospermatogenesis. The potential future use of these two biomarkers (ECM1 and TEX101) in the reproductive clinics has the potentiality to avoid testicular biopsy for TESE retrieval in cases of pure SCOS as well as to improve the NOA differential diagnosis, thus reducing the cost of azoospermia counseling. Other authors have proposed other proteins such as clusterin (CLU), prolactin-inducible protein (PIP), galectin-3-binding protein (LGALS3BP), L-lactate dehydrogenase C chain (LDHC), phosphoglycerate kinase 2 (PGK2), and transketolase-like protein 1 (TKTL1) as complementary spermatogenic biomarkers.[54,59]

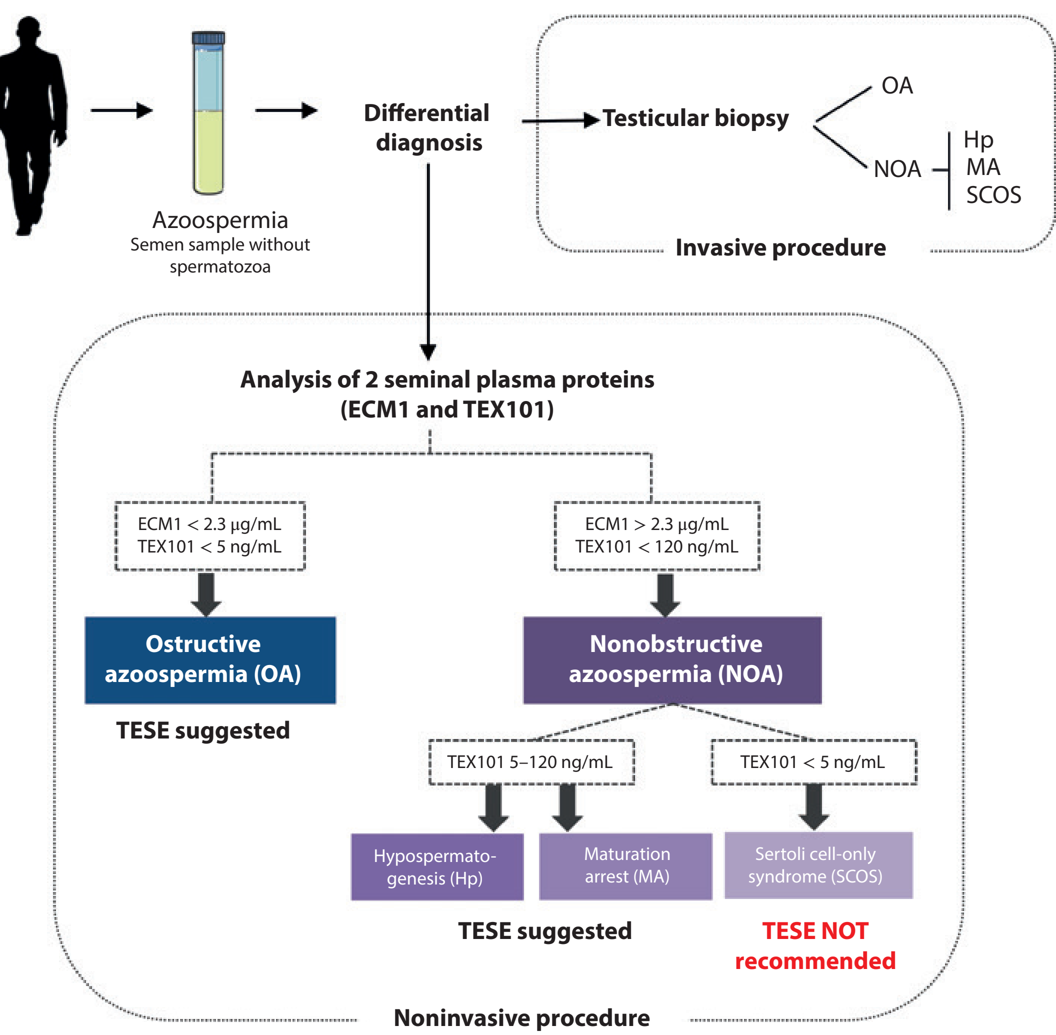

FIGURE 18.5 Semen protein biomarkers in the differential diagnosis of azoospermia. Testicular biopsy is the current main diagnostic methodology to diagnose obstructive azoospermia (OA) versus nonobstructive azoospermia (NOA) and its subtypes: hypospermatogenesis (Hp), maturation arrest (MA), and Sertoli cell-only syndrome (SCOS). Also, molecular tests such as karyotyping, Y-chromosome microdeletion studies or CFTR testing, and physical examination and ultrasounds are useful in some but not all azoospermic cases to assist in the differential diagnosis of azoospermia. A new diagnostic tool using two semen proteome biomarkers (epididymis-expressed protein ECM1 and the testis-expressed protein TEX101) is able to discern the different subtypes of azoospermia. Seminal plasma levels of ECM1 protein <2.3 µg/mL suggest an epididymal or vas deferens blockage, whereas values >2.3 µg/mL are suggestive of NOA. Low seminal plasma levels of germ-cell protein TEX101 <5 ng/mL indicate azoospermia due to lack of spermatogenesis for the presence of SCOS or OA. On the other hand, NOA men with affected spermatogenesis (Hp and MA) have TEX101 seminal levels between 5 and 120 ng/mL. NOA patients presenting >5 ng/mL of TEX101 protein in seminal plasma suggest the presence of spermatogenesis, and sperm retrieval by testicular sperm extraction (TESE) is allowed. In cases of SCOS (<5 ng/mL), TESE should be avoided.[60]

Improvement of Clinical Processes

Cryopreservation of human sperm is widely applied in the field of reproductive biology and medicine. Sperm cryopreservation has enabled preservation of male fertility particularly for those men who will undergo potentially sterilizing anticancer treatments. Furthermore, cryopreservation allows the creation of sperm donor banks including the storage of sperm recovered by TESE for future uses, thus avoiding repetitive biopsies in azoospermic patients. An initial comparative proteomic study of fresh and cryopreservated sperm detected a total of 27 differentially expressed sperm proteins.[61] Protein degradation and alteration of posttranslational modifications such as phosphorylation were proposed as the potential cryoinjury mechanisms involved. Proteomic studies may help design new cryogenic strategies to improve the sperm protection against freezing. In a more recent study, the application of TMT

