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Advanced sperm selection methods: effects on sperm parameters and IVF outcomes

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1. Abstract

To date, human infertility is considered by World Health Organization (WHO) as a serious global problem. It affects 10-15% of couples worldwide (Simon *et al.*, 2017) and approximately 20-30% of infertility cases are related to a male infertility factor. Semen parameters have a direct relation to male infertility and the periodic updates of the WHO manual for the examination and processing of human semen highlight an important decline in semen quality during the last 50 years (WHO laboratory manual, 2021). Moreover, increasing evidence are suggesting that seminal fluids with normal sperm parameters can conceal underlying molecular abnormalities, as DNA damages, able to negatively affect in vitro fertilization (IVF) outcomes (Sedò *et al*., 2017; Borges *et al*., 2019; Ribas-Maynou *et al*., 2021). DNA fragmentation may already exist in semen samples due to different conditions or can be induced or increased during assisted reproductive technologies (ART) semen manipulations. Traditional techniques of sperm selection aim to isolate the healthiest sperm in terms of vitality, motility and morphology, but can be inefficient in selecting spermatozoa without DNA fragmentation; furthermore, they often include centrifugation steps which may enhance DNA damages. In an attempt to optimize ART outcomes, alternative sperm selection methods have been proposed in the last years. This study evaluates the effect of two emerging sperm selection procedures, magnetic activated cell sorting (MACS) and microfluidics, on ART cycles in terms of sperm motility, fertilization rate, cleavage rate and pregnancy rate. Reported data are from couples who underwent one or more homologous or oocyte donation ART cycle at the Chianciano Salute clinic. The results from novel sperm selection techniques are compared to those of conventional methods. Collected data may inform ART clinical practice by clarifying the effectiveness of various sperm selection approaches, optimizing routinary used IVF laboratory protocols and improving ART treatments outcomes.

2. Introduction

2.1 Male reproductive system

The male reproductive system is responsible for the production and delivery of spermatozoa, the male gametes, into the female reproductive tract. The structures of the male reproductive system include the testes, the epididymis, the penis and the ducts and glands that produce and carry semen (Figure 1). Testes are crucial, as they produce both sperm and androgens, primarily testosterone, which support male reproductive physiology.

Several accessory organs and ducts aid the process of sperm maturation and transport the sperm and other seminal components to the penis, which delivers sperm to the female reproductive tract. The seminal vesicles and prostate gland add fluids to the sperm to create the semen (Moore *et al.*, 2010).

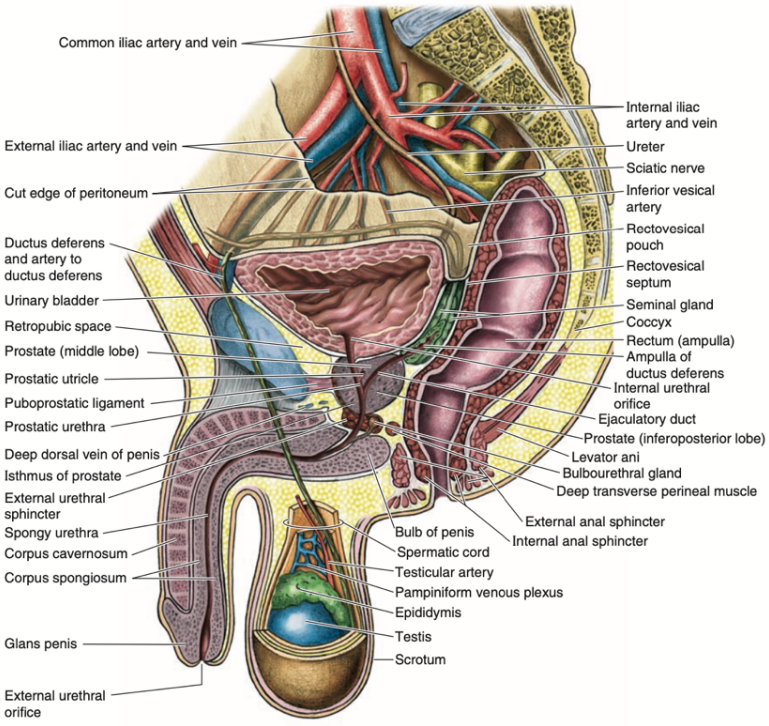


Figure 1. Male reproductive system, lateral view. (Moore *et al.*, 2010)

