**Name of Journal: *World Journal of Translational Medicine***

**ESPS Manuscript NO: 23595**

**Manuscript Type: Review**

**New insights in sperm biology: How benchside results in the search for molecular markers may help understand male infertility**

Marchiani *et al*. Molecular markers of male infertility

**Sara Marchiani, Lara Tamburrino, Monica Muratori, Elisabetta Baldi**

**Sara Marchiani**, **Lara Tamburrino**, **Monica Muratori**, **Elisabetta Baldi**, Department of Experimental and Clinical Biomedical Sciences, University of Florence, 50139 Florence, Italy

**Author contributions:** Marchiani S wrote the manuscript; Tamburrino L participated in drafting the article; Muratori M and Baldi E critically revised the manuscript; Marchiani S and Baldi E provided final approval of the article.

**Conflict-of-interest statement:** Authors declare no conflict of interests for this article.

**Open-Access:** This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/4.0/

**Correspondence to: Sara Marchiani, PhD,** Department of Experimental and Clinical Biomedical Sciences, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy.sara.marchiani@unifi.it

**Telephone:** +39-055-2758235

**Received:** December 4, 2015

**Peer-review started:** December 5, 2015

**First decision:** December 28, 2015

**Revised:** January 22, 2016

**Accepted:** February 16, 2016

**Article in press:**

**Published online:**

**Abstract**

Themale factor is responsible for about40% of couple infertility cases and such percentage is expected to increase in the future because of several likely factors including the presence of endocrine disruptors in the environment, changes in lifestyle habits and advanced couple aging. How such factors affect male fertility status, however, should be clarified. Most studies on male fertility status have focused on parameters analyzed using a spermiogram test, the primary diagnostic tool in the routine assessment of male infertility, which is, however, poorly predictive of both natural and medically assisted conception. For these reasons it is mandatory for the scientific community to identify new molecular markers to incorporate into the existing diagnostic tests of male fertility. Ideally, such markers would be detected in mature spermatozoa to avoid invasive procedures for the patient. This review summarizes the recent advancements in benchside approaches that appear most promising for the development of new diagnostic sperm fertility tests, or identification of therapeutic targets, and, illustrates their advantages and limits.

**Key words:** Sperm markers; Male infertility; Genetic and epigenetic approaches; Proteomic approach; Ion channels

© **The Author(s) 2016**. Published by Baishideng Publishing Group Inc. All rights reserved.

**Core tip:** This review focuses on genetic, epigenetic, proteomic, and post-translational protein modification and ion channel studies present thus far in the literature to identify possible sperm markers that could be helpful for new diagnostic tests or represent possible therapeutic targets for male infertility.

Marchiani S, Tamburrino L, Muratori M, Baldi E. New insights in sperm biology: How benchside results in the search for molecular markers may help understand male infertility. *World J Transl Med* 2016; In press

**INTRODUCTION**

Infertility is a worldwide health problem affecting about 15% of couples[1]. Although the World Health Organization (WHO, 5th edition[2]) defines it as a disease of the reproductive system, infertility also influences emotional, social and psychological spheres. The male factor is involved in about 40% of couple infertility cases, with the highest incidence rates in Eastern Europe and Africa[1]. Male infertility, affecting presently 7% of the worldwide population, is expected to double over the coming years. Some possible explanations reside in the rise in hectic lifestyles, in the increase of pollution and in socio-economic changes that delay couples in starting a new family. Currently, how all these factors affect male fertility status is not clear.

The increase in reproductive age is becoming an important social problem, which can be particularly noted in industrialized countries. The role of advanced maternal age in the lower success of natural and medically assisted reproduction has been well established[3]. A recent trend among young women is to freeze their oocytes for social reasons, such as desire to have a career, delaying the age of the first conception. Not surprisingly, some multinational American corporations offer to pay for an oocyte preservation procedure for their female employees to allow for career advancement. In contrast with the maternal age, whether paternal age affects fertility is still highly debated. Despite some authors not finding correlations between paternal age and infertility[4,5], others have shown that a forward shift in male age represents a further risk factor for the failure to conceive[6,7], for the success of assisted reproductive techniques (ART) and for the health of offspring[8]. Advanced age may lead to changes in hormonal profile[9] and germinal epithelium disorders with the consequent alterations in seminal parameters[10,11]. Decreased sperm quality may be due to alterations in the expression of some proteins[11,12],as well as an increase in sperm DNA fragmentation (sDF)[13,14] or of other types of DNA damage[11]. In addition, it has been demonstrated that the higher number of de novo mutations found in offspring of increasingly older fathers can mostly be attributed to paternal transmission[15-17].

As mentioned above, besides male aging, there are several other factors contributing to the decrease in male fertility potential with similar pathogenic mechanisms, such as the ever increasing presence of endocrine disrupting chemicals in the environment[18] and the changes in lifestyle with an increased prevalence of obesity and metabolic syndrome[19].

Pharmacological treatment of the male partner can only be successfully applied to non-idiopathic causes (such as hypogonadropic hypogonadism), whereas for idiopathic infertility, despite many attempts, virtually no effective treatment is currently available[1]. A recent meta-analysis has concluded that gonadotropin therapy is a possible choice to improve fertility, especially in case of post-pubertal onset hypogonadotropic hypogonadism[20]. Efforts to treat idiopathic male infertility, for instance using gonadotropins, or anti-aromatase, anti-estrogen and anti-oxidant drugs, have not demonstrated a conclusive, beneficial effect of said therapies[21]. Until robust results are obtained, ARTs remain, for idiopathic male infertility, the option with the highest chance of achieving pregnancy.

Although ARTs have expanded globally over the last few decades, these procedures remain inaccessible in many parts of the world and are quite expensive. Moreover, despite ARTs’ success rate having approved greatly over the past few years, the current live birth outcome remains low, averaging just 34%[22], with important economic and psychological consequences for couples. For these reasons it is mandatory, for the scientific community, to identify the causes of infertility in order to find effective treatments and new sperm markers to improve the accuracy of diagnosis.

The primary diagnostic tool in the routine assessment of male infertility is semen analysis (spermiogram), which consists in the evaluation of the macroscopic (volume, pH, liquefaction) and microscopic (number, motility and morphology) characteristics of seminal fluid. Despite the fact that WHO issued detailed laboratory guidelines to standardize the methods and has established normal reference values[2], spermiogram has a high operator variability, high intra-individual variation[23] and is not highly predictive of the fertility status[24,25]. The diagnosis of infertility results as being accurate only in the case of azoospermia and severe oligozoospermia. Semen analysis does not provide information about the molecular status of spermatozoon and the functions necessary for oocyte fertilization. For this reason, identification of new semen or sperm molecular markers able to discriminate between fertile and infertile men is one of the main goals of current research. Markers that single out spermatozoa with a higher fertilizing ability could lead also, in the future, to a better sperm selection for ARTs. Indeed, although new advanced tools for sperm selection have been developed based on sperm surface charge, apoptotic or maturity sperm markers and sperm ultramorphology, more studies are needed before introducing advanced sperm selection methods in ART[26]. Based on current published data, sperm selection using real-time motile sperm organelle morphology examination at high magnification coupled with intracytoplasmic morphologically selected sperm injection seems to be a promising method with benefits for late ART outcomes (pregnancy, live birth and abortion rates)[27].

This review will focus on the recent advancements of benchside approaches that appear most promising for the identification of new sperm/germ cells as molecular markers of infertility.

**GENETIC AND EPIGENETIC STUDIES ON TESTICULAR GERM CELLS AND MATURE SPERMATOZOA**

At least 15% of male infertility cases are due to genetic alterations[28], including Y chromosome microdeletions, present in about 20% of cases of azoospermia or severe oligozoospermia[29]. Innovative approaches implying whole-genome analysis, such as the evaluation of single nucleotide polymorphisms and copy number variations, could be helpful in the search for new gene candidates having a role in male infertility[30-32]. For instance, a recent study by Yatsenko *et al*[33] identified hemizygous mutations in the TEX11 gene as one of the causes of meiotic arrest and azoospermia in infertile men. A microarray study found a different expression of genes linked to spermatogenesis in testis RNA from non-obstructive azoospermic (NOA) men when compared to commercial RNA from normal testicular tissue[34,35]. We expect that other genes responsible for azoospermic/severe oligozoospermic phenotypes will be discovered in the future.

Whereas genetic studies are of great help in identifying the genes involved in testicular disorders that lead to severe alterations in sperm number, the search for genetic modifications leading to sperm dysfunctions in idiopathic infertility appears to be a sort of “fishing expedition”. Conversely, the use of genetic, epigenetic and proteomic approaches on ejaculated spermatozoa could allow researchers to characterize the complete spectrum of sperm phenotypes present in infertile subjects better and, accordingly, to understand the leading causes of infertility in depth.

