European Review, Vol. 32, No. 4, 346–378

doi:10.1017/S1062798724000231

How Relevant are Male Factors for Fertilization and Early Embryo Development? Looking into the (Epi)genome, Proteome and Metabolome

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Infertility affects 10–15% of couples at the age of conception. Mounting evidence supports that not only are paternal factors crucial during fertilization, but also for embryogenesis. This review aims to provide some clues about the contribution of male factors to reproductive success and live birth, as such contributions can be as important as that of the female. Semen is composed of two fractions: sperm and seminal plasma. Regarding the former, the integrity of sperm components (i.e., centrioles, DNA integrity and methylation, histone-to-protamine ration, specific proteins, etc.) has been proven to be essential for some of the events occurring upon engulfment of the spermatozoon into the oocyte cytoplasm. The metabolic status of sperm also seems to shape their potential fertilizing capacity. Furthermore, seminal plasma appears to modulate the female reproductive tract, and has been suggested to support embryo implantation. In spite of the aforementioned, it remains largely unaddressed how paternal factors interact with maternal ones, and whether the latter may mask the former. While assisted reproductive techniques (ART) are useful to rescue infertility, a better understanding about the contribution of semen to fertilization, embryo development and implantation can increase the efficiency of these techniques, and address further the causes of total fertilization failure, implantation deficiency and recurrent miscarriage.

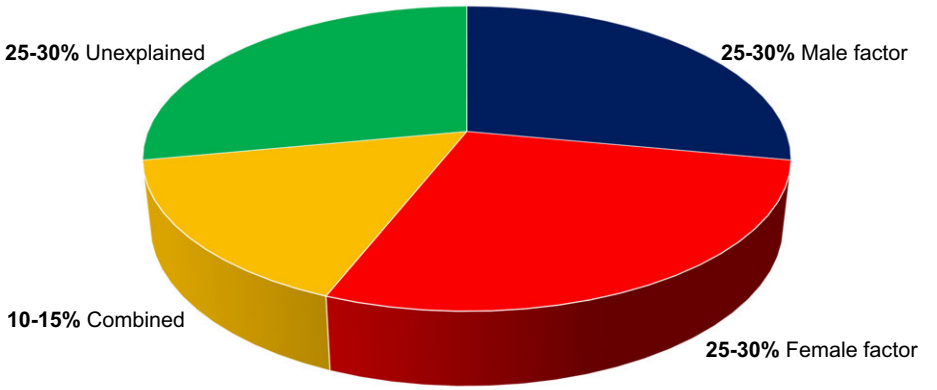

Figure 1. Causes of human infertility. About 25–30% of infertility cases are exclusively due to male factors, and a similar figure of cases is related to female factors (25–30%). In addition, about 10–15% of infertility cases are due to a combination of male and female factors, and between 25% and 30% of cases are unexplained. Percentages are given in ranges, as there are some differences between clinical studies.

Introduction

The incidence of infertility, which is defined as the inability of a couple at reproductive age of conceiving after one year of unprotected intercourse, is around 10–15% worldwide (Simon *et al.* 2017a). Primary infertility can be distinguished from secondary infertility depending on whether the couple has never been able to conceive (primary), or was able to conceive but is no longer capable to do so (Zegers-Hochschild *et al.* 2008). The causes of each type of infertility differ. In the case of male infertility, the contribution of male factors can be as important as that of the female. Indeed, while single male factors account for 30–35% of cases, as too do single female factors, there are still about 30–40% of cases that could arise from both male and female sides, and about of 20% of unexplained/idiopathic infertility that could also involve the participation of male factors (Figure 1) (Yeste *et al.* 2016).

In order to understand the contribution of semen, one needs to dissect each of its fractions and components, as their integrity may have an influence. In most cases, diagnosis of male infertility is based on the evaluation of spermiogram variables (ejaculate volume, pH, concentration, motility, morphology) which, despite being very useful, does not provide a complete picture of what can be wrong with male gamete components (Colaco and Sakkas 2018; Bashiri *et al.* 2021; Tarozzi *et al.* 2021). In effect, great attention to motility and concentration has been paid, as sperm have been regarded as a means of bringing the haploid genome of the father to the female's oocyte. The shortcoming of this approach is that it neglects that other paternal factors can also play a crucial role during fertilization and, even, at the start of embryogenesis, as emerging research supports (reviewed in Yeste *et al.* 2016; Vallet-Buisan *et al.* 2023).

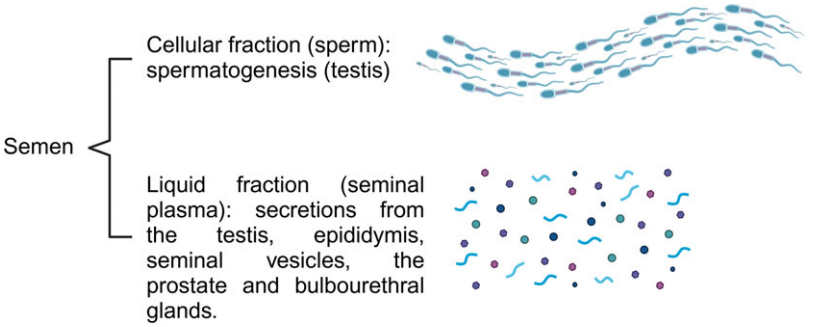

Figure 2. Semen composition. The semen is composed of cellular (sperm) and liquid (seminal plasma, also known as seminal fluid) fractions. Sperm are produced in the testis through spermatogenesis and then enter the efferent duct and epididymis, where they mature. The seminal plasma (SP) comprises secretions from the testis, epididymis, seminal vesicles, the prostate and bulbourethral glands.

While great advances in rescuing male infertility through assisted reproductive techniques have been made over the last 40 years, allowing for the birth of more than eight million babies until 2018, according to figures released by the European Society of Human Reproduction and Embryology (Niederberger *et al.* 2018; ESHRE 2018), a full comprehension about which paternal factors can underlie fertilization failure, impaired embryo development and miscarriage has not been achieved. The aim of this review is to discuss the paternal factors that can potentially contribute to the events taking place during fertilization and beyond (including their influence on embryo development and implantation, and on offspring health), and how this may help improve diagnosis and treatment of male infertility. For this purpose, the following sections approach sperm and seminal plasma separately.

Composition of Semen: Sperm and Seminal Plasma

The contribution of paternal factors, which, as aforementioned, is at least 30%, comes from the semen. This differs from the case of the female, where the respective contribution of 30–35% does not only involve ovarian dysfunction and advancing maternal age, which have repercussions on oocyte quality and competence, but also problems derived from uterine, pelvic and Fallopian tube alterations (including endometriosis), and other factors such as psychosexual ones (Child 2013).