Testes are two oval-shaped organs, each approximately 4 to 5 cm in length and are suspended in the scrotum by the spermatic cords, with the left testis usually suspended more inferiorly than the right testis. The scrotum is a skin-covered, highly pigmented muscular sack that extends from the body behind the penis. The scrotal wall consists of a thin outer layer of skin followed by several underlying layers, arranged from the outside in as follows: the dartos muscle, external spermatic

fascia, cremaster muscle, internal spermatic fascia, and the parietal layer of the tunica vaginalis (Kuchakulla *et al.*, 2021). This multilayered structure serves to shield the testes from external trauma and plays a vital role in maintaining proper temperature conditions. The position of the testes outside the body allows them to remain at a temperature approximately 2–4°C lower than the body's core temperature, an essential factor for effective sperm production (Ivell, R. 2007; Moore *et al.*, 2010). Among these layers, the cremasteric muscle, which envelops both the testicles and the spermatic cord, has a significant function in thermal regulation. When this muscle contracts, it pulls the testicles upward toward the body, increasing warmth to support optimal temperature. Conversely, when the body needs to release heat, the muscle relaxes, allowing the testes to descend and cool away from the body's core heat (De Jong, M. R. 2020).

Internally, testes are surrounded by two distinct layers of protective connective tissue: the outer tunica vaginalis, with a parietal and a visceral layer, and the tunica albuginea. The tunica albuginea, from its internal ridge, invaginates to form septa that divide the testis into 300 to 400 structures called lobules. Inside the lobules are present from 3 to 10 long and highly coiled seminiferous tubules, in which spermatozoa develop. The seminiferous tubules are joined by straight tubules to the rete testis, a network of canals in the mediastinum of the testis (Figure 2) (Moore *et al.*, 2010; Houda *et al*., 2021).

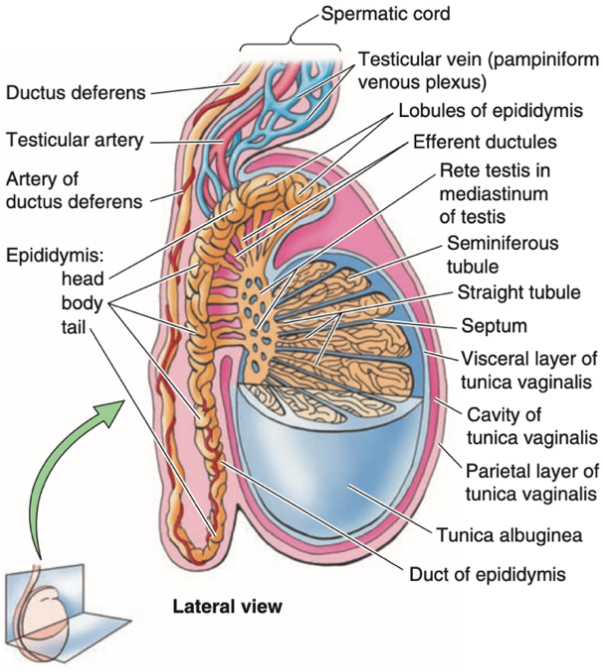


Figure 2. Internal structure of testicle and epididymis. (Moore *et al.*, 2010)

Seminiferous tubules consist of a basement membrane, Sertoli cells and germ cells at varying stages of maturation. The seminiferous tubule is surrounded by a peritubular tissue composed of layers of myoid cells, fibrocyte-like adventitial cells and collagen matrix (Figure 3). Together with Sertoli cells, this peritubular tissue forms the blood testis barrier (BTB) (Hermo *et al.*, 1977).