Epigenetic alterations derived from environmental pollution, toxicants and nutritional habits could impair both sperm quality and embryo development[36,37], increasing the risk in offspring of developing chronic diseases, such as type 2 diabetes, obesity, cardiovascular disease and cancer[38,39]. Evidence in animal models suggests that some epigenetic markers can be inherited by the offspring through parents’ gametes[39]. Rodent studies have demonstrated that paternal diet affects pregnancy achievement and offspring metabolism[40,41]. In two recent studies evaluating genome wide sperm DNA methylation, such an epigenetic pattern was found to differ significantly between *in-vitro* fertilization (IVF) patients and normozoospermic fertile men [42] and between men achieving pregnancy within two months and men who did not obtain pregnancy within twelve months, despite similar semen quality[43]. These studies identified candidate methylation loci to be explored in future studies in order to consolidate the results. Epigenetic inheritance related to spermatozoa includes not only DNA methylation but also other epigenetic factors such as histone retention or non-coding RNA (ncRNA). In view of the recent observation that histone retention in specific loci is important for subsequent embryo development[44,45], new sperm diagnostic tests based on histone enrichment in specific genes could be developed in the future. Alterations in ncRNAs may also impair embryo development and transgenerational inheritance. Among ncRNA, the occurrence of miRNA in sperm, seminal fluid and testicular tissue has been reported recently[46]. The fundamental role of miRNA during spermatogenesis is demonstrated by the fact that the knockout of the Dicer enzyme, which is responsible for the cleavage from immature to mature forms of miRNA, leads to infertility[47]. What remains to be determined is whether miRNAs are required also for human spermatogenesis. Recently, an alteration of five miRNAs in subfertile and NOA subjects has been shown[48]. Similarly, employing next generation sequencing, Jodar *et al*[49] found a set of sperm RNA elements required to achieve live births in couples with idiopathic infertility undergoing non-invasive fertility treatments, such as timed intercourse or intrauterine insemination (IUI). However, the absence of such RNA elements does not appear to be critical when ARTs are employed.

Whereas the above described potentially new tools for male infertility diagnosis are still a long way off from use in clinical practice, sDF tests are utilized at present in many ART laboratories in support of traditional semen analysis. Many studies, summarized in the meta-analysis by Zini *et al*[13], have evaluated the effect of high sDF levels on the outcomes of both natural conception and ART. The meta-analysis concluded that pregnancy rate is negatively associated with sDF in natural insemination, IUI and IVF but not in intra-cytoplasmatic sperm injection (ICSI). These results were confirmed in a later meta-analysis[50]. Even more disturbing, the risk of miscarriage resulted as being strongly related to sDF levels in couples undergoing both IVF and ICSI[13]. Also these results were confirmed in recent meta-analyses[51,52]. Interestingly, the review by Robinson *et al*[51], pointed out the importance of the methodology used to evaluate sDF, as a subgroup analysis demonstrated that the association with miscarriage is strongest for studies employing the TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay.

The methodology used in sDF studies represents an important issue. Among the various techniques employed to detect sDF[53], Sperm Chromatin Dispersion assay (SCSA) is the only standardized one and the only one for which there is enough agreement on the reference values across studies. Conversely, for the other methods, such as the widely employed TUNEL or COMET assays, standardization is lacking and established cut-off levels for fertility differ in the various studies. Recently, our group has set up a new refined flow cytometric method, TUNEL/PI (Propidium Iodide), which allows a more accurate measure of sDF[54,55], eliminating all semen confounders[56]. Employing such a method, we have established a cut-off level for fertile subjects and demonstrated that sDF is able to discriminate between fertile men and patients regardless of age and semen quality[57]. sDF analysis in live sperm[58,59] is an advancement of the TUNEL technique allowing clinicians to detect the damage in the sperm population which participates in the fertilization process. Another advancement is the possibility of assessing, in the same COMET slides, sDF and the presence of oxidative damage[60].

Despite the presence, in the literature, of many studies evaluating the impact of sDF on reproduction, a position report from the European Society of Human Reproduction and Embryology[61] and the guidelines for male infertility drafted by the American Society for Reproductive Medicine Practice Committee[62] claim that evaluation of sDF cannot be considered as a diagnostic test until “randomized, well-designed, adequately powered studies comparing infertile couples to a population of men with demonstrated recent fertility, and excluding cases with female infertility” are conducted in great number*.* However, as has recently been, introducing sDF among the diagnostic tests of male infertility could improve IVF success rate[63].

Finding the causes responsible for the generation of sperm DNA breaks could be the basis for the development of new therapeutic strategies to prevent the onset of sDF in infertile men. As oxidative stress is considered the main insult generating DNA damage in spermatozoa[64] and infertile men have lower levels of antioxidants and higher reacting oxygen species (ROS) amount in their semen compared to fertile men[65-67], many studies have investigated the effect of antioxidant administration on sDF. A recent Cochrane review[68] concluded that the current body of evidence does not allow for the deducing of clear conclusions regarding the role of antioxidants in the treatment of idiopathic infertility. Further well-designed randomized controlled trials are necessary in order, on one hand, to demonstrate the real efficacy of antioxidants and, on the other hand, to evaluate any eventual adverse events and their side effects[69]. Interestingly, we have recently demonstrated that sDF is mostly established in the testis as a result of an apoptotic process, whereas oxidative DNA damage occurs mostly during transit in the male genital tracts[70]. Accordingly, testis apoptosis should be the primarily target of therapies aimed to reduce sDF. Among these, treatment with follicle-stimulating hormone appears promising[71-73]. However, the complex role of apoptosis in human health makes it difficult to develop anti-apoptotic treatments for male infertility, whereas antioxidants remain an interesting object of study.

**PROTEOMIC STUDIES ON MATURE SPERMATOZOA**

In recent years, proteomic studies have been conducted in order to define sperm protein profiles and to characterize the role of different proteins in sperm functions. Over the years multiple strategies have been set up to study sperm proteome. In general, the first step is the isolation of spermatozoa from the complex semen matrix, then proteins are separated by various methods (Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis, two dimensional-gel electrophoresis, two dimensional fluorescence difference gel elettrophoresis), analyzed by liquid chromatography-mass spectroscopy and identified by a database. Isolation of spermatozoa from semen matrix is a tricky step, representing a major limitation of these studies, as density gradient centrifugation or swim up (*i.e.*, the collection of a fraction of motile spermatozoa moving from semen to an upper medium) procedures, although they eliminate most immature germ cells and leukocytes, may lead to selection of a sperm population which is not representative of the entire sperm population present in the ejaculate. Another stumbling block in performing proteomic analysis is the poor amount of available sperm material in cases of oligozoospermia, thus leaving out a considerable portion of infertile subjects, as in many cases oligozoospermia is accompanied by other sperm defects, such as low motility and abnormal morphology.

In initial studies, few sperm proteins were detected, but the optimization of proteomic technologies has allowed, in recent years, to characterize more than 6000 proteins[74], even though proteins whose concentration is under the dynamic range of instruments remain undetected.

To investigate the roles of sperm proteins in male infertility, studies comparing proteomic profiles of different sperm samples have been performed. They compared infertile *vs* fertile subjects[75-78], asthenozoospermic *vs* normozoospermic men[79-83], male partners of couples undergoing successful ART *vs* those who failed[84-86], subjects with high sDF *vs* low sDF[87,88], men displaying elevated *vs* low ROS levels[89,90], and patients with metabolic disorders *vs* healthy men[91-93]. Overall, these studies led to the identification of a variable number of proteins, likely implicated in male infertility, that are down- or up-regulated in specific sperm defects. Results are, however, often inconsistent among the various studies, probably because of a high intra- and inter-variability of proteomic sperm profiles[94,95], the frequent use of pooled samples and problems related to sperm isolation (see above).

 A further progression of proteomic studies is the isolation of proteins from specific sperm compartments leading to the association of the identified protein with its cellular localization and thus with its specific function. Using these approaches, several proteins have been assigned to the main compartments, including histone variants, transcription factors and zinc finger proteins in the nucleus[96,97], several receptors (progesterone receptor, metabotropic glutamate receptor, transforming beta growth factor receptor, Neurotensin receptor 3) to the sperm head[98] and proteins related to energetic metabolism, structure, and motility to the tail[82]. In the latter compartment, also proteins involved in lipid metabolism, mitochondrial oxidation and ADP/ATP carriers[99,100] have been found. Further studies are needed to understand if these proteins are differentially expressed or mislocalized in spermatozoa from men with defects in motility or morphology.