The semen is composed of a cellular fraction (sperm) and a liquid one (seminal plasma, also known as seminal fluid) (Figure 2). Regarding the former, three parts can be distinguished in a spermatozoon: head (composed of nucleus and acrosome), neck (or connecting piece), and tail (where mitochondrial, principal and end pieces are distinguished). Remarkably, the spermatozoon is a very peculiar, complex and differentiated cell, as it is the only one that performs its mission (i.e., bring the father haploid genome to the oocyte) in another body (the female's). It is formed through

spermatogenesis, and then completes its maturation during the transit along the epididymis. This transit is featured by the remodelling of the plasma membrane, nuclear compaction, and incorporation of proteins, including cytosine-rich secretory protein 1 (CRISP1) (Cohen *et al.* 2011; Weigel Muñoz *et al.* 2018), acrosin binding protein (ACRBP) (Nagdas *et al.* 2010; Yin *et al.* 2018), and disintegrins/metalloproteinases (ADAM) (Inoue *et al.* 2005; Nishimura *et al.* 2007), through extracellular vesicles (James *et al.* 2020; Barrachina *et al.* 2022). In addition, motile ability is acquired in the epididymis cauda (Tourzani *et al*. 2021). As discussed later, epididymosomes also contain non-coding RNAs, and could be involved in passing the paternal epigenetic inheritance to the offspring (Zhang *et al.* 2018; Chan *et al.* 2020).

In humans, sperm are deposited in the vagina, and then enter the cervical canal, where they must pass though the cervical mucus. Active and passive (myometrium) contractions let sperm travel along the uterus. In the uterus, sperm trigger NETosis, which is a process through which polymorphonuclear granulocytes, a type of white blood cells, release their DNA into the extracellular environment to generate neutrophil extracellular traps (NETs), which can capture and kill bacteria and sperm (Zambrano *et al.* 2016). NETosis appears to have a selective mission, removing the excess of sperm, particularly those that have less motility and potentially less fertility, as studies in animal models indicate (Alghamdi and Foster 2005; Alghamdi *et al.* 2009; Zambrano *et al.* 2021). Those sperm that escape from leukocytes reach the utero-tubal junction (UTJ) (Ishimoto and Gaffney 2016). This junction has a selection role, as it presents different folds that allow for the retention of poor-quality sperm. Sperm cells capable of overcoming the UTJ then enter the fallopian tube, where, based on observations performed in animal models and in vitro studies in humans (Pacey *et al.* 1995; Ziskind *et al.* 2000; Massa *et al*. 2019), they appear to attach to the epithelial cells of the isthmus, one of the fallopian tube's sections, and form a 'reservoir' (Sharif *et al.* 2022). Only sperm in good shape can bind these epithelial cells; hence, this reservoir has been posited to exert a selective function (Rath *et al.* 2016), and would drive the transient storage of sperm, maintaining their survival until the oocyte is available (Teijeiro and Marini 2012). Moreover, an in-vitro study conducted in humans reported that binding of sperm to OE-E6/E7, an immortalized human cell line of fallopian tube cells, triggers a transcriptomic response in these epithelial cells. This results in a downregulation of inflammatory molecules (cytokines and chemokines) that ultimately allow for a greater immunological tolerance with regard to sperm (Mousavi *et al*. 2021). When, in contrast, sperm incubated with the OE-E6/E7 cell line have high levels of DNA fragmentation (evaluated with the TUNEL assay), they rather activate a toll-like receptor signalling pathway in these cells, upregulating the expression of inflammatory cytokines and chemokines (Mohammadi *et al*. 2022).

Around ovulation, sperm detach from the epithelial cells of the isthmus and capacitate. Only capacitated sperm are able to trigger the acrosome reaction, which is required for the male gamete to pass through the cumulus cells and the zona pellucida (Molina *et al.* 2018; Gupta 2021). Some defective sperm cells are unable to

undergo capacitation and acrosome reaction, and/or penetrate the oocyte, whereas others – even when they fuse with the oocyte – are not able to activate the oocyte and drive embryo development (Colombero *et al.* 1999). For example, microinjection of parts of the sperm (such as the head or the tail) gives rise to an embryo that is not able to withstand several mitotic divisions (Moomjy *et al.* 1999).

The following sections scrutinize what we know about sperm components that are potentially involved in the interaction with the oocyte (i.e., gamete fusion), and what happens thereafter (oocyte activation and onset of embryogenesis). This includes: sperm proteome, centrioles, chromatin condensation and DNA integrity, epigenome and metabolome. The seminal plasma is approached in a separate section.

The Sperm Proteome

The sperm proteome differs between fertile and infertile men (Légaré *et al.* 2014). Castillo *et al*. (2018) conducted a meta-analysis, identifying 103 sperm proteins that could be involved in fertilization and 93 that could play a role during early embryo development. This section is split two different subsections, referring to sperm proteins that could play a role (i) during, and (ii) after fertilization.

Sperm Proteins Involved in Fertilization

The fusion of sperm and oocyte membranes is a complex process that appears to involve many proteins; some of the protein–protein interactions have been established in the last decade. From the sperm side, the proteins that interact with their oolemma counterparts are located in the equatorial region. One of these proteins is IZUMO1 (Inoue *et al.* 2005; Hirohashi and Yanagimachi 2018), which interacts with JUNO, an oocyte membrane protein; this interaction is needed for gamete fusion and the subsequent engulfment of the spermatozoon into the oocyte (Bianchi *et al.* 2014; Jean *et al.* 2019).

In addition to IZUMO1, other sperm proteins have been suggested to mediate gamete fusion. For example, tetraspanins CD9, CD81 and CD151 are located in the equatorial region of sperm, and have been found to be involved in the fusion of oocyte and sperm membranes (Jankovicova *et al*. 2020). Another protein found in the sperm plasmalemma, TMEM95, appears to play a similar role (Lamas-Toranzo *et al.* 2020).

Sperm Proteins Involved after Fertilization

Whether the male proteome can contribute to early embryo development has been a subject of research, despite the technical difficulties to address the matter. From the 93 sperm proteins potentially involved in post-fertilization events, 11 have been suggested to have a mission during the first embryo divisions (up to eight-cell stage), 29 in the formation of morulae, and 19 in blastocyst development.