technology coupled to LC-MS/MS led to the detection of substantial changes in the sperm proteome at every stage of the cryopreservation process, including the effect of the cryoprotectant itself, which may ultimately impair the sperm fertilizing capability.[72]

Design of Potential Fertility Therapies

Proteomics might help identify the key proteins for male fertility. New fertility enhancers might be developed based on these key proteins. Toward this objective it has been described that around 20%–40% of infertile males present high levels of oxidative stress[62] and antioxidant intake therapy is showing beneficial effects on these infertile males. However, several types of antioxidant therapies exist and the optimal type and dose have not been established yet.[63] Proteomic studies comparing infertile males presenting different levels of reactive oxygen species (ROS) with fertile males have resulted in the identification of several proteins involved in the oxidative stress.[64–66] These differential proteins might help standardize the antioxidant therapies as well as predict those patients who are going to positively respond to the therapy.

Design of Potential Anticonceptive Strategies

Proteomics may also help identify new targets for male contraception. Hormonal methods such as the administration of exogenous testosterone have shown the partial or total suppression of spermatogenesis resulting in oligozoospermia or azoospermia, respectively. Although hormonal anticonceptive methods are reversible they have several side effects. Proteomic analysis of human testicular biopsies in men before and after exogenous testosterone treatment resulted in the detection of 13 differential expressed proteins. Those differential proteins, probably crucial for a normal spermatogenesis, might be good candidates for new potential reversible male contraception methodology, although their clinical use should be elucidated.[67] Using a different approach proteomics has also been applied to the study of sperm immunogenic antigens, both with a view to understand immunologic infertility and also to identify potential immunocontraceptive candidates.[68–70]

Test Availability

A recent patent application on the use of identified germ cell-specific proteins in an antibody-based assay (Fertichip™) to predict the successful testicular biopsy outcomes in human nonobstructive azoospermia is being developed based on a combination of different proteins, although the test is not yet available in the market.[59] The SpermCHECK® Male Fertility Test is an example of an application based on the detection of a single protein. It works by detecting the concentrations of the acrosomal protein SP-10 (ACRV1), known to be present in the sperm head cell membrane, to determine sperm count number or presence.[71] This is an at-home sperm test and is already available on the market (http://www.spermcheck.com/) through different retailers in the United States, Canada, UK, Hong Kong, Macau, and France. As the field of sperm cell proteomics further advances it can be expected that many applications will become available based on the detection of single proteins or on the combination of proteins.