**POST-TRANSLATIONAL PROTEIN MODIFICATIONS IN MATURE SPERMATOZOA**

Another point that increases the complexity of proteomic analysis is post-translational protein modifications (PTMs) that carry out an important role in the regulation of functions of mature spermatozoa which, being transcriptionally and translationally silent, mostly rely on PTMs to accomplish important and complex processes necessary for oocyte fertilization, such as capacitation, development of hyperactivated motility and acrosome reaction[101]. For this reason, expression levels *per se* could not have biological relevance for those proteins undergoing PTMs for their functionality. Phosphorylation is a well described PTM in spermatozoa and human phosphoproteomic studies found numerous differently regulated phosphoproteins involved in sperm capacitation[102] and motility[80]. Early studies by Buffone *et al*[103] demonstrated that spermatozoa from asthenozoospermic men showed a reduced protein tyrosine phosphorylation during capacitation *in vitro*, which may be related to a decrease in membrane fluidity leading to the inability to achieve a hyperactivated motility[104]. Among the proteins that are highly phosphorylated in tyrosine during the process of capacitation, A-kinase-anchoring proteins (for review see[105]), structural proteins of the sperm tail, represent an interesting target of these studies, in light of their involvement in motility.

Although ubiquitination is another important PTM, which most likely acts as a sperm quality control system during epididymal transit[106,107] and is related positively to normal sperm morphology[108], most ubiquitin-modified proteins in spermatozoa are still unknown. A similar PTM to ubiquitination is sumoylation, which is associated with poor motility, occurrence of DNA damage and recognition of morphologically defective spermatozoa[109,110]. Recently, Vigodner *et al*[109] identified by mass spectrometry several sumoylated proteins, whose role in sperm functions remains undefined.

Clearly, proteomic studies on in spermatozoa are still in their infancy and need to be further validated in field trials before drafting a complete list of sperm proteins that may differentiate fertile and infertile subjects.

**SPERM ION CHANNELS**

In the attempt to find new male infertility markers, researchers have focused their attention on sperm ion channels having a central role in sperm physiology and in the fertilization process[111]. In particular, proton voltage-gated ion channels (Hv1) induce intracellular pH (pHi) modification involved in the capacitation process[112]. pHi regulation and the role of Hv1 channels has assumed importance with the discovery of two pHi- and voltage-sensitive ion channels, namely Slo3 and Cation channel of sperm (CatSper), that may be connected functionally to the regulation of important sperm activities. Slo3 is a sperm-specific potassium channel involved in mouse sperm capacitation[113], whose role in human sperm functions has yet to be defined. Recent studies have shown that Slo3 channel activity may be regulated also by intracellular calcium increase[114]. Calcium is a well-studied sperm second messenger, whose role in the fertilization process has been widely demonstrated over the last 15 years. Many different types of calcium channels have been described in spermatozoa. Among them, the CatSper calcium channel[115] appears to play a key role in intracellular calcium regulation. CatSper knock-out mice are unable to develop hyperactivated motility, and, for this reason, to reach and fertilize the oocyte[115-117]. Similarly, men with CatSper gene mutations leading to a lack of expression of the protein are infertile[118,119]. CatSper gained further importance when, in 2011, two independent groups of research[120,121] demonstrated that it is activated, in human spermatozoa, by progesterone which is considered the main candidate for stimulating the acrosome reaction process in the fertilizing spermatozoon[122,123]. We have demonstrated recently that sperm CatSper expression is lower in asthenozoospermic men and correlates positively with progressive and hyperactivated motility[124,125]. In addition, we found that CatSper (but none of the parameters evaluated by routine semen analysis) accurately predicts the ability of the sample to hyperactivate[125]. Conversely, the involvement of CatSper in the acrosome reaction process, although expected, is debated in the literature[124,126,127]. CatSper and Slo3 expression and activity may be related to the fertility status of the patient and may be involved in the pathogenesis of asthenozoospermia. However, introduction of CatSper or Slo3 evaluation in the diagnosis of male infertility is presently unlikely. Indeed, the techniques to evaluate their function or expression (patch clamping, flow cytometry and Western blot) are costly and/or need skilled personnel, becoming unsuitable for routine clinical practice. Studies on CatSper gene mutations or polymorphisms[118,128], if conducted in a large cohort of infertile men, could help to identify novel gene candidates for male infertility. In addition, both channels represent an attractive target for development of a male contraceptive[129,130], being expressed only in germ cells[114,115].

**CONCLUSION**

Follow-up studies reveal that ART children present an increased incidence of birth defects, prematurity and low birth weight[131], congenital malformations[132] and imprinting disorders[133] when compared to naturally conceived children. A large study conducted in Australian ART couples demonstrated that, after multivariate adjustments for male and female factors of infertility, the risk for any birth defect retained statistical significance only for ICSI, hypothesizing that differences in male infertility factors, which lead to the use of ICSI, may underlie the phenomenon. Similarly, a recent large and well-designed retrospective study demonstrated that ICSI children have an increased incidence of neurodevelopmental disorders[134]. Identifying the possible causes of male infertility may lead, in the future, to a decrease in ART children’s anomalies, not only because of the possible development of new therapeutic strategies for male infertility but also because of the establishment of new technologies for a better sperm selection for ARTs. However, despite the urgency of establishing new diagnostic tests and defining new sperm markers of male infertility to be used in conjunction with semen analysis, new tests based on “omics” studies or in evaluating sDF (Table 1), are not routinely made a part of the diagnosis of infertile men, mainly because of a lack of standardized procedures, the need to validate the results, and the establishment of clinically accepted cut-off values.

Researchers’ efforts should be devoted to gradually translating their acquired knowledge to clinical practice. In this respect, a continuous discussion between clinicians and researchers is desirable, so that basic research will be conducted on the real needs of the medical practice. This will allow for research innovations to be transformed into new diagnostic or therapeutic methods in order to achieve a more successful natural or assisted conception and delivery of healthy babies. The inclusion in clinical practice of new markers, employing advanced technologies, could be more expensive and may require skilled personnel compared to semen analysis, however, once such predictive markers are validated and, consequently, widely employed to diagnose male infertility, their costs will likely decrease, allowing a breakthrough in the management of infertile couples.

**REFERENCES**

1 **Agarwal A**, Mulgund A, Hamada A, Chyatte MR. A unique view on male infertility around the globe. *Reprod Biol Endocrinol* 2015; **13**: 37 [PMID: 25928197 DOI: 10.1186/s12958-015-0032-1]

2 **World Health Organization.** Laboratory manual for the examination and processing of human semen. 5th ed. Geneva: WHO Press, 2010

3 **Belloc S**, Cohen-Bacrie P, Benkhalifa M, Cohen-Bacrie M, De Mouzon J, Hazout A, Ménézo Y. Effect of maternal and paternal age on pregnancy and miscarriage rates after intrauterine insemination. *Reprod Biomed Online* 2008; **17**: 392-397 [PMID: 18765010 DOI: [10.1016/S1472-6483(10)60223-4](http://dx.doi.org/10.1016/S1472-6483%2810%2960223-4%22%20%5Ct%20%22_blank)]

4 **Paulson RJ**, Milligan RC, Sokol RZ. The lack of influence of age on male fertility. *Am J Obstet Gynecol* 2001; **184**: 818-22; discussion 822-4 [PMID: 11303188 DOI: [10.1067/mob.2001.113852](http://dx.doi.org/10.1067/mob.2001.113852%22%20%5Ct%20%22_blank)]

5 **Bellver J**, Garrido N, Remohí J, Pellicer A, Meseguer M. Influence of paternal age on assisted reproduction outcome. *Reprod Biomed Online* 2008; **17**: 595-604 [PMID: 18983742 DOI: [10.1016/S1472-6483(10)60305-7](http://dx.doi.org/10.1016/S1472-6483%2810%2960305-7%22%20%5Ct%20%22_blank)]

6 **Ford WC**, North K, Taylor H, Farrow A, Hull MG, Golding J. Increasing paternal age is associated with delayed conception in a large population of fertile couples: evidence for declining fecundity in older men. The ALSPAC Study Team (Avon Longitudinal Study of Pregnancy and Childhood). *Hum Reprod* 2000; **15**: 1703-1708 [PMID: 10920089 DOI: [10.1093/humrep/15.8.1703](http://dx.doi.org/10.1093/humrep/15.8.1703%22%20%5Ct%20%22_blank)]

7 **Hassan MA**, Killick SR. Effect of male age on fertility: evidence for the decline in male fertility with increasing age. *Fertil Steril* 2003; **79 Suppl 3**: 1520-1527 [PMID: 12801554 DOI: [10.1016/S0015-0282(03)00366-2](http://dx.doi.org/10.1016/S0015-0282%2803%2900366-2%22%20%5Ct%20%22_blank)]

8 **Lewis SE**, Kumar K. The paternal genome and the health of the assisted reproductive technology child. *Asian J Androl* 2015; **17**: 616-622 [PMID: 25926606 DOI: 10.4103/1008-682X.153301]