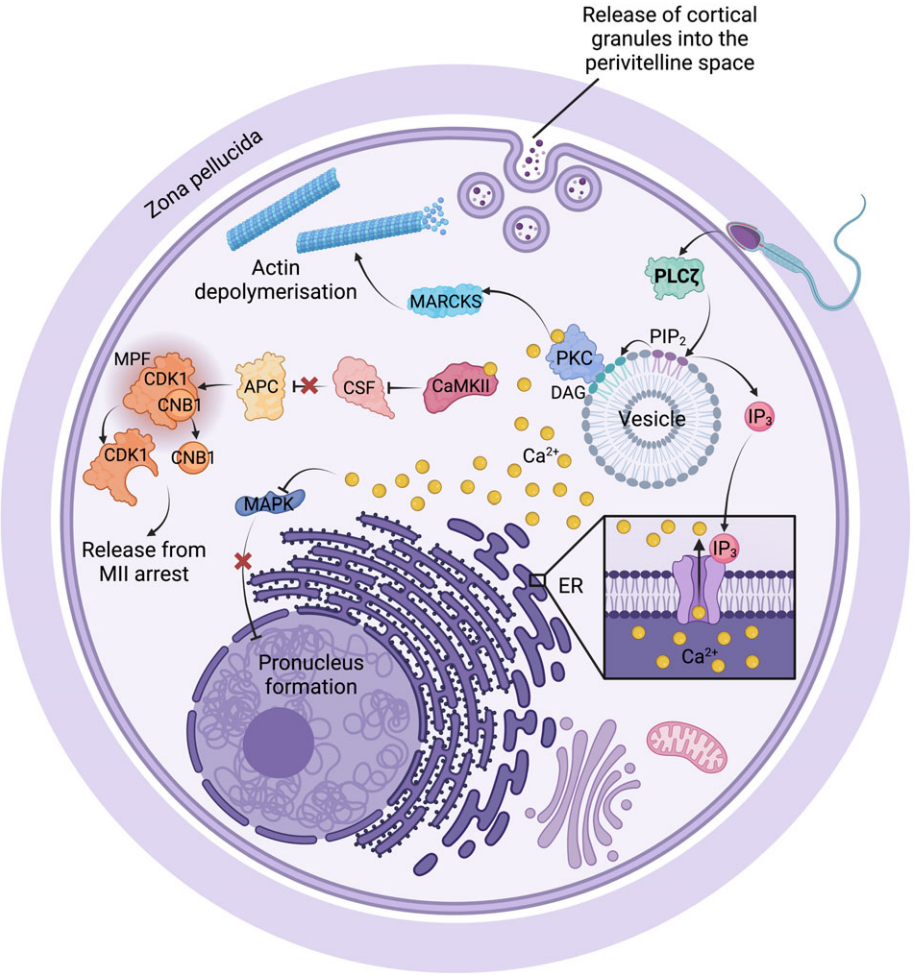

Figure 3. Protein phospholipase C zeta (PLCζ). Sperm-specific protein PLCζ is involved in oocyte activation. Upon gamete fusion, it is released into the ooplasm, where it triggers the signalling pathway that alleviates the oocyte from the metaphase-II arrest. Abbreviations: APC, Anaphase-promoting complex/cyclosome; CaM/CaMKII, Calmodulin/Calmodulin-dependent protein kinase II; CSF, Cytostatic factor; CNB1, Cyclin B1; CDK1, Cyclin-dependent kinase 1; DAG, Diacylglycerol; IP3, Inositol 1,4,5-trisphosphate; IP_3R, IP3 receptor; MAPK, Mitogen-activated protein kinase; PIP2, Phosphatidylinositol 4,5-bisphosphate; PKC, Protein kinase C. Reproduced from Yeste et al. (2023) with permission.

A sperm-borne protein that has been largely confirmed to be relevant for what occurs after gamete fusion is phospoholipase C Zeta (PLCζ), a sperm-specific phospholipase C located in the equatorial and post-acrosomal regions (Figure 3) (Yeste *et al.* 2023). Mounting evidence supports that this protein is involved in oocyte activation, which is the process that releases the oocyte from its metaphase-II arrest, then allows for the onset of embryogenesis, and is featured by Ca^{2+} oscillations in the

oocyte cytoplasm (Saunders *et al.* 2002; Horner and Wolfner 2008; Yeste *et al.* 2016, 2023). Sperm devoid of PLCζ are unable to trigger Ca^{2+} oscillations, which leads to oocyte activation deficiency and lower fertilization rates (Heytens *et al.* 2009; Heindryckx *et al*. 2013; Kashir *et al.* 2011, 2013; Yelumalai *et al.* 2015). Mutations in the coding sequence of this gene result in an impaired activity of this protein and total fertilization failure (Escoffier *et al.* 2016). A study conducted by Hachem *et al.* (2017) used a *Plcz1* knock-out male mouse model and confirmed the crucial role of this protein for oocyte activation, although the sperm from *Plcz1* knock-out males were still able to initiate embryogenesis after oocyte fertilization. In addition, total fertilization failure has been found to occur when sperm present the adequate levels of PLCζ. All these data suggest that sperm proteins other than PLCζ could be involved in oocyte activation. For this reason, other sperm proteins, such as post-acrosomal sheath WW domain-binding protein (WBP2NL), have been suggested to play a role during oocyte activation (Wu *et al.* 2007; Aarabi *et al.* 2010, 2014; Kennedy *et al.* 2014; Kaya *et al.* 2022). Yet, the exact function of WBP2NL has not been ascertained, as other studies did not observe the same results (Nomikos *et al.* 2014, 2015; Freour *et al.* 2017).

Other proteins that could play a role after fertilization are desmocollin 3 (DSC3), which is delivered via sperm to the zygote (Den *et al.* 2006) and has been proposed to regulate cell adhesion in blastomeres before embryo genome activation (EGA) (Castillo *et al.* 2018); lactosylceramide 1,3-N-acetyl-beta-D-glucosaminyltransferase (B3GNT5), which would be implied in morula formation (Biellmann *et al.* 2008); and choline-phosphate cytidyltransferase A (PCYT1A), which could be implicated in blastocyst development and embryo implantation (Wang *et al.* 2005).

The Centrioles

Centrioles are crucial organelles in eukaryotic cells, and are involved in the organization of the cytoskeleton, cell division, and formation of flagellum. Mature sperm have two centrioles located in the neck: the proximal (PC) and the distal centriole (DC) (Figure 4) (Khanal *et al.* 2021; Leung *et al.* 2021). In most eutherian mammals except rodents, paternal centrioles are inherited by the fertilized oocyte, which does not have centrioles. For this reason, paternal centrioles organize the cytoskeleton of the zygote, mediate pronuclei migration (Scheffler *et al.* 2021) and drive cell cleavage (Rawe *et al.* 2002; Amargant *et al.* 2021; Avidor-Reiss *et al.* 2019; 2022). Sperm from infertile men have been identified to have abnormal centrioles (Turner *et al.* 2021), and the fact that paternal centrioles in humans have a crucial function after fertilization has been suggested to be one of the reasons for the greater proportion of aneuploid embryos in humans compared with rodents (Cannarella *et al.* 2020).