Acknowledgments

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LLISTAT D'ABREVIATURES I ANGLICISMES

1D-LC	Cromatografia líquida monodimensional/*One-dimensional líquid chromatography*
1D-PAGE	Electroforesi en gels de poliacrilamida en una dimensió/*One-dimensional Polyacrylamide Gel Electrophoresis*
25-30%UC	Ultracentrifugació en coixins de sacarosa al 25-30%/*Ultracentrifugation in a 25-30% sucrose gradient*
2D-LC	Cromatografia líquida bidimensional/*Two-dimensional liquid chromatography*
2D-PAGE	Electroforesi en gels de poliacrilamida en dues dimensions/*Two-dimensional Polyacrylamide Gel Electrophoresis*
ABP	Proteïna transportadora d’andrògens
acH4	Acetilació de la histona H4
ACR	*Acrosin*
ADAM7	*Disintegrin and metalloproteinase domain-containing protein 7*
ALDOA	*Fructose-bisphosphate aldolase A*
ALDOC	*Fructose-bisphosphate aldolase C*
ANOVA	*One-way analysis of variance*
ANPEP	*Aminopeptidase N*
ARSA	*Arylsulfatase A*
AS	Astenozoospèrmics
AZ	Azoospèrmics
BC	Cèl·lules basals/*Basal cells*
BCA	*Bicinchoninic acid*
BPH	*Benign prostatic hyperplasia*
CASA	*Computer-Assisted Sperm Analysis*
CC	Cèl·lules clares/*Clear cells*
CCT2	*T-complex protein 1 subunit beta*
CCT3	*T-complex protein 1 subunit gamma*
CCT4	*T-complex protein 1 subunit delta*
CCT7	*T-complex protein 1 subunit eta*
CCT8	*T-complex protein 1 subunit theta*
CD	*Cytoplasmic droplet*
CD117	*CD177 antigen*
CD9	*CD9 antigen*
CEACAM1	*Carcinoembryonic antigen-related cell adhesion molecule 1*
CID	Dissociació induïda per col·lisió/*Collision-induced dissociation*
CLGN	*Calmegin*
CRISP1	*Cysteine-rich secretory protein 1*
cTid	Espermàtides condensants
CTL	Control
DDA	*Data-dependent acquisition*

DEFA1 *Neutrophil defensin 1*
DHT *Dihydrotestosterone*
DPP4 *Dipeptidyl peptidase 4*
DTT *Dithiothreitol*
E2 *Estradiol*
ECD Dissociació per captura d'electrons/*Electron-capture dissociation*
ECM1 *Extracellular matrix protein 1*
EDDM3B *Epididymal secretory protein E3-beta*
ELSPBP1 *Epididymal sperm-binding protein 1*
ENO1 *Alpha-enolase*
ENO2 *Gamma-enolase*
ESE Endosomes primerencs/*Early sorting endosome*
ESI Ionització per electrospray/*Electrospray Ionisation*
ETD Dissociació per transferència d'electrons/*Electron-transfer dissociation*
eTid Espermàtides elongades
EVs *Extracellular vesicles*
FA *Formic acid*
FDR *False discovery rate*
FSH Hormona fol·liculoestimulant/*Follicle-stimulating hormone*
FSH-R Receptors FSH
GAPDH *Glyceraldehyde-3-phosphate dehydrogenase*
GDF15 *Growth/differentiation factor 15*
GnRH Factor alliberador de gonadotropines
GO *Gene Ontology*
GPI *Glucose-6-phosphate isomerase*
HCD Dissociació per col·lisió d'alta energia/*Higher energy collision dissociation*
HH Hipogonadisme hipogonadotròpic/*Hypogonadotropic hypogonadism*
HPA *Human Protein Atlas*
HPLC Cromatografia líquida d'alta resolució/*High performance liquid chromatography*
HYPO *Hypogonadic patients*
IAA *Iodoacetamide*
IEF Enfocament isoelèctric/*Isoelectric focusing*
ILV Vesícules intraluminals/*Intraluminal vesicles*
KRT19 *Keratin type I cytoskeletal 19*
LC-MS/MS Cromatografia líquida amb espectrometria de masses en tàndem/*Liquid Chromatography coupled with Tandem Mass Spectrometry*
LH Hormona luteïnitzant/*Luteinizing hormone*
LSE Endosoma tardà/*Late-sorting endosome*
m/z Massa-càrrega
MALDI-TOF *Matrix Assisted Laser Desorption Ionization - Time Of Flight*