9 **Feldman HA**, Longcope C, Derby CA, Johannes CB, Araujo AB, Coviello AD, Bremner WJ, McKinlay JB. Age trends in the level of serum testosterone and other hormones in middle-aged men: longitudinal results from the Massachusetts male aging study. *J Clin Endocrinol Metab* 2002; **87**: 589-598 [PMID: 11836290 DOI: [10.1210/jcem.87.2.8201](http://dx.doi.org/10.1210/jcem.87.2.8201%22%20%5Ct%20%22_blank)]

10 **Kidd SA**, Eskenazi B, Wyrobek AJ. Effects of male age on semen quality and fertility: a review of the literature. *Fertil Steril* 2001; **75**: 237-248 [PMID: 11172821 DOI: [10.1016/S0015-0282(00)01679-4](http://dx.doi.org/10.1016/S0015-0282%2800%2901679-4%22%20%5Ct%20%22_blank)]

11 **Sharma R**, Agarwal A, Rohra VK, Assidi M, Abu-Elmagd M, Turki RF. Effects of increased paternal age on sperm quality, reproductive outcome and associated epigenetic risks to offspring. *Reprod Biol Endocrinol* 2015; **13**: 35 [PMID: 25928123 DOI: 10.1186/s12958-015-0028-x]

12 **Liu X**, Liu FJ, Jin SH, Wang YW, Liu XX, Zhu P, Wang WT, Liu J, Wang WJ. Comparative proteome analysis of human testis from newborn, young adult, and aged men identified spermatogenesis-associated proteins. *Electrophoresis* 2015 [PMID: 26031402 DOI: 10.1002/elps.201500135]

13 **Zini A**. Are sperm chromatin and DNA defects relevant in the clinic? *Syst Biol Reprod Med* 2011; **57**: 78-85 [PMID: 21208147 DOI: 10.3109/19396368.2010.515704]

14 **Moskovtsev SI**, Willis J, Mullen JB. Age-related decline in sperm deoxyribonucleic acid integrity in patients evaluated for male infertility. *Fertil Steril* 2006; **85**: 496-499 [PMID: 16595239]

15 **Kong A**, Frigge ML, Masson G, Besenbacher S, Sulem P, Magnusson G, Gudjonsson SA, Sigurdsson A, Jonasdottir A, Jonasdottir A, Wong WS, Sigurdsson G, Walters GB, Steinberg S, Helgason H, Thorleifsson G, Gudbjartsson DF, Helgason A, Magnusson OT, Thorsteinsdottir U, Stefansson K. Rate of de novo mutations and the importance of father's age to disease risk. *Nature* 2012; **488**: 471-475 [PMID: 22914163 DOI: 10.1038/nature11396]

16 **Templado C**, Vidal F, Estop A. Aneuploidy in human spermatozoa. *Cytogenet Genome Res* 2011; **133**: 91-99 [PMID: 21282942 DOI: 10.1159/000323795]

17 **Hassold T**, Hunt P. To err (meiotically) is human: the genesis of human aneuploidy. *Nat Rev Genet* 2001; **2**: 280-291 [PMID: 11283700]

18 **Knez J**. Endocrine-disrupting chemicals and male reproductive health. *Reprod Biomed Online* 2013; **26**: 440-448 [PMID: 23510680 DOI: 10.1016/j.rbmo.2013.02.005]

19 **Michalakis K**, Mintziori G, Kaprara A, Tarlatzis BC, Goulis DG. The complex interaction between obesity, metabolic syndrome and reproductive axis: a narrative review. *Metabolism* 2013; **62**: 457-478 [PMID: 22999785 DOI: 10.1016/j.metabol.2012.08.012]

20 **Rastrelli G**, Corona G, Mannucci E, Maggi M. Factors affecting spermatogenesis upon gonadotropin-replacement therapy: a meta-analytic study. *Andrology* 2014; **2**: 794-808 [PMID: 25271205 DOI: 10.1111/andr.262]

21 **Garg H**, Kumar R. Empirical Drug Therapy for Idiopathic Male Infertility: What is the New Evidence? *Urology* 2015; **86**: 1065-1075 [PMID: 26255035 DOI: 10.1016/j.urology.2015.07.030]

22 **Wright VC**, Schieve LA, Reynolds MA, Jeng G; Centers for Disease Control and Prevention (CDC). Assisted reproductive technology surveillance--United States, 2002. *MMWR Surveill Summ* 2005; **54**: 1-24 [PMID: 15931153]

23 **Filimberti E**, Degl'Innocenti S, Borsotti M, Quercioli M, Piomboni P, Natali I, Fino MG, Caglieresi C, Criscuoli L, Gandini L, Biggeri A, Maggi M, Baldi E. High variability in results of semen analysis in andrology laboratories in Tuscany (Italy): the experience of an external quality control (EQC) programme. *Andrology* 2013; **1**: 401-407 [PMID: 23307477 DOI: 10.1111/j.2047-2927.2012.00042.x]

24 **Guzick DS**, Overstreet JW, Factor-Litvak P, Brazil CK, Nakajima ST, Coutifaris C, Carson SA, Cisneros P, Steinkampf MP, Hill JA, Xu D, Vogel DL; National Cooperative Reproductive Medicine Network. Sperm morphology, motility, and concentration in fertile and infertile men. *N Engl J Med* 2001; **345**: 1388-1393 [PMID: 11794171]

25 **Leushuis E**, van der Steeg JW, Steures P, Repping S, Bossuyt PM, Mol BW, Hompes PG, van der Veen F. Semen analysis and prediction of natural conception. *Hum Reprod* 2014; **29**: 1360-1367 [PMID: 24795091]

26 **Said TM**, Land JA. Effects of advanced selection methods on sperm quality and ART outcome: a systematic review. *Hum Reprod Update* 2011; **17**: 719-733 [PMID: 21873262 DOI: 10.1093/humupd/dmr032]

27 **Setti AS**, Paes de Almeida Ferreira Braga D, Iaconelli A, Aoki T, Borges E. Twelve years of MSOME and IMSI: a review. *Reprod Biomed Online* 2013; **27**: 338-352 [PMID: 23948449 DOI: 10.1016/j.rbmo.2013.06.011]

28 **Krausz C**, Escamilla AR, Chianese C. Genetics of male infertility: from research to clinic. *Reproduction* 2015; **150**: R159-R174 [PMID: 26447148 DOI: 10.1530/REP-15-0261]

29 **Hotaling J**, Carrell DT. Clinical genetic testing for male factor infertility: current applications and future directions. *Andrology* 2014; **2**: 339-350 [PMID: 24711280 DOI: 10.1111/j.2047-2927.2014.00200.x]

30 **Aston KI**, Krausz C, Laface I, Ruiz-Castané E, Carrell DT. Evaluation of 172 candidate polymorphisms for association with oligozoospermia or azoospermia in a large cohort of men of European descent. *Hum Reprod* 2010; **25**: 1383-1397 [PMID: 20378615 DOI: 10.1093/humrep/deq081]

31 **Tüttelmann F**, Simoni M, Kliesch S, Ledig S, Dworniczak B, Wieacker P, Röpke A. Copy number variants in patients with severe oligozoospermia and Sertoli-cell-only syndrome. *PLoS One* 2011; **6**: e19426 [PMID: 21559371 DOI: 10.1371/journal.pone.0019426]

32 **Krausz C**, Giachini C, Lo Giacco D, Daguin F, Chianese C, Ars E, Ruiz-Castane E, Forti G, Rossi E. High resolution X chromosome-specific array-CGH detects new CNVs in infertile males. *PLoS One* 2012; **7**: e44887 [PMID: 23056185 DOI: 10.1371/journal.pone.0044887]

33 **Yatsenko AN**, Georgiadis AP, Röpke A, Berman AJ, Jaffe T, Olszewska M, Westernströer B, Sanfilippo J, Kurpisz M, Rajkovic A, Yatsenko SA, Kliesch S, Schlatt S, Tüttelmann F. X-linked TEX11 mutations, meiotic arrest, and azoospermia in infertile men. *N Engl J Med* 2015; **372**: 2097-2107 [PMID: 25970010 DOI: 10.1056/NEJMoa1406192]

34 **Malcher A**, Rozwadowska N, Stokowy T, Kolanowski T, Jedrzejczak P, Zietkowiak W, Kurpisz M. Potential biomarkers of nonobstructive azoospermia identified in microarray gene expression analysis. *Fertil Steril* 2013; **100**: 1686-94.e1-7 [PMID: 24012201 DOI: 10.1016/j.fertnstert.2013.07.1999]