The Relevance of Nuclear Integrity: Chromatin and DNA

In contrast to prokaryotic cells, DNA is bound to proteins in the eukaryotic ones forming a structure called chromatin, which, during interphase, is isolated from the

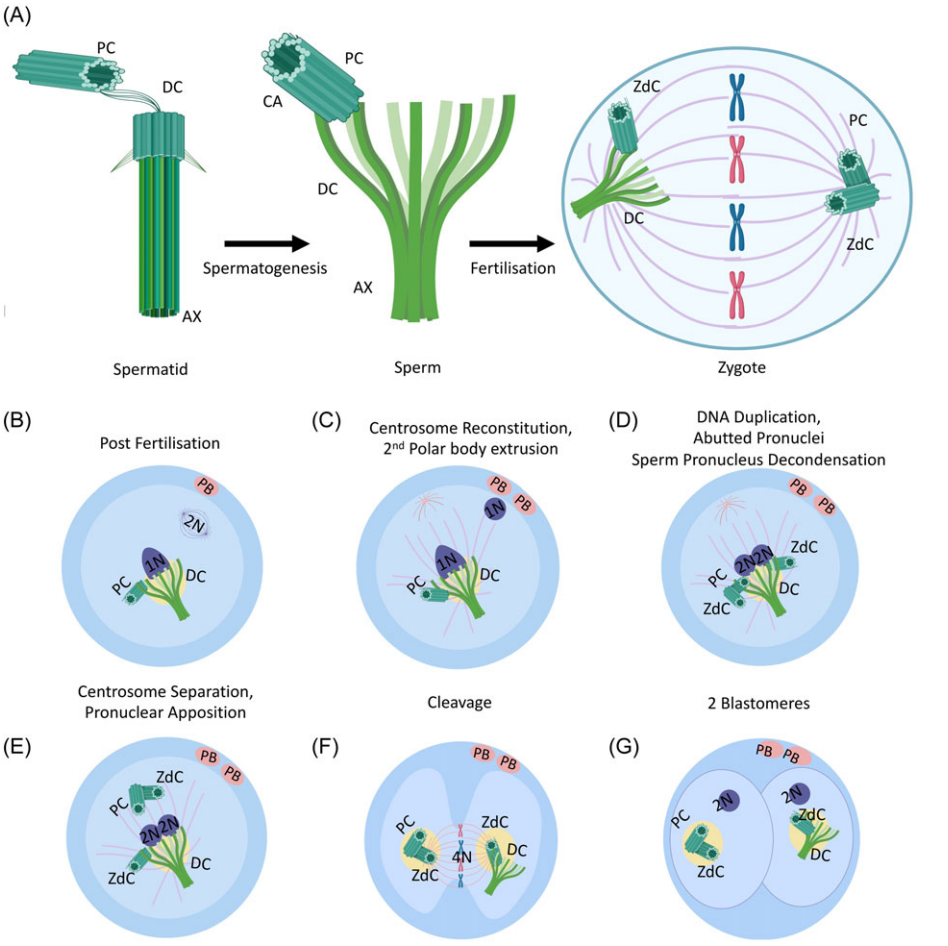

Figure 4. Sperm centrioles. Centrioles are crucial organelles in eukaryotic cells, which are involved in the organization of the cytoskeleton, cell division, and flagellum formation. Mature sperm have two centrioles located in the connecting piece: the proximal (PC) and the distal centriole (DC) **(A)**. After gamete fusion (B), the sperm centrosome forms an aster while the oocyte completes meiosis II and the second polar body is extruded **(C)**. Thereafter, centrioles begin to duplicate to produce two daughter centrioles **(D)**, which is followed by the separation of the zygote centrosomes **(E).** Subsequently, the zygote undergoes mitosis **(F)**, leading to the formation of two blastomeres. Abbreviations: Ax, Axoneme; Ca, Centriole adjunct; DC, Distal centriole; PC, Proximal centriole; PCL, Proximal centriole-like structure; ZdC, Zygotic daughter centriole; PB, Polar body; N, Ploidy. Reproduced from Vallet-Buisan *et al.* (2023) with permission.

rest of the cytoplasm thanks to the nuclear envelope. While chromatin is formed by DNA and histones (which may bear epigenetic signatures) in somatic cells (Bartosovic *et al.* 2021), mature sperm get most of these histones (85–90%) replaced by protamines (protamine 1 and protamine 2, in the case of humans) (Figure 5)

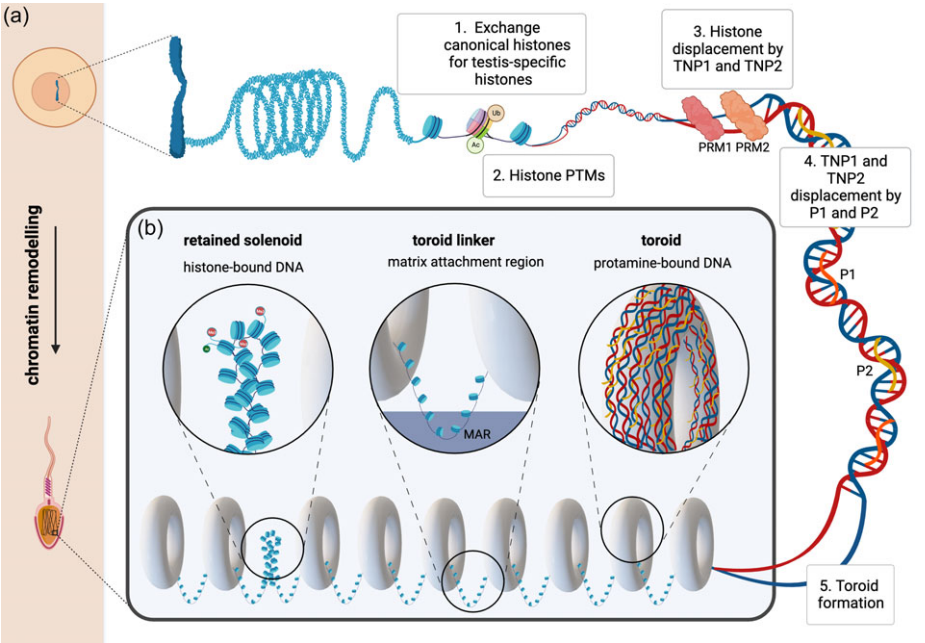

Figure 5. (a) Chromatin remodelling during spermiogenesis. Some histones are replaced with testis-specific histone variants. Histones then undergo post-translational modifications. Following this, histones are replaced by transition nuclear proteins, and these by protamines, which ultimately increases chromatin packaging. (b) **Organization of chromatin in sperm.** In sperm, chromatin is organized into three components: the most abundant, which consists of DNA bound to protamines; histone-bound DNA; and nuclear matrix attachment regions (MAR). Protamine-bound DNA is coiled into toroids, and histone-bound DNA is present in retained solenoids and toroid linkers. Abbreviations: MAR, Matrix attachment region; PTM, post-translational modification; P1, protamine 1; P2, protamine 2; TNP, transition nuclear protein. Reproduced from Balder *et al.* (2024) with permission.