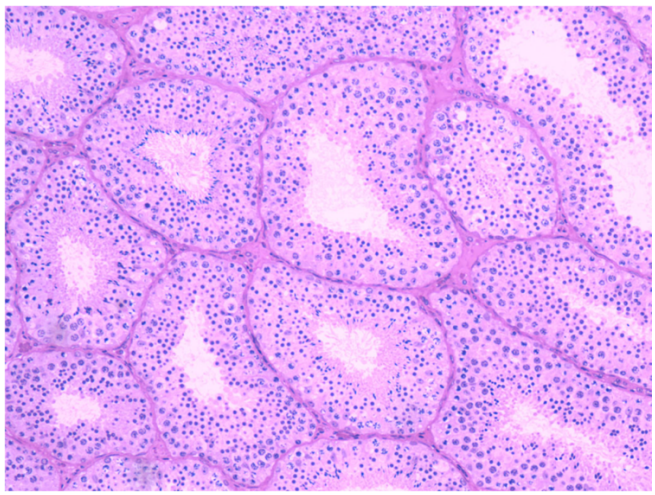


Figure 3. Histological section of seminiferous tubules. (www.medcell.org)

Peritubular myoid cells (PMC) are large, flat cells arranged in discontinuous cell layers and thanks to their ability to contract, they allow the propulsion of the testicular fluid containing immotile spermatozoa to the rete testis (Rossi *et al.*, 2002; Schell *et al.*, 2010). PMC are involved in the paracrine regulation of Sertoli cells functions, since they modulate the secretion of transferrin, inhibin and androgen-binding protein by Sertoli cells (Phillips et al., 2010). Through the secretion of fibronectin, collagens, proteoglycans and entactin, PMC have also a role in the production and maintenance of the BTB (Cigorraga *et al.*, 1994; Verhoeven *et al.*, 2000).

Together with PMC, outside the seminiferous tubules also Leydig cells are present. Leydig cells are polygonal cells arranged in clusters between blood vessels and seminiferous tubules. Their main product is testosterone, synthesized from cholesterol, and their secretion accounts for more than 95% of circulating testosterone in post-pubertal men. Testosterone (T) is the primary hormone produced by the testes, with a daily output ranging from 3 to 10 mg; it can either be stored within the testes or released into the bloodstream. This hormone plays a crucial role in regulating spermatogenesis as well as the development of secondary sexual characteristics. After puberty, Leydig cells become the main source of estrogen within the testes, particularly estradiol, which is synthesized from

testosterone through the action of the enzyme aromatase P450. In addition to steroid hormones, Leydig cells also produce insulin-like factor 3 (INSL3), which is essential for testicular descent during foetal development, influences bone metabolism and supports the migration of germ cells into the scrotum. They also secrete oxytocin, which contributes to the contraction of peritubular myoid cells (Phillips *et al.*, 2010).

The epithelium of the seminiferous tubules is occupied for 17-20% by Sertoli cells, irregular shaped cells with the base on the basal membrane and the apex in the lumen of the seminiferous tubule (Schulze *et al.*, 1976.). Sertoli cells are indispensable in the spermatogenesis process, since they support, thanks to their vast cytoplasmic ramifications, up to 30-50 germ cells at different stages of development (Cheng *et al.*, 2010). The interaction between germ cells and Sertoli cells plays a fundamental role in maintaining and regulating spermatogenesis in a highly structured and coordinated way. Driven by the expression of the *SRY* gene, present only in the Y chromosome, Sertoli cells undergo proliferation during the perinatal and neonatal stages. They then enter a prolonged quiescent phase, resuming proliferation shortly before puberty: at this point, they begin to differentiate and acquire the ability to fully support spermatogenesis (Houda *et al*., 2021). Once differentiated, Sertoli cells produce a variety of essential proteins for reproductive function: androgen-binding protein (ABP), inhibin, transferrin, vitamin transporters, lactate, acetate, transforming growth factor-beta (TGF-β) and interleukins. ABP, in particular, binds with high affinity to testosterone and dihydrotestosterone, concentrating these hormones within the testes and thereby creating an optimal environment for spermatogenesis. Moreover, Sertoli cells act as macrophages to clear degenerating germ cells or residual bodies from spermatids, a crucial event for sperm production (Nakagawa *et al.*, 2005).