35 **Malcher A**, Rozwadowska N, Stokowy T, Jedrzejczak P, Zietkowiak W, Kurpisz M. The gene expression analysis of paracrine/autocrine factors in patients with spermatogenetic failure compared with normal spermatogenesis. *Am J Reprod Immunol* 2013; **70**: 522-528 [PMID: 23869807 DOI: 10.1111/aji.12149]

36 **Sharpe RM**. Environmental/lifestyle effects on spermatogenesis. *Philos Trans R Soc Lond B Biol Sci* 2010; **365**: 1697-1712 [PMID: 20403879 DOI: 10.1098/rstb.2009.0206]

37 **Hamlin HJ**. Prenatal stress and development: beyond the single cause and effect paradigm. *Birth Defects Res C Embryo Today* 2012; **96**: 289-298 [PMID: 24203918 DOI: 10.1002/bdrc.21023]

38 **Gluckman PD**, Hanson MA, Beedle AS. Early life events and their consequences for later disease: a life history and evolutionary perspective. *Am J Hum Biol* 2007; **19**: 1-19 [PMID: 17160980]

39 **Wei Y**, Schatten H, Sun QY. Environmental epigenetic inheritance through gametes and implications for human reproduction. *Hum Reprod Update* 2015; **21**: 194-208 [PMID: 25416302 DOI: 10.1093/humupd/dmu061]

40 **Binder NK**, Hannan NJ, Gardner DK. Paternal diet-induced obesity retards early mouse embryo development, mitochondrial activity and pregnancy health. *PLoS One* 2012; **7**: e52304 [PMID: 23300638 DOI: 10.1371/journal.pone.0052304]

41 **Ng SF**, Lin RC, Laybutt DR, Barres R, Owens JA, Morris MJ. Chronic high-fat diet in fathers programs β-cell dysfunction in female rat offspring. *Nature* 2010; **467**: 963-966 [PMID: 20962845 DOI: 10.1038/nature09491]

42 **Aston KI**, Uren PJ, Jenkins TG, Horsager A, Cairns BR, Smith AD, Carrell DT. Aberrant sperm DNA methylation predicts male fertility status and embryo quality. *Fertil Steril* 2015; **104**: 1388-1397.e5 [PMID: 26361204 DOI: 10.1016/j.fertnstert.2015.08.019]

43 **Jenkins TG**, Aston KI, Meyer TD, Hotaling JM, Shamsi MB, Johnstone EB, Cox KJ, Stanford JB, Porucznik CA, Carrell DT. Decreased fecundity and sperm DNA methylation patterns. *Fertil Steril* 2016; **105**: 51-57.e3 [PMID: 26453269 DOI: 10.1016/j.fertnstert.2015.09.013]

44 **Hammoud SS**, Nix DA, Zhang H, Purwar J, Carrell DT, Cairns BR. Distinctive chromatin in human sperm packages genes for embryo development. *Nature* 2009; **460**: 473-478 [PMID: 19525931 DOI: 10.1038/nature08162]

45 **Yu B**, Zhou H, Liu M, Zheng T, Jiang L, Zhao M, Xu X, Huang Z. Epigenetic Alterations in Density Selected Human Spermatozoa for Assisted Reproduction. *PLoS One* 2015; **10**: e0145585 [PMID: 26709917 DOI: 10.1371/journal.pone.0145585]

46 **Kotaja N**. MicroRNAs and spermatogenesis. *Fertil Steril* 2014; **101**: 1552-1562 [PMID: 24882619 DOI: 10.1016/j.fertnstert.2014.04.025]

47 **Maatouk DM**, Loveland KL, McManus MT, Moore K, Harfe BD. Dicer1 is required for differentiation of the mouse male germline. *Biol Reprod* 2008; **79**: 696-703 [PMID: 18633141 DOI: 10.1095/biolreprod.108.067827]

48 **Abu-Halima M**, Hammadeh M, Backes C, Fischer U, Leidinger P, Lubbad AM, Keller A, Meese E. Panel of five microRNAs as potential biomarkers for the diagnosis and assessment of male infertility. *Fertil Steril* 2014; **102**: 989-997.e1 [PMID: 25108464 DOI: 10.1016/j.fertnstert.2014.07.001]

49 **Jodar M**, Sendler E, Moskovtsev SI, Librach CL, Goodrich R, Swanson S, Hauser R, Diamond MP, Krawetz SA. Absence of sperm RNA elements correlates with idiopathic male infertility. *Sci Transl Med* 2015; **7**: 295re6 [PMID: 26157032 DOI: 10.1126/scitranslmed.aab1287]

50 **Zhao J**, Zhang Q, Wang Y, Li Y. Whether sperm deoxyribonucleic acid fragmentation has an effect on pregnancy and miscarriage after in vitro fertilization/intracytoplasmic sperm injection: a systematic review and meta-analysis. *Fertil Steril* 2014; **102**: 998-1005.e8 [PMID: 25190048 DOI: 10.1016/j.fertnstert.2014.06.033]

51 **Robinson L**, Gallos ID, Conner SJ, Rajkhowa M, Miller D, Lewis S, Kirkman-Brown J, Coomarasamy A. The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. *Hum Reprod* 2012; **27**: 2908-2917 [PMID: 22791753 DOI: 10.1093/humrep/des261]

52 **Osman A**, Alsomait H, Seshadri S, El-Toukhy T, Khalaf Y. The effect of sperm DNA fragmentation on live birth rate after IVF or ICSI: a systematic review and meta-analysis. *Reprod Biomed Online* 2015; **30**: 120-127 [PMID: 25530036 DOI: 10.1016/j.rbmo.2014.10.018]

53 **Tamburrino L**, Marchiani S, Montoya M, Elia Marino F, Natali I, Cambi M, Forti G, Baldi E, Muratori M. Mechanisms and clinical correlates of sperm DNA damage. *Asian J Androl* 2012; **14**: 24-31 [PMID: 22138903 DOI: 10.1038/aja.2011.59]

54 **Muratori M**, Marchiani S, Tamburrino L, Tocci V, Failli P, Forti G, Baldi E. Nuclear staining identifies two populations of human sperm with different DNA fragmentation extent and relationship with semen parameters. *Hum Reprod* 2008; **23**: 1035-1043 [PMID: 18326515 DOI: 10.1093/humrep/den058]

55 **Muratori M**, Tamburrino L, Tocci V, Costantino A, Marchiani S, Giachini C, Laface I, Krausz C, Meriggiola MC, Forti G, Baldi E. Small variations in crucial steps of TUNEL assay coupled to flow cytometry greatly affect measures of sperm DNA fragmentation. *J Androl* 2010; **31**: 336-345 [PMID: 19959824 DOI: 10.2164/jandrol.109.008508]

56 **Marchiani S**, Tamburrino L, Forti G, Baldi E, Muratori M. M540 bodies and their impact on flow cytometric analyses of human spermatozoa. *Soc Reprod Fertil Suppl* 2007; **65**: 509-514 [PMID: 17644988]

57 **Muratori M**, Marchiani S, Tamburrino L, Cambi M, Lotti F, Natali I, Filimberti E, Noci I, Forti G, Maggi M, Baldi E. DNA fragmentation in brighter sperm predicts male fertility independently from age and semen parameters. *Fertil Steril* 2015; **104**: 582-90.e4 [PMID: 26151619 DOI: 10.1016/j.fertnstert.2015.06.005]

58 **Aitken RJ**, De Iuliis GN, Finnie JM, Hedges A, McLachlan RI. Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: development of diagnostic criteria. *Hum Reprod* 2010; **25**: 2415-2426 [PMID: 20716559 DOI: 10.1093/humrep/deq214]

59 **Mitchell LA**, De Iuliis GN, Aitken RJ. The TUNEL assay consistently underestimates DNA damage in human spermatozoa and is influenced by DNA compaction and cell vitality: development of an improved methodology. *Int J Androl* 2011; **34**: 2-13 [PMID: 20158539 DOI: 10.1111/j.1365-2605.2009.01042.x]

60 **Simon L**, Lutton D, McManus J, Lewis SE. Sperm DNA damage measured by the alkaline Comet assay as an independent predictor of male infertility and in vitro fertilization success. *Fertil Steril* 2011; **95**: 652-657 [PMID: 20864101 DOI: 10.1016/j.fertnstert.2010.08.019]

61 **Barratt CL**, Aitken RJ, Björndahl L, Carrell DT, de Boer P, Kvist U, Lewis SE, Perreault SD, Perry MJ, Ramos L, Robaire B, Ward S, Zini A. Sperm DNA: organization, protection and vulnerability: from basic science to clinical applications--a position report. *Hum Reprod* 2010; **25**: 824-838 [PMID: 20139429 DOI: 10.1093/humrep/dep465]

62 **ASRM Practice Committee**. The clinical utility of sperm DNA integrity testing: a guideline. *Fertil Steril* 2013; **99**: 673-677 [PMID: 23391408 DOI: 10.1016/j.fertnstert.2012.12.049]