(Brykczynska *et al.* 2010; Jung *et al.* 2017). This occurs during spermiogenesis, when the canonical organization of chromatin with nucleosomes changes to a toroidal one around protamines (Barral *et al.* 2017). The different toroids, each containing about 200 kb of DNA, are linked with toroid-linker regions, which contain histones and mediate the attachment to the proteinaceous nuclear matrix (Narwade *et al.* 2019). Unlike histones, protamines contain arginine to allow for stronger binding to DNA in order to form a toroid-like ridged structure. As sperm do not have all the machinery required for repairing DNA damage, the greater degree of condensation provided by protamines has been suggested as a strategy to maintain DNA integrity better (Ni *et al.* 2016). Remarkably, the 10–15% of histones retained in the sperm chromatin may bear epigenetic marks, such as H3K23me, and are organized in nucleosomes (Champroux *et al.* 2018). The potential contribution of these epigenetic signatures brought into the zygote by sperm is discussed later (see the section about the sperm epigenome).

Chromatin Protamination and Condensation

An insufficient replacement of histones by protamines is associated with chromatin immaturity, and may lead to an impaired embryo development (Ribas-Maynou *et al.* 2023); the relevance of this sperm protamination may be masked by oocyte factors. Specifically, when the histone-to-protamine ratio is lower than 6% or greater than 26%, embryo development is impaired (Fournier *et al.* 2018). On the other hand, and as aforementioned, chromatin in human sperm contains two protamines (P1 and P2), which should be in a 1:1 ratio (Sarasa *et al.* 2020). Alterations in this ratio are related to male infertility and poor embryo quality (Rogenhofer *et al.* 2017; Amor *et al.* 2019).

On the other hand, chromatin condensation can be evaluated through the integrity of disulphide bridges between protamines, and has been found to be a consequence of deficient protamination. A lower degree of chromatin condensation is linked to male infertility (Ribas-Maynou *et al.* 2023).

DNA Integrity

Damage in sperm DNA occurs in one (single) or two (double) DNA strands and underlies male infertility and impaired embryo, and may negatively affect offspring health (Fernández-González *et al.* 2008; Sedó *et al.* 2017; Borges *et al.* 2019; Ribas-Maynou *et al.* 2021b; 2022a). Sperm DNA damage can arise from different factors including lifestyle habits, such as nutrition (Jurewicz *et al.* 2018) and smoking (Cui *et al.* 2016; Muñoz *et al.* 2024); diseases, such as diabetes (Condorelli *et al.* 2018), obesity (Fullston *et al.* 2015), cancer (Meseguer *et al.* 2008), and male genital tract infections (Han *et al.* 2021); advancing male age (Evenson *et al.* 2020; Vaughan *et al.* 2020; Guo *et al.* 2023); altered histone-to-protamine ratio (Yoshida *et al.* 2018); insufficient chromatin condensation; abortive apoptotic-like changes (Shukla *et al.* 2012), and oxidative stress (Dorostghoal *et al.* 2017). The detrimental impact of sperm DNA damage on the embryo can be more apparent after embryo genome activation – which includes the expression of paternal-inherited genes – occurring at the 4/8-cell stage (Wong *et al.* 2010).

As aforementioned, sperm do not have all the machinery required for repairing DNA breaks. While the higher degree of chromatin condensation is intended to prevent it, DNA fragmentation may still occur (Smith *et al.* 2013). Although, in this scenario, the oocyte plays a crucial role in restoring the integrity of paternal DNA (Shimura *et al*. 2002; Lord and Aitken 2015), its repair capacity is limited, so that it may not be able to fix all paternal DNA damage. Thus, and as clinical data support, alterations in paternal DNA may be passed on to the embryo, which increases the mutational load, and may lead to embryo development arrest, failure to implant, miscarriage, and reduced clinical pregnancy and birth rate (Avendaño *et al.* 2010; Robinson *et al.* 2012; Lane *et al.* 2014; Ohno *et al.* 2014; Zhao *et al.* 2014; Simon *et al.* 2017b; Haddock *et al.* 2021; Ribas-Maynou *et al.* 2021b, 2022a). It is worth noting that the effects of sperm DNA fragmentation on embryo development and

other reproductive outcomes have been found to not completely agree across studies, as a meta-analysis compiling data from 25,000 IVF and ICSI cycles indicated (Ribas-Maynou *et al.* 2021b). These disparities could be explained by the different tests – the most used being sperm chromatin structure assay, sperm chromatin dispersion test, single-cell gel electrophoresis, also known as Comet, and TUNEL – employed to evaluate sperm DNA damage (Vallet-Buisan *et al.* 2023). The ART utilized also matters, as a negative correlation between sperm DNA fragmentation and reproductive outcomes (blastocyst development and pregnancy rates) was observed in the case of IVF but not in that of ICSI (Ruvolo *et al.* 2013; Cankut *et al.* 2019; Ribas-Maynou *et al.* 2021b). All this evidence supports the need for testing sperm DNA integrity when total fertilization failure and/or impaired embryo development occur, in order to discard male factor infertility.

Other Influences from Altered Paternal Genome

Alteration in paternal DNA can also lead to aneuploid embryos, although in most cases aneuploidy results from problems in the first meiotic division of the oocyte (Oldereid *et al.* 2018, Wang *et al.* 2020). Aneuploidies in sperm result from aberrant spermatogenesis, which manifests in poor morphology (e.g., macrocephalic sperm, teratozoospermia, oligoasthenoteratozoospermia) (Mehdi *et al.* 2012; Braham *et al.* 2019, Nayel *et al.* 2021; Saei *et al.* 2021).