Tight junctions, gap junctions, desmosomes and basal ectoplasmic specializations of adjacent Sertoli cells are the main components of the blood testis barrier, an anatomical and functional barrier that restricts the paracellular transit of substances and divides the seminiferous epithelium in two separate regions, the basal and the adluminal compartment (Cheng *et al.*, 2012). BTB is necessary since germ cells need to be kept in a tightly regulated microenvironment because to their high immunogenicity.

From the seminiferous tubules, the efferent ductules of the testis transport spermatozoa from the rete testis to the epididymis, which has an important role in storage function, transport and maturation of spermatozoa, with the development of progressive motility and fertilizing ability. During ejaculation, sperm exit the tail of the epididymis and reach to the ductus deferens thanks to smooth muscle contractions. The ejaculate is composed of sperm, accounting for approximately the 2-5% of the total volume, and seminal plasma, the liquid component. Seminal plasma is a mixture release by different glands with a slightly basic pH. Seminal vesicles give the main contribution, approximately the 65-75% percent of the total volume, and secrete an alkaline fluid rich in fructose, ascorbic acid, semenogelin I and II and prostaglandins. Fructose is the energy source used by sperm mitochondria to generate adenosine triphosphate (ATP) and allow flagellar movement. The prostate gland produces approximately the 20-30% percent of the ejaculate volume and release high concentrations of citric acid, prostatic acid phosphatase (PAP), the prostate specific antigen (PSA) and inositol (Batruch *et al.*, 2011; Kumar *et al.*, 2017).

2.2 Spermatogenesis and spermiogenesis

Spermatogenesis is a complex process occurring in the seminiferous tubules, that require approximately 74 days (Heller *et al.*, 1963) and goes from the onset of proliferation of a stem cell (spermatogonium) to the production and the release of mature male gametes into the lumen of the seminiferous tubule. Testes produce around 200-300 millions of spermatozoa daily and only about 100 million become mature viable gametes. During the process of spermatogenesis, several testicular structures and cells play a determinant role.

Male germ cells are located in the seminiferous tubules and they have a highly organized distribution, with less matured cells in the basal compartment progressing to the adluminal compartment as they mature (Heller *et al.*, 1964).

Primordial germ cells arise from extra-embryonic tissues around the yolk sac and between 3 and 5 weeks of development migrate to the gonadal ridge and differentiate into gonocytes under the stimulus of Sertoli cells factors. Gonocytes enters in the G0 phase and remain mitotically inactive until after birth. Between birth and 6 months, gonocytes go on with the differentiation in spermatogonia, which remain quiescent up to 5-7 years of age, when they increase in number through

mitosis. At puberty, spermatogonia begin the differentiation process to become spermatozoa (Paniagua *et al.*, 1984). The basal compartment of the seminiferous tubules with the basement membrane, PMC, Sertoli cells, together with the vascular network and local soluble factors, form the complex spermatogonial stem cell's niche microenvironment (Figure 4). Depending on the stimulus, a balance between self-renewal and differentiation factors regulates the fate of spermatogonial stem cells (SSCs) that can go through self-renewal, differentiation and/or apoptosis (Houda *et al.*, 2021).

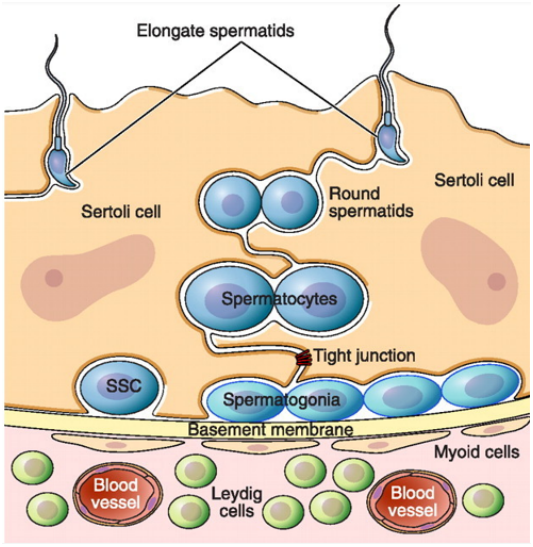


Figure 4 The spermatogonial stem cell's niche (SSCs) (Oatley, J. M. & Brinster, 2012).