MB	*Dynabeads® magnetic beads*
MBCD63	Ultracentrifugació en coixins de sacarosa al 25-30% seguit d'una immunoselecció mitjançant l'ús de boles magnètiques unides a l'anticòs específic de VEs CD63/*Ultracentrifugation in a 25-30% sucrose gradient followed by the use of magnetic beads coated with the CD63 antibody*
Micro-TESE	Microdissecció testicular per a l'obtenció d'espermatozoides/ *Microdissection Testicular Sperm Extraction*
MS	Espectrometria de masses/*Mass spectrometry*
MS/MS	Espectrometria de masses en tàndem/*Tandem mass spectrometry*
MSMB	*Beta-microseminoprotein*
MVB	Cossos multivesiculars/*Multivesicular bodies*
MVs	Multivesícules
NC	Cèl·lules estretes/*Narrow cells*
NH	Domini nucleo-histona
NIH	*National Institutes of Health*
NOA	Azoospèrmia no obstructiva
NP	Domini nucleo-protamina
NPC2	*NPC intracellular cholesterol transporter 2*
NTA	Anàlisi de seguiment de nanopartícules/*Nanoparticle Tracking Analysis*
NZ	Normozoospèrmics
OA	Azoospèrmia obstructiva
OMS	Organització Mundial de la Salut
OZ	Oligozoospèrmics
P1	Protamina 1
P2	Protamina 2
PAS	*Periodic acid and schiff's reagent*
PBS	*Phosphate-buffered saline*
PC	Cèl·lules principals/*Principal cells*
PCI	*Protein C inhibitor*
PEPC	*Gastricsin*
PFA	*Paraformaldehyde*
PFKL	*ATP-dependent 6-phosphofructokinase, liver type*
PFKM	*ATP-dependent 6-phosphofructokinase, muscle type*
PFKP	*ATP-dependent 6-phosphofructokinase, platelet type*
PGK1	*Phosphoglycerate kinase 1*
PIP	*Prolactin-inducible protein*
PKM	*Pyruvate kinase PKM*
PMF	Empremta peptídica/*Peptide mass fingerprint*
PPAP	Fosfatasa àcida prostàtica/*Prostatic acid phosphatase*
PPE	Parells de proteïnes estables
pre-P2	Precursor de la protamina 2

PRM1 Gen que codifica per la protamina 1
PRM2 Gen que codifica per la protamina 2
PSA/KLK3 Antigen prostàtic específic/*Prostate-specific antigen*
PSMs *Peptide spectrum matches*
PTMs *Post-translational modifications*
RAB3B *Ras-related protein Rab-3B*
RIA *Radioimmunoassay*
RT *Room temperature*
rTid Espermàtides rodones
SC Cèl·lules de Sertoli
SEMG1 *Semenogelin-1*
SEMG2 *Semenogelin-2*
SEMGs *Semenogelins*
Serpina 5/IPSP *Plasma serine protease inhibitor*
SHBG Globulina fixadora d'hormones sexuals/*Sex hormone-binding globulin*
SLC27A2/FATP2 *Very long-chain acyl-CoA synthetase*
SNAREs Receptors de proteïnes solubles d'unió al factor sensible a N-etilmaleimida/*Soluble N-ethylmaleimide-sensitive factor attachment protein receptors*
Spc Espermatòcits
Spg Espermatogònia
T *Testosterone*
TBST *TBS with 0.1% (v/v) Tween 20*
TCEP *Tris (2-carboxyethyl) phosphine*
TCP1 *T-complex protein 1 subunit alpha*
TEAB *Triethyl ammonium bicarbonate*
TESA Aspiració d'espermatozoides testiculars/*Testicular Sperm Aspiration*
TESE Extracció testicular d'espermatozoides/*Testicular Sperm Extraction*
TFA *Trifluoroacetic acid*
TMT *Tandem mass tags*
tPA *Tissue plasminogen activator*
TPI1 *Triosephosphate isomerase*
TRFL *Lactotransferrin*
TRiC *8-membered chaperonin-containing T-complex*
TRT Teràpia de reemplaçament de testosterona/*Testosterone replacement treatment*
UC Ultracentrifugació/*Ultracentrifugation*
uPA *Urokinase*
VEs Vesícules extracel·lulars
WFDC8 *WAP four-disulfide core domain protein 8*
WHO *World Health Organization*