The Sperm Transcriptome

Although mammalian sperm are widely regarded as transcriptionally silent cells and they have about 600 times less mRNA than somatic cells (Zhao *et al.* 2006; Ren *et al.* 2017), their nuclei exhibit residual DNA and RNA polymerase activity (Bianchi *et al.* 2018). Whether sperm transcripts could play a function is, however, not clear. Many have suggested that sperm mRNAs could be delivered to the oocyte, where they could be involved in the onset of embryogenesis, perhaps before embryo genome is activated (Ko *et al.* 2000; Hayashi *et al.* 2003; Ostermeier *et al.* 2004; Jodar *et al.* 2015; Ntostis *et al.* 2017). Different sperm-borne transcripts, such as *PSG1* and *HLA-E,* have been hypothesized to be involved in embryo implantation (Avendaño *et al.* 2008), perhaps because they play an immunotolerance function, with the sperm levels of *PSG1* and *HLA-E* mRNA being higher in fertile than in infertile men (Avendaño *et al.* 2009). Other transcripts, such as *SSFA2* and *SESN1,* have been suggested to be involved in the development of pig embryos until the 4-cell stage, when EGA occurs (Guo *et al.* 2017). Embryo development is also related to the levels of protamine-1 (*PRM1*), protamine-2 (*PRM2*), POU domain class 5 transcription factor 1 (*POU5F1*, also known as *OCT4*), glutathione peroxidase 4 (*GPX4*), and heat shock protein 90 (*HSP90AA1*) transcripts in sperm (Cho *et al.* 2003; Meseguer *et al.* 2006; Hwang *et al.* 2013; Rogenhofer *et al.* 2017; Sadakierska-Chudy *et al.* 2020). As most of these studies assume that sperm transcripts play a role during embryogenesis

because they are present in the embryo, further research to ascertain their specific function is needed. It also remains unaddressed when and how they are degraded, as in the mouse this happens as early as the zygote stage (Hayashi *et al.* 2003).

The Sperm Epigenome

Epigenetics involve a series of regulatory mechanisms that modify gene expression without altering the DNA. In sperm, the epigenome includes DNA methylation, histone modifications and non-coding RNAs (Figure 6); all these elements can also contribute to embryo development (Smith *et al.* 2014).

DNA Methylation

Methylation of DNA is one of the epigenetic modifications that modulates gene expression. Methylation and demethylation are mediated through DNA-methyltransferases (DNMTs) and TET enzymes (Kaneda *et al.* 2004; Yagi *et al.* 2020). Sperm DNA methylation is established during spermatogenesis, and active demethylation of paternal DNA by dioxygenase ten-eleven translocation 3 (TET3) enzyme occurs in the early embryo before EGA (Lee *et al.* 2018; Cheng *et al.* 2019). Alterations in sperm DNA methylation profile are linked to male infertility, impaired embryo development and miscarriage (Aston *et al.* 2015; Cao *et al.* 2020; Richard Albert *et al.* 2020). Paternal DNA regions linked to H3K9me2 are not demethylated, and can be related to imprinted loci, as in the case of *H19* and *RASGRF1* genes (Nakamura *et al.* 2007). Alterations in the imprinting of these genes are associated with congenital syndromes, including Angelman, Prader-Willi and Beckwith-Wiedemann syndromes (Kobayashi *et al.* 2009; Hattori *et al.* 2019; Inoue *et al.* 2020). In addition, altered methylation patterns in the regions of sperm DNA that have retained histone CpG islands are linked to impaired embryo development (Denomme *et al.* 2017). Specifically, high levels of hypomethylated DNA are related to reduced fertilizing ability (Pacheco *et al.* 2011).

Modifications in Histones

Acetylation, methylation, phosphorylation and chronotylation are post-translation modifications in histones that regulate gene expression. As aforementioned, retained paternal histones can bear epigenetic signatures and be inherited by the embryo (Ozturk *et al.* 2021). These retained histones have been found to be associated with developmental genes – such as HOX gene clusters – in human sperm (Hammoud *et al.* 2009). In addition, these retained histones could be located in the linker regions (Ribas-Maynou *et al.* 2021a; 2022b), and their alterations could be related to impaired embryo development (Hammoud *et al.* 2011; Vieweg *et al.* 2015; Glanzner *et al.* 2017; Huang *et al.* 2019). Specific lysine modifications of sperm-borne H3, such as H3K4me3 (Deng *et al.* 2020; Lambrot *et al.* 2021) and H3K27me3 (Sun *et al.*

2021), are involved in EGA. Also, before EGA, the male chromatin packaged into protamines is reorganized, thanks to the involvement of nucleoplasmins; and maternal histones, such as H3.3, TH2A and TH2B, are deposited onto paternal chromatin (Ishiuchi *et al*. 2021). Interestingly, sperm from infertile men have reduced levels of six histone variants, which has been proposed as one of the reasons for impaired embryogenesis (Azpiazu *et al.* 2014).

Non-coding RNAs

Non-coding mRNAs include miRNAs, siRNAs, piRNAs, circular (circ) RNAs and lnc-RNAs, and are found in human sperm, where they could be involved in fertilization and embryo development (Salas-Huetos *et al.* 2020). Most of the evidence comes from microRNAs, which are small single-strand RNAs that regulate gene expression, and are associated with male infertility (Lian *et al.* 2010; Marcet et al. 2011; Romero *et al.* 2011; Comazzetto *et al.* 2014; Gou *et al.* 2017). miR-34c, one of the most abundant miRNAs in human sperm (Salas-Huetos *et al.* 2014), plays an essential role during the first cleavage in mice (Liu *et al.* 2012b). Another important sperm miRNAs is miR-216b, which regulates the expression of a protein that participates in cell proliferation and differentiation in two-cell embryos, and is known as KRAS (Alves *et al.* 2019). Most of these miRNAs could be acquired during epididymal maturation, via extracellular vesicles, and could be one of the ways of passing epigenetic marks from the father to the offspring (Zhang *et al.* 2018; Chan *et al.* 2020). Other ncRNAs, such as tRNAs, can also be passed onto the offspring from the father (Sharma *et al.* 2018; Chen *et al.* 2016; Zhang *et al.* 2018). Furthermore, the miRNA profile differs between fertile and infertile patients (Liu *et al.* 2012a).

On the other hand, lncRNAs and circRNAs in sperm, which are circular single-strand molecules of RNA, can regulate the function of proteins, mRNAs and miRNAs, and even have a role in embryo development (Dang *et al.* 2016; Corral-Vazquez *et al.* 2021; Li *et al.* 2021). For instance, circCNOT6L is brought by sperm to the oocyte, where it participates in the transition from zygote to 2-cell stage (Chioccarelli *et al.* 2021). It is worth mentioning that lncRNA and circRNA cargo in sperm differs between men with good and poor sperm quality (Chioccarelli *et al.* 2019; Manfrevola *et al.* 2020).