The process of spermatogenesis can be subdivided in (1) proliferation of spermatogonial stem cells through mitosis, (2) differentiation of spermatogonia into spermatocytes, (3) meiotic divisions producing haploid spermatids, (4) morphological transformation of round spermatids into spermatozoa in a process known as spermiogenesis, and (5) release of mature sperm into the lumen of the seminiferous tubules, termed spermiation.

The process begins in the basal compartment of the seminiferous epithelium, where spermatogonial stem cells (SSCs) are located in close contact with Sertoli cells and the basement membrane. SSCs possess long-term self-renewal potential and are capable of both mitotic proliferation and differentiation and are categorized into three main subtypes: type A dark (A_{dark}), type A pale (A_{pale}), and type B spermatogonia (Clermont, Y. 1966; Larose *et al.*, 2019).

After their final mitotic division, type B spermatogonia give rise to primary preleptotene spermatocytes (Larose *et al.*, 2019).

Preleptotene spermatocytes can pass through the BTB since they are no single cells, but form syncytia in which the cells are connected through cytoplasmic bridges which allows the synchronization of the germ cell maturation (Houda *et al.*, 2021). As soon as the primary preleptotene spermatocytes are passed, the BTB undergoes reconstruction. Preleptotene spermatocytes finish the meiosis I and II, spermiogenesis and spermiation in the specialized adluminal compartment behind the BTB.

During meiosis I (reduction division) each diploid primary spermatocyte produces two haploid secondary spermatocytes over approximately 16 days. These secondary spermatocytes quickly enter meiosis II, wherein each divides into two haploid round spermatids, resulting in a total of four spermatids from a single primary spermatocyte (Figure 5).

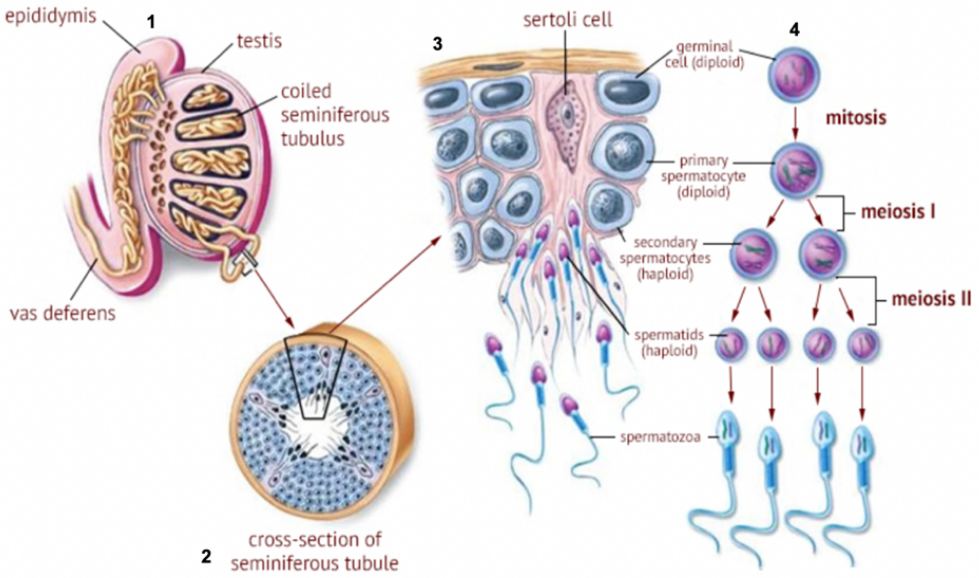


Figure 5. Structure and organization of the testicle: 1. Testicle; 2. Transversal section of the seminiferous tubule; 3. Magnification of the seminiferous tubule structure; 4. Main events of spermatogenesis. (Adapted from buffonescience9.wikispaces)

The subsequent spermiogenesis phase involves the transformation of these round spermatids into highly specialized spermatozoa. This process is marked by dramatic nuclear, cytoplasmic and structural changes and occurs without further cellular division. Spermiogenesis is subdivided into four morphological phases: the Golgi phase, the cap phase, the acrosome phase, and the maturation phase.