63 **Lewis SE**. Should sperm DNA fragmentation testing be included in the male infertility work-up? *Reprod Biomed Online* 2015; **31**: 134-137 [PMID: 26096033 DOI: 10.1016/j.rbmo.2015.05.006]

64 **Aitken RJ**, Smith TB, Jobling MS, Baker MA, De Iuliis GN. Oxidative stress and male reproductive health. *Asian J Androl* 2014; **16**: 31-38 [PMID: 24369131 DOI: 10.4103/1008-682X.122203]

65 **Bykova M,** Athayde K, Sharma R, Jha R, Sabanegh E, Agarwal A. Defining the reference value of seminal reactive oxygen species in a population of infertile men and normal healthy volunteers. *Fertil Steril* 2007; **88:** 305

66 **Tremellen K**. Oxidative stress and male infertility--a clinical perspective. *Hum Reprod Update* 2008; **14**: 243-258 [PMID: 18281241 DOI: 10.1093/humupd/dmn004]

67 **Aktan G**, Doğru-Abbasoğlu S, Küçükgergin C, Kadıoğlu A, Ozdemirler-Erata G, Koçak-Toker N. Mystery of idiopathic male infertility: is oxidative stress an actual risk? *Fertil Steril* 2013; **99**: 1211-1215 [PMID: 23254182 DOI: 10.1016/j.fertnstert.2012.11.045]

68 **Showell MG**, Mackenzie-Proctor R, Brown J, Yazdani A, Stankiewicz MT, Hart RJ. Antioxidants for male subfertility. *Cochrane Database Syst Rev* 2014; **12**: CD007411 [PMID: 25504418 DOI: 10.1002/14651858.CD007411]

69 **Menezo Y**, Evenson D, Cohen M, Dale B. Effect of antioxidants on sperm genetic damage. *Adv Exp Med Biol* 2014; **791**: 173-189 [PMID: 23955679 DOI: 10.1007/978-1-4614-7783-9\_11]

70 **Muratori M**, Tamburrino L, Marchiani S, Cambi M, Olivito B, Azzari C, Forti G, Baldi E. Investigation on the Origin of Sperm DNA Fragmentation: Role of Apoptosis, Immaturity and Oxidative Stress. *Mol Med* 2015; **21**: 109-122 [PMID: 25786204 DOI: 10.2119/molmed.2014.00158]

71 **Palomba S**, Falbo A, Espinola S, Rocca M, Capasso S, Cappiello F, Zullo F. Effects of highly purified follicle-stimulating hormone on sperm DNA damage in men with male idiopathic subfertility: a pilot study. *J Endocrinol Invest* 2011; **34**: 747-752 [PMID: 21606671 DOI: 10.3275/7745]

72 **Colacurci N**, Monti MG, Fornaro F, Izzo G, Izzo P, Trotta C, Mele D, De Franciscis P. Recombinant human FSH reduces sperm DNA fragmentation in men with idiopathic oligoasthenoteratozoospermia. *J Androl* 2012; **33**: 588-593 [PMID: 21868752 DOI: 10.2164/jandrol.111.013326]

73 **Ruvolo G**, Roccheri MC, Brucculeri AM, Longobardi S, Cittadini E, Bosco L. Lower sperm DNA fragmentation after r-FSH administration in functional hypogonadotropic hypogonadism. *J Assist Reprod Genet* 2013; **30**: 497-503 [PMID: 23435529 DOI: 10.1007/s10815-013-9951-y]

74 **Codina M**, Estanyol JM, Fidalgo MJ, Ballescà JL, Oliva R. Advances in sperm proteomics: best-practise methodology and clinical potential. *Expert Rev Proteomics* 2015; **12**: 255-277 [PMID: 25921224 DOI: 10.1586/14789450.2015.1040769]

75 **Zhao C**, Huo R, Wang FQ, Lin M, Zhou ZM, Sha JH. Identification of several proteins involved in regulation of sperm motility by proteomic analysis. *Fertil Steril* 2007; **87**: 436-438 [PMID: 17074334]

76 **Martínez-Heredia J**, de Mateo S, Vidal-Taboada JM, Ballescà JL, Oliva R. Identification of proteomic differences in asthenozoospermic sperm samples. *Hum Reprod* 2008; **23**: 783-791 [PMID: 18281682 DOI: 10.1093/humrep/den024]

77 **Chan CC**, Shui HA, Wu CH, Wang CY, Sun GH, Chen HM, Wu GJ. Motility and protein phosphorylation in healthy and asthenozoospermic sperm. *J Proteome Res* 2009; **8**: 5382-5386 [PMID: 19678645 DOI: 10.1021/pr9003932]

78 **Thacker S**, Yadav SP, Sharma RK, Kashou A, Willard B, Zhang D, Agarwal A. Evaluation of sperm proteins in infertile men: a proteomic approach. *Fertil Steril* 2011; **95**: 2745-2748 [PMID: 21536282 DOI: 10.1016/j.fertnstert.2011.03.112]

79 **Siva AB**, Kameshwari DB, Singh V, Pavani K, Sundaram CS, Rangaraj N, Deenadayal M, Shivaji S. Proteomics-based study on asthenozoospermia: differential expression of proteasome alpha complex. *Mol Hum Reprod* 2010; **16**: 452-462 [PMID: 20304782 DOI: 10.1093/molehr/gaq009]

80 **Parte PP**, Rao P, Redij S, Lobo V, D'Souza SJ, Gajbhiye R, Kulkarni V. Sperm phosphoproteome profiling by ultra performance liquid chromatography followed by data independent analysis (LC-MS(E)) reveals altered proteomic signatures in asthenozoospermia. *J Proteomics* 2012; **75**: 5861-5871 [PMID: 22796355 DOI: 10.1016/j.jprot.2012.07.003]

81 **Shen S**, Wang J, Liang J, He D. Comparative proteomic study between human normal motility sperm and idiopathic asthenozoospermia. *World J Urol* 2013; **31**: 1395-1401 [PMID: 23455884 DOI: 10.1007/s00345-013-1023-5]

82 **Amaral A**, Paiva C, Attardo Parrinello C, Estanyol JM, Ballescà JL, Ramalho-Santos J, Oliva R. Identification of proteins involved in human sperm motility using high-throughput differential proteomics. *J Proteome Res* 2014; **13**: 5670-5684 [PMID: 25250979 DOI: 10.1021/pr500652y]

83 **Giacomini E**, Ura B, Giolo E, Luppi S, Martinelli M, Garcia RC, Ricci G. Comparative analysis of the seminal plasma proteomes of oligoasthenozoospermic and normozoospermic men. *Reprod Biomed Online* 2015; **30**: 522-531 [PMID: 25779018 DOI: 10.1016/j.rbmo.2015.01.010]

84 **Zhu Y**, Wu Y, Jin K, Lu H, Liu F, Guo Y, Yan F, Shi W, Liu Y, Cao X, Hu H, Zhu H, Guo X, Sha J, Li Z, Zhou Z. Differential proteomic profiling in human spermatozoa that did or did not result in pregnancy via IVF and AID. *Proteomics Clin Appl* 2013; **7**: 850-858 [PMID: 24115602 DOI: 10.1002/prca.201200078]

85 **Azpiazu R**, Amaral A, Castillo J, Estanyol JM, Guimerà M, Ballescà JL, Balasch J, Oliva R. High-throughput sperm differential proteomics suggests that epigenetic alterations contribute to failed assisted reproduction. *Hum Reprod* 2014; **29**: 1225-1237 [PMID: 24781426 DOI: 10.1093/humrep/deu073]

86 **Légaré C**, Droit A, Fournier F, Bourassa S, Force A, Cloutier F, Tremblay R, Sullivan R. Investigation of male infertility using quantitative comparative proteomics. *J Proteome Res* 2014; **13**: 5403-5414 [PMID: 25355644 DOI: 10.1021/pr501031x]

87 **Behrouzi B**, Kenigsberg S, Alladin N, Swanson S, Zicherman J, Hong SH, Moskovtsev SI, Librach CL. Evaluation of potential protein biomarkers in patients with high sperm DNA damage. *Syst Biol Reprod Med* 2013; **59**: 153-163 [PMID: 23634713 DOI: 10.3109/19396368.2013.775396]

88 **Intasqui P**, Camargo M, Del Giudice PT, Spaine DM, Carvalho VM, Cardozo KH, Zylbersztejn DS, Bertolla RP. Sperm nuclear DNA fragmentation rate is associated with differential protein expression and enriched functions in human seminal plasma. *BJU Int* 2013; **112**: 835-843 [PMID: 23890255 DOI: 10.1111/bju.12233]