The Sperm Metabolome

Metabolomics has been emerging over the last decade and has been proved to have many applications. Most of the studies focused on semen investigated the metabolites of seminal plasma. One of these studies revealed that men suffering from oligoasthenospermia had significantly lower values of free L-carnitine, polyunsaturated fatty acids, glutamate, aspartate, methionine, tryptophan, proline, and alanine; and four biogenic amines (spermine, spermidine, serotonin,

and alpha-aminoadipate). This deficiency in L-carnitine could be related to a decreased activity of β-oxidation of fatty acids, and oxidative phosphorylation, whereas reduced levels of amino acids and biogenic amines could be related to dysregulated signalling pathways (Boguenet *et al.* 2020).

Other research, which also targeted seminal plasma, compared fertile and infertile patients and identified up to 40 molecules (metabolomics/lipidomics approach) differing between fertile and infertile men. Acylcarnitines, phosphatidylserine (PS) (40:2) and lactate were lower, and PE (18:1; 18:1), phosphatidic acid (PA) (O-19:2; 18:1), lysophosphatidylethanolamine (LPE) (O-16:1) and phosphatidylcholine (PC) (O-16:2; 18:1)-CH3 were higher in infertile patients (Correnti *et al.* 2023). In a systematic review, Llavanera *et al.* (2022) compiled a list of metabolites whose levels in seminal plasma were significantly different between fertile and infertile men, including lactate, alanine, choline, citrate, glycero-phosphocholine, glutamine, tyrosine, histidine, phenylalanine, and uridine. Another study found that smoking alters the metabolism of sperm, which could potentially affect fertilizing ability, as it decreases the uptake of fatty acids by sperm mitochondria, which in turn decreases energy supply (Engel *et al.* 2021).

On the other hand, and given the events occurring during gamete fusion and thereafter, it seems quite unrealistic that sperm metabolites have a direct influence on fertilization and early embryo development. In spite of this, Guo *et al.* (2023) compared sperm from young and aged men, and identified 129 differentially expressed metabolites; from these, four, such as pipamperone, 2,2-bis(hydroxymethyl)-2,2',2''-nitrilotriethanol, Arg-Pro and triethyl phosphate, were more abundant in aged men. These findings are supported by investigations performed in animal models. In cattle, a previous study found that from 3704 metabolites identified in sperm, bulls with different fertility differed in the levels of 33 metabolites. These metabolites were related to taurine and hypotaurine, whose levels were reduced in bulls with low fertility, and also with different routes, such as glycolysis, β-oxidation of fatty acids, and synthesis of pyrimidines (Talluri *et al.* 2022).

Also in cattle, a study using liquid chromatography–mass spectrometry (LC-MS) identified up to 3704 metabolites in sperm, and identified five metabolites (hypotaurine, selenocysteine, l-malic acid, d-cysteine, and chondroitin 4-sulfate) that differed between bulls of high and low fertility (Saraf *et al.* 2020). In pigs, sperm with higher quality and fertilizing ability, and giving rise to more embryos at day 6 having greater levels of glycolysis-derived metabolites than those with poor quality, were found to have greater amounts of metabolites related to oxidative phosphorylation. This suggests the basal metabolism in mammalian sperm could be relevant for the ability of sperm to fertilize the oocyte and give rise to a viable embryo (Mateo-Otero *et al.* 2023). Finally, another study also conducted in pigs found that alteration of sperm mitochondrial activity, which impacts oxidative phosphorylation and metabolism, could increase ROS levels, which could in turn affect sperm DNA integrity and ultimately embryo development (Mateo-Otero *et al.* 2024).

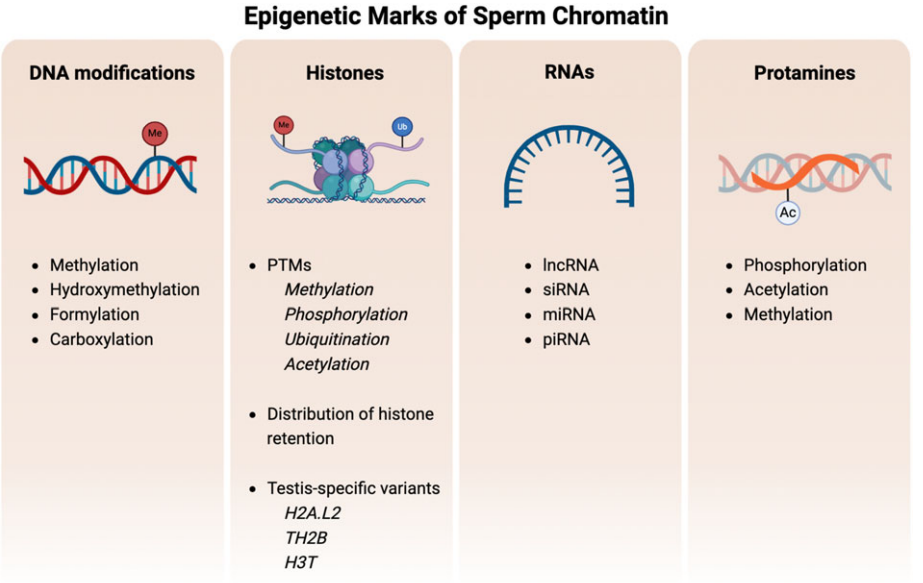

Figure 6. Epigenetic signatures in sperm. The contributors to the sperm epigenome include modifications to DNA and histones, RNAs and protamines. Abbreviations: lncRNA, long noncoding RNA; miRNA, microRNA; piRNA, piwi-interacting RNA; PTMs, post-translational modifications; siRNA, small interfering RNA. Reproduced from Balder *et al.* (2024) with permission.

All this compiled evidence, therefore, indicates that sperm metabolic fate influences fertilizing ability and could be even relevant while giving rise to a viable embryo, as the yet few studies in humans and animal models suggest.

Does the Seminal Plasma have Any Role?

Seminal plasma is the liquid part of semen, and is produced by epididymis and male accessory glands (prostate, seminal vesicles) (Figure 7). While this fluid has largely been regarded as a mere vehicle for sperm upon ejaculation, growing evidence supports that it interacts with the female reproductive tract and can be involved in the modulation of the uterine environment, embryo development, and foetal growth (Bromfield *et al.* 2014; Watkins *et al.* 2018). In mice, seminal plasma modulates the expression of tumour necrosis factor (TNF), interleukin-1β (IL-1β), interferon-γ (IFN-γ), microphage inflammatory protein 1-α (MIP-1α) and colony stimulating factor 3 (CSF3), and the expression of regulatory T-cell genes (Watkins *et al.* 2018) in the endometrium, which supports its immunomodulation role. This would increase the maternal tolerance to paternal and foetal antigens, and facilitate embryo implantation in mice (Robertson *et al.* 1996, 2001; Bromfield *et al.* 2014).