During the Golgi phase starts the development of several granules, the pre-acrosomal granules, rich in carbohydrates, which converge to form the acrosomal vesicle (Gupta, 2005). These pre-acrosomal granules are guided by microtubules to the apical region of the nucleus, where their membrane attaches to the nuclear envelope. As the acrosomal vesicle begins to develop, the centrioles positioned near to the Golgi apparatus translocate to the pole opposite to the developing acrosome, establishing a longitudinal polarity of the cell. The distal centriole became perpendicularly oriented to the cell surface and gives rise to the formation of the flagellum. The proximal centriole attaches to the nuclear envelope and eventually gives rise to the connecting piece in the neck region of the sperm. Concurrently cytoplasmic tubules arise and form a cylindrical structure called manchette. The elongation of spermatid is attributed to the movement of cytoplasm back along the manchette. The manchette is one of the numerous transient organelles developing during spermiogenesis and disappearing without any residue in spermatozoa. During the Golgi phase mitochondria migrate from the peri-nucleolar region to the periphery of the cell close to the plasma membrane.

During the cap phase of spermiogenesis, just after the acrosomal vesicle binds to the nuclear envelope, the attachment site begins to enlarge towards the caudal end of the cell until it covers half of the nuclear surface (Gupta, 2005).

The cap phase is followed by the acrosome phase, during which acrosome finishes its differentiation and mitochondria move from cell periphery to come closer to the developing flagellum. Simultaneously to the movement of mitochondria and the development of the mitochondrial sheath occur the organization the axoneme, the annulus and the outer dense fibers. The axoneme is constituted of two central single microtubules surrounded by nine peripheral doublets in continuation with the wall of distal centriole. During the differentiation of the tail, nine longitudinally oriented segmented columns arise around the central doublet. As manchette disappears, mitochondria organize themself around the portion of the flagellum between the annulus and the nucleus and start to be distributed helically around it to complete the differentiation of the middle piece of the flagellum. While these developmental events are in progress, a series of circumferentially oriented ribs are deposited around the tail to form the fibrous sheath of principal piece (Figure 6).

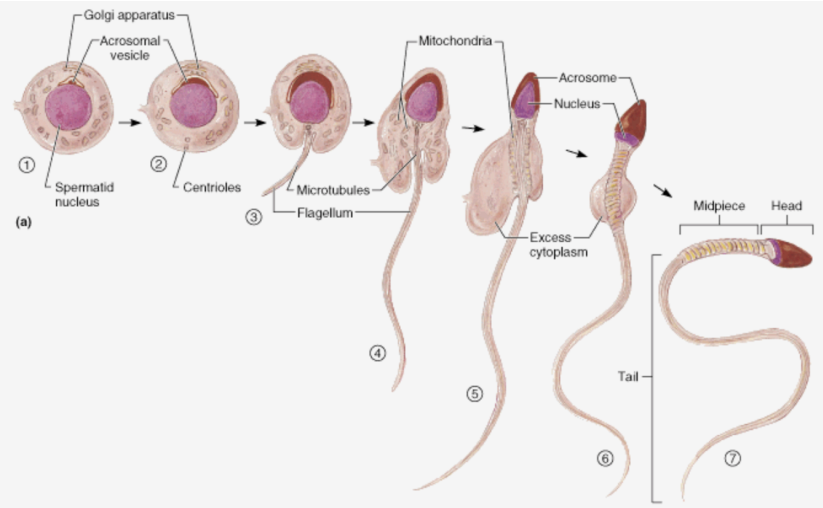


Figure 6. Main events of spermiogenesis. (Benjamin Cumming, an imprint of Addison Wesley Longman Inc., 2001)

In the maturation phase, excess cytoplasm is discarded as residual bodies, which are phagocytosed by Sertoli cells. Concurrently, chromatin modifications take place achieving the level of organization and compression necessary to fit the haploid genome into the compact sperm head and allowing its protection during all its journey through the male and female reproductive tracts (Houda *et al*., 2021).