89 **Sharma R**, Agarwal A, Mohanty G, Du Plessis SS, Gopalan B, Willard B, Yadav SP, Sabanegh E. Proteomic analysis of seminal fluid from men exhibiting oxidative stress. *Reprod Biol Endocrinol* 2013; **11**: 85 [PMID: 24004880 DOI: 10.1186/1477-7827-11-85]

90 **Hamada A**, Sharma R, du Plessis SS, Willard B, Yadav SP, Sabanegh E, Agarwal A. Two-dimensional differential in-gel electrophoresis-based proteomics of male gametes in relation to oxidative stress. *Fertil Steril* 2013; **99**: 1216-1226.e2 [PMID: 23312230 DOI: 10.1016/j.fertnstert.2012.11.046]

91 **Kriegel TM**, Heidenreich F, Kettner K, Pursche T, Hoflack B, Grunewald S, Poenicke K, Glander HJ, Paasch U. Identification of diabetes- and obesity-associated proteomic changes in human spermatozoa by difference gel electrophoresis. *Reprod Biomed Online* 2009; **19**: 660-670 [PMID: 20021714]

92 **Paasch U**, Heidenreich F, Pursche T, Kuhlisch E, Kettner K, Grunewald S, Kratzsch J, Dittmar G, Glander HJ, Hoflack B, Kriegel TM. Identification of increased amounts of eppin protein complex components in sperm cells of diabetic and obese individuals by difference gel electrophoresis. *Mol Cell Proteomics* 2011; **10**: M110.007187 [PMID: 21525168 DOI: 10.1074/mcp.M110.007187]

93 **Liu Y**, Guo Y, Song N, Fan Y, Li K, Teng X, Guo Q, Ding Z. Proteomic pattern changes associated with obesity-induced asthenozoospermia. *Andrology* 2015; **3**: 247-259 [PMID: 25293813 DOI: 10.1111/andr.289]

94 **Pixton KL**, Deeks ED, Flesch FM, Moseley FL, Björndahl L, Ashton PR, Barratt CL, Brewis IA. Sperm proteome mapping of a patient who experienced failed fertilization at IVF reveals altered expression of at least 20 proteins compared with fertile donors: case report. *Hum Reprod* 2004; **19**: 1438-1447 [PMID: 15105389]

95 **Kichine E**, Di Falco M, Hales BF, Robaire B, Chan P. Analysis of the sperm head protein profiles in fertile men: consistency across time in the levels of expression of heat shock proteins and peroxiredoxins. *PLoS One* 2013; **8**: e77471 [PMID: 24204839 DOI: 10.1371/journal.pone.0077471]

96 **Xu W**, Hu H, Wang Z, Chen X, Yang F, Zhu Z, Fang P, Dai J, Wang L, Shi H, Li Z, Qiao Z. Proteomic characteristics of spermatozoa in normozoospermic patients with infertility. *J Proteomics* 2012; **75**: 5426-5436 [PMID: 22771312 DOI: 10.1016/j.jprot.2012.06.021]

97 **de Mateo S**, Castillo J, Estanyol JM, Ballescà JL, Oliva R. Proteomic characterization of the human sperm nucleus. *Proteomics* 2011; **11**: 2714-2726 [PMID: 21630459 DOI: 10.1002/pmic.201000799]

98 **Baker MA**, Naumovski N, Hetherington L, Weinberg A, Velkov T, Aitken RJ. Head and flagella subcompartmental proteomic analysis of human spermatozoa. *Proteomics* 2013; **13**: 61-74 [PMID: 23161668 DOI: 10.1002/pmic.201200350]

99 **Kim YH**, Haidl G, Schaefer M, Egner U, Mandal A, Herr JC. Compartmentalization of a unique ADP/ATP carrier protein SFEC (Sperm Flagellar Energy Carrier, AAC4) with glycolytic enzymes in the fibrous sheath of the human sperm flagellar principal piece. *Dev Biol* 2007; **302**: 463-476 [PMID: 17137571]

100 **Amaral A**, Castillo J, Estanyol JM, Ballescà JL, Ramalho-Santos J, Oliva R. Human sperm tail proteome suggests new endogenous metabolic pathways. *Mol Cell Proteomics* 2013; **12**: 330-342 [PMID: 23161514 DOI: 10.1074/mcp.M112.020552]

101 **Muratori M**, Marchiani S, Tamburrino L, Forti G, Luconi M, Baldi E. Markers of human sperm functions in the ICSI era. *Front Biosci (Landmark Ed)* 2011; **16**: 1344-1363 [PMID: 21196236]

102 **Ficarro S**, Chertihin O, Westbrook VA, White F, Jayes F, Kalab P, Marto JA, Shabanowitz J, Herr JC, Hunt DF, Visconti PE. Phosphoproteome analysis of capacitated human sperm. Evidence of tyrosine phosphorylation of a kinase-anchoring protein 3 and valosin-containing protein/p97 during capacitation. *J Biol Chem* 2003; **278**: 11579-11589 [PMID: 12509440]

103 **Buffone MG**, Calamera JC, Verstraeten SV, Doncel GF. Capacitation-associated protein tyrosine phosphorylation and membrane fluidity changes are impaired in the spermatozoa of asthenozoospermic patients. *Reproduction* 2005; **129**: 697-705 [PMID: 15923385]

104 **Yunes R**, Doncel GF, Acosta AA. Incidence of sperm-tail tyrosine phosphorylation and hyperactivated motility in normozoospermic and asthenozoospermic human sperm samples. *Biocell* 2003; **27**: 29-36 [PMID: 12847912]

105 **Luconi M**, Forti G, Baldi E. Pathophysiology of sperm motility. *Front Biosci* 2006; **11**: 1433-1447 [PMID: 16368527]

106 **Sutovsky P**, Moreno R, Ramalho-Santos J, Dominko T, Thompson WE, Schatten G. A putative, ubiquitin-dependent mechanism for the recognition and elimination of defective spermatozoa in the mammalian epididymis. *J Cell Sci* 2001; **114**: 1665-1675 [PMID: 11309198]

107 **Ozanon C**, Chouteau J, Sutovsky P. Clinical adaptation of the sperm ubuquitin tag immunoassay (SUTI): relationship of sperm ubiquitylation with sperm quality in gradient-purified semen samples from 93 men from a general infertility clinic population. *Hum Reprod* 2005; **20**: 2271-2278 [PMID: 15817585]

108 **Muratori M**, Marchiani S, Forti G, Baldi E. Sperm ubiquitination positively correlates to normal morphology in human semen. *Hum Reprod* 2005; **20**: 1035-1043 [PMID: 15705629]

109 **Vigodner M**, Shrivastava V, Gutstein LE, Schneider J, Nieves E, Goldstein M, Feliciano M, Callaway M. Localization and identification of sumoylated proteins in human sperm: excessive sumoylation is a marker of defective spermatozoa. *Hum Reprod* 2013; **28**: 210-223 [PMID: 23077236 DOI: 10.1093/humrep/des317]

110 **Marchiani S**, Tamburrino L, Ricci B, Nosi D, Cambi M, Piomboni P, Belmonte G, Forti G, Muratori M, Baldi E. SUMO1 in human sperm: new targets, role in motility and morphology and relationship with DNA damage. *Reproduction* 2014; **148**: 453-467 [PMID: 25118297 DOI: 10.1530/REP-14-0173]

111 **Darszon A**, Labarca P, Nishigaki T, Espinosa F. Ion channels in sperm physiology. *Physiol Rev* 1999; **79**: 481-510 [PMID: 10221988]

112 **Lishko PV**, Botchkina IL, Fedorenko A, Kirichok Y. Acid extrusion from human spermatozoa is mediated by flagellar voltage-gated proton channel. *Cell* 2010; **140**: 327-337 [PMID: 20144758 DOI: 10.1016/j.cell.2009.12.053]

113 **Santi CM**, Martínez-López P, de la Vega-Beltrán JL, Butler A, Alisio A, Darszon A, Salkoff L. The SLO3 sperm-specific potassium channel plays a vital role in male fertility. *FEBS Lett* 2010; **584**: 1041-1046 [PMID: 20138882 DOI: 10.1016/j.febslet.2010.02.005]

114 **Zheng LP**, Wang HF, Li BM, Zeng XH. Sperm-specific ion channels: targets holding the most potential for male contraceptives in development. *Contraception* 2013; **88**: 485-491 [PMID: 23845210 DOI: 10.1016/j.contraception.2013.06.002]

115 **Ren D**, Navarro B, Perez G, Jackson AC, Hsu S, Shi Q, Tilly JL, Clapham DE. A sperm ion channel required for sperm motility and male fertility. *Nature* 2001; **413**: 603-609 [PMID: 11595941]