Since humans do not ejaculate in the uterus, as mice do, but in the vagina, the role that seminal plasma could play in this species is less clear, as it remains unknown

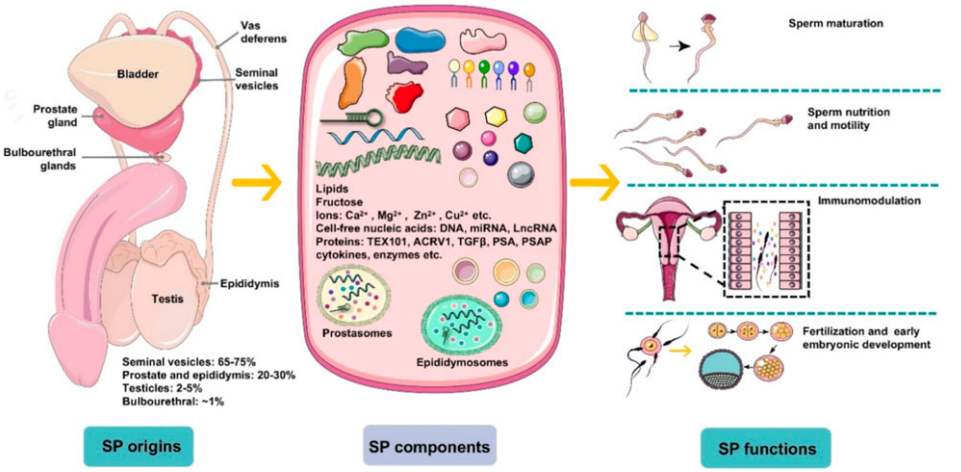

Figure 7. Origin, components, and functions of seminal plasma (SP). Seminal plasma represents more than 95% of the semen volume, whereas testicular secretions containing sperm account for 2–5%. The seminal plasma is composed of a complex set of heterogeneous molecules, such as proteins (enzymes, cytokines, TEX101, ACRV1, TGFββ, prostate-specific antigen (PSA), prostatic-specific acid phosphatase (PSAP), etc.), lipids, sugars (fructose), cell-free nucleic acid (DNA, microRNA, and LncRNA), ions (Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, etc.), and small-molecule metabolites. Not only does SP modulate sperm function, but some of its components, such as cytokines, also recognize receptors on epithelial cells lining the cervix and uterus and induce the synthesis of pro-inflammatory cytokines and chemokines that recruit and activate inflammatory leukocytes. The SP also modulates the release of cytokines and growth factors, which appear to regulate embryo development in the oviduct and uterus before implantation. Reproduced from Wang *et al.* (2020), which was published under the terms and conditions of a Creative Commons Attribution license (CC BY 4.0).

whether it reaches the uterus. Studies in other animal models that, like cattle, also ejaculate in the vagina indicate that seminal plasma factors could adhere onto the sperm surface, thereby influencing the uterine environment (Recuero *et al.* 2020), as in this species they also induce the production of cytokines and chemokines in the female genital tract (Schjenken *et al.* 2015; Nongbua *et al.* 2020). These findings would match those in humans, where seminal plasma has been proposed to prime the maternal endometrium for implantation (Ibrahim *et al.* 2019; George *et al.* 2020; Ajdary *et al.* 2021), as it increases pregnancy rate after embryo transfer (Wolff *et al.* 2009; Chicea *et al.* 2013; Friedler *et al.* 2013; Crawford *et al.* 2015).

Seminal plasma contains extracellular vesicles, mainly epididymosomes and prostasomes. These vesicles are membrane-bound particles released by cells (Yáñez-Mó *et al.* 2015). They have been found to be synthesized by the epididymis and prostate. In the case of the epididymis, these extracellular vesicles, called epididymosomes, fuse with epididymal sperm (Păunescu *et al.* 2014). Thanks to this fusion, epididymal cells can transfer proteins and miRNA to sperm (Twenter *et al.* 2020). Proteins identified in

these extracellular vesicles include enzymes, and others having a function for sperm motility (Barrachina *et al.* 2022), and even galactin-3, which is a lectin transferred from the seminal plasma to the sperm surface, that is involved in the binding of capacitated sperm to the zona pellucida (Mei *et al.* 2019).

Prostasomes, which are produced by prostate epithelial cells (Brody *et al.* 1983), also transfer proteins to sperm (Ronquist *et al.* 2011), and have been suggested to modify the composition of sperm plasmalemma (Dubois *et al.* 2015). Prostasomes cargo also seems to regulate sperm motility (Andrews *et al.* 2015; García-Rodríguez *et al.* 2018), capacitation (Pons-Rejraji *et al.* 2011; Aalberts *et al.* 2013), and even protect sperm from the female immune system (Milutinović *et al.* 2019; Paktinat *et al.* 2019). In humans, seminal plasma vesicles have been suggested to fuse with endometrial stromal cells, induce decidualization, and increase the secretion of prolactin (Rodriguez-Caro *et al.* 2019). All these data indicate that these seminal plasma vesicles play a role with regard to both sperm function and the female reproductive tract, and should be further investigated in the future.

Conclusions

Mounting evidence supports that semen, which is composed of cellular (sperm) and liquid (seminal plasma) fractions, does not only play a role during oocyte fertilization, but may even be relevant to embryo development and implantation, and modulate the female reproductive tract. Sperm components such as centrioles, chromatin condensation/protamination, DNA integrity, transcriptome, and epigenome appear to be important for post-fertilization events, including implantation. In addition, the sperm metabolome tells about their fertilizing ability, and some specific metabolomics signatures may indicate that the male gamete is more capable of giving rise to a viable embryo. All these findings contribute to shedding light onto the paternal factors underlying infertility and suggest that further research is needed to address to what extent these factors are crucial for embryo development and implantation, and whether they may influence offspring health.

Acknowledgements

The author appreciates the invitations from the Young Academy of Europe, and Academia Europaea, for giving him the opportunity to present his research at the 34th Annual Conference of Academia Europaea 'Building Bridges 2023'. The author also acknowledges the support from the Regional Government of Catalonia, Spain (2017-SGR-1229) and the Catalan Institution for Research and Advanced Studies (ICREA).

Conflict of Interest

The author declares no conflict of interest.

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