In somatic cells, chromatin is organized into nucleosomes composed of DNA organized around histone proteins. Each nucleosome typically consists of nearly two loops of DNA wrapped around an octamer of histones. These histones can undergo various post-translational modifications that influence transcriptional regulation and gene accessibility (Bartosovic *et al*., 2021). During spermiogenesis most histones are removed and replaced with transition proteins (TP1 and TP2), and ultimately with protamines (PRM1 and PRM2) (Figure 7). Protamines are proteins rich in arginine, similar to histones but more positively charged, which condense the DNA into tightly packaged units called toroids, also known as doughnut loops. Protamine toroids are unique of the mature sperm cell and are connected one to another by a toroid linker called matrix attached region (MAR), chromatin domains more susceptible to DNA fragmentation.

This specialized packaging system protects the sperm DNA from potential damage, ensuring its structural integrity (Ni *et al*., 2016; Schneider *et al*., 2020).

Throughout this transition, the primary protamines involved, PRM1 and PRM2, typically substitute the canonical histones H2A, H2B, H3, and H4 (Wang *et al*., 2019). Despite this widespread histone replacement, certain histones such as H1

(linker histone), H3, and H4 are selectively retained and may carry epigenetic information (Govin *et al*., 2007; Kasimanickam *et al*., 2019).

During this compaction, physiological DNA strand breaks are introduced to facilitate protamination: these breaks are normally repaired before sperm maturation concludes. However, since late spermatids and mature spermatozoa lack DNA repair mechanisms, any persistent damage can result in spermatozoa with fragmented DNA being released into the ejaculate. Notably, these protamines are removed and replaced by histones shortly after fertilization, allowing for zygotic genome activation. Furthermore, some studies highlighted the potential of using histone–protamine ratios as biomarkers for predicting embryo quality at the blastocyst stage. Optimal development appears to occur when histone content ranges between 6% and 26%: exceeding this threshold may lead to an imbalance in paternal genome regulation, thereby impairing proper embryonic development up to the blastocyst stage (Fournier *et al*., 2018).

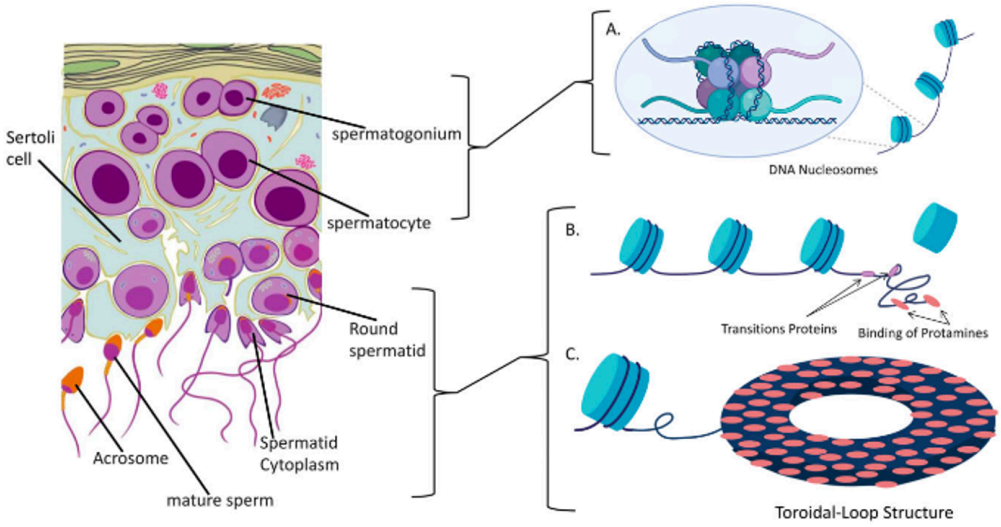


Figure 7. Histone to protamine transition. **A.** In spermatogonia and primary spermatocytes eight histones are packed together in a nucleosome. **B.** In round spermatids transition proteins help remove chromatin from the nucleosomes and bind it to protamines. **C.** Protamines form a toroidal loop structure (Montserrat *et al.,* 2023).