116 **Qi H**, Moran MM, Navarro B, Chong JA, Krapivinsky G, Krapivinsky L, Kirichok Y, Ramsey IS, Quill TA, Clapham DE. All four CatSper ion channel proteins are required for male fertility and sperm cell hyperactivated motility. *Proc Natl Acad Sci USA* 2007; **104**: 1219-1223 [PMID: 17227845]

117 **Jin J**, Jin N, Zheng H, Ro S, Tafolla D, Sanders KM, Yan W. Catsper3 and Catsper4 are essential for sperm hyperactivated motility and male fertility in the mouse. *Biol Reprod* 2007; **77**: 37-44 [PMID: 17344468]

118 **Hildebrand MS**, Avenarius MR, Fellous M, Zhang Y, Meyer NC, Auer J, Serres C, Kahrizi K, Najmabadi H, Beckmann JS, Smith RJ. Genetic male infertility and mutation of CATSPER ion channels. *Eur J Hum Genet* 2010; **18**: 1178-1184 [PMID: 20648059 DOI: 10.1038/ejhg.2010.108]

119 **Smith JF**, Syritsyna O, Fellous M, Serres C, Mannowetz N, Kirichok Y, Lishko PV. Disruption of the principal, progesterone-activated sperm Ca2+ channel in a CatSper2-deficient infertile patient. *Proc Natl Acad Sci USA* 2013; **110**: 6823-6828 [PMID: 23530196 DOI: 10.1073/pnas.1216588110]

120 **Strünker T**, Goodwin N, Brenker C, Kashikar ND, Weyand I, Seifert R, Kaupp UB. The CatSper channel mediates progesterone-induced Ca2+ influx in human sperm. *Nature* 2011; **471**: 382-386 [PMID: 21412338 DOI: 10.1038/nature09769]

121 **Lishko PV**, Botchkina IL, Kirichok Y. Progesterone activates the principal Ca2+ channel of human sperm. *Nature* 2011; **471**: 387-391 [PMID: 21412339 DOI: 10.1038/nature09767]

122 **Baldi E**, Luconi M, Muratori M, Marchiani S, Tamburrino L, Forti G. Nongenomic activation of spermatozoa by steroid hormones: facts and fictions. *Mol Cell Endocrinol* 2009; **308**: 39-46 [PMID: 19549590 DOI: 10.1016/j.mce.2009.02.006]

123 **Jin M**, Fujiwara E, Kakiuchi Y, Okabe M, Satouh Y, Baba SA, Chiba K, Hirohashi N. Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during in vitro fertilization. *Proc Natl Acad Sci USA* 2011; **108**: 4892-4896 [PMID: 21383182 DOI: 10.1073/pnas.1018202108]

124 **Tamburrino L**, Marchiani S, Minetti F, Forti G, Muratori M, Baldi E. The CatSper calcium channel in human sperm: relation with motility and involvement in progesterone-induced acrosome reaction. *Hum Reprod* 2014; **29**: 418-428 [PMID: 24430778 DOI: 10.1093/humrep/det454]

125 **Tamburrino L**, Marchiani S, Vicini E, Muciaccia B, Cambi M, Pellegrini S, Forti G, Muratori M, Baldi E. Quantification of CatSper1 expression in human spermatozoa and relation to functional parameters. *Hum Reprod* 2015; **30**: 1532-1544 [PMID: 25983333 DOI: 10.1093/humrep/dev103]

126 **Sagare-Patil V,** Galvankar M, Satiya M, Bhandari B, Gupta SK, Modi D.Differential concentration and time dependent effects of progesterone on kinaseactivity, hyperactivation and acrosome reaction in human spermatozoa. *Int JAndrol* 2012; **35:** 633-644

127 **Sagare-Patil V**, Galvankar M, Satiya M, Bhandari B, Gupta SK, Modi D. Differential concentration and time dependent effects of progesterone on kinase activity, hyperactivation and acrosome reaction in human spermatozoa. *Int J Androl* 2012; **35**: 633-644 [PMID: 22775762 DOI: 10.1111/j.1365-2605.2012.01291.x]

128 **Avenarius MR**, Hildebrand MS, Zhang Y, Meyer NC, Smith LL, Kahrizi K, Najmabadi H, Smith RJ. Human male infertility caused by mutations in the CATSPER1 channel protein. *Am J Hum Genet* 2009; **84**: 505-510 [PMID: 19344877 DOI: 10.1016/j.ajhg.2009.03.004]

129 **Navarro B**, Kirichok Y, Chung JJ, Clapham DE. Ion channels that control fertility in mammalian spermatozoa. *Int J Dev Biol* 2008; **52**: 607-613 [PMID: 18649274 DOI: 10.1387/ijdb.072554bn]

130 **Carlson AE**, Burnett LA, del Camino D, Quill TA, Hille B, Chong JA, Moran MM, Babcock DF. Pharmacological targeting of native CatSper channels reveals a required role in maintenance of sperm hyperactivation. *PLoS One* 2009; **4**: e6844 [PMID: 19718436 DOI: 10.1371/journal.pone.0006844]

131 **Jackson S**, Hong C, Wang ET, Alexander C, Gregory KD, Pisarska MD. Pregnancy outcomes in very advanced maternal age pregnancies: the impact of assisted reproductive technology. *Fertil Steril* 2015; **103**: 76-80 [PMID: 25450294 DOI: 10.1016/j.fertnstert.2014.09.037]

132 **Bonduelle M**, Wennerholm UB, Loft A, Tarlatzis BC, Peters C, Henriet S, Mau C, Victorin-Cederquist A, Van Steirteghem A, Balaska A, Emberson JR, Sutcliffe AG. A multi-centre cohort study of the physical health of 5-year-old children conceived after intracytoplasmic sperm injection, in vitro fertilization and natural conception. *Hum Reprod* 2005; **20**: 413-419 [PMID: 15576393]

133 **Sutcliffe AG**, Ludwig M. Outcome of assisted reproduction. *Lancet* 2007; **370**: 351-359 [PMID: 17662884]

134 **Kissin DM**, Zhang Y, Boulet SL, Fountain C, Bearman P, Schieve L, Yeargin-Allsopp M, Jamieson DJ. Association of assisted reproductive technology (ART) treatment and parental infertility diagnosis with autism in ART-conceived children. *Hum Reprod* 2015; **30**: 454-465 [PMID: 25518976 DOI: 10.1093/humrep/deu338]

**P- Reviewer:** Bai G, Carter WG, Chui YL **S- Editor:** Song XX **L- Editor:** **E- Editor:**

**Table 1 Promising sperm markers of male infertility based on so far published literature**

|  |  |  |  |
| --- | --- | --- | --- |
| **Approach type** | **Main outcomes** | **Ref.** | **Advantages (+)****disadvanteges (-)** |
| **Semen analysis** | Macroscopic and microscopic evaluation of semen according WHO guidelines | [2] | (+) established reference values(-) high operator variability(-) poorly predictive of fertility |
| **Genetic and****epigenetic** | NGS: Found a set of sperm RNA elements required to achieve live births | [47] | (+) broad-spectrum analysis(-) lack of validation(-) not independently predictive of fertility(-) too early for diagnostic purpose |
| miRNA: Alteration of 5 miRNAs in subfertile and NOA subjects compared to controls | [46] |
| DNA methylation: Different methylation pattern between fertile and infertile subjects | [42,43] |
| sDF: Discrimination between fertile and infertile subjects | [55,56,58] | (+) presently adopted in many ART laboratories(+) prediction of fertility independent from semen quality(-) employment of different techniques to detect sDF(-) lack of agreement on cutoff values |
| **Proteomic** | > 6000 proteins (histone variants, transcription factors, zinc finger proteins, receptors, proteins related to metabolism, structure and motility, carriers) | [80,95-98] | (+) broad-spectrum analysis(-) isolation of spermatozoa(-) low available sperm material in oligozoospermic subjects(-) intra- and inter-variability of proteomic profiles |
| **PTMs** | Phosphorylation: Reduced tyrosine phosphorylation in asthenozoospermic subjects | [101] | (+) higher biological relevance compared to gene or protein expression *per se*(-) no target proteins identified(-) too early for diagnostic purpose |
| Ubiquitination: Sperm quality control system | [104] |
| Sumoylation: Marker of defective sperm | [107,108] |
| **Ion channels** | Slo3: Involved in hyperpolarization during sperm capacitation | [111,112] | (+) analysis free from confounders(-) skilled personnel and advanced instruments are required(-) too early for diagnostic purpose |
| CatSper: Involved in sperm progressive and hyperactivated motility | [123] |

PTMs: Post-translational protein modifications; WHO: World Health Organization; NGS: Next-generation sequencing; NOA: Non-obstructive azoospermia; sDF: Sperm DNA fragmentation; ART: Assisted reproduction technique.