In humans, an equal ratio of protamine 1 (PRM1) to protamine 2 (PRM2) is generally considered normal (Sarasa *et al.*, 2020). However, disruptions in the timing of protamine gene expression can disturb this ratio: alterations in the PRM1:PRM2 balance, as well as a higher-than-normal histone-to-protamine ratio, have been strongly associated to male infertility (Aoki *et al.*, 2006; Rogenhofer *et al.*, 2013).

Deviations in protamine expression, observed at both the protein and mRNA levels, have also been linked to poor IVF outcomes and reduced embryo quality (Rogenhofer *et al*., 2017; Amor *et al*., 2019).

A perfect coordination of the hypothalamic-pituitary-gonadal axis is needed for the normal testicular function and for spermatogenesis. The gonadotropin releasing hormone (GnRH), an hypothalamic hormone, is secreted in a pulsatile manner and acts on specific membrane receptors located on the gonadotropic cells of the anterior pituitary gland. Upon stimulation, these cells release two gonadotropins: the luteinizing hormone (LH) and the follicle stimulating hormone (FSH) into the bloodstream. Both LH and FSH are glycoprotein hormones composed of two subunits: an alpha subunit, which is identical across LH, FSH, thyroid-stimulating hormone (TSH), and human chorionic gonadotropin (hCG), and a beta subunit that is unique and confers biological specificity to each hormone (Matsumoto *et al.*, 1987).

Once in circulation, LH primarily targets Leydig cells in the testes binding the LH/hCG membrane receptor with high affinity and stimulating the steroidogenic activity of these cells (Eacker *et al*., 2008). This process involves the enzymatic conversion of cholesterol into testosterone. Following puberty, Leydig cells respond to LH by controlling testicular growth, as well as the development of external genitalia and accessory sex organs. Produced testosterone binds to androgen receptors (ARs), located in a range of testicular cells and tissues, including Sertoli cells, peritubular myoid cells, Leydig cells themselves, vascular smooth muscle and endothelial cells.

FSH, on the other hand, primarily acts on Sertoli cells, regulating both their number and function, and plays a key role in the initiation and maintenance of spermatogenesis. Upon activation of the FSH receptor, Sertoli cells begin synthesizing several important proteins and factors, such as androgen-binding protein (ABP), which is essential for concentrating testosterone within the seminiferous tubules, and inhibins A and B, which are involved in hormonal feedback regulation. FSH also induces the expression of aromatase, the enzyme responsible for converting testosterone into estradiol, the main estrogen in males.

Sertoli cells rely on both FSH and testosterone signaling to support and regulate spermatogenesis: they are the only somatic cells within the seminiferous tubules that directly interact with germ cells and support them secreting essential growth

factors and glycoproteins required for germ cell development (Griswold, 1998). For instance, glycoproteins such as androgen-binding protein (ABP) and transferrin allows the transport of ions and hormones, while anti-Müllerian hormone (AMH) is involved in the regression of Müllerian ducts during male fetal development (Kuchakulla *et al.*, 2021). Testosterone stimulation of Sertoli cells also plays a pivotal role in maintaining the blood–testis barrier.

In addition to endocrine signals, Sertoli cells and peritubular myoid cells secrete glial cell line–derived neurotrophic factor (GDNF), a paracrine factor essential for the self-renewal and maintenance of spermatogonial stem cells (SSCs) (Kuchakulla *et al.,* 2021). These cells also play a role in modulating the local testicular microenvironment through the secretion of additional paracrine and autocrine factors.

The regulatory framework of spermatogenesis also includes negative feedback mechanisms. Testosterone, upon reaching certain threshold concentrations, inhibits the release of GnRH from the hypothalamus and reduces LH secretion from the pituitary. Meanwhile, inhibin B produced by Sertoli cells primarily regulates FSH secretion through feedback inhibition (Corradi *et al.*, 2016) (Figure 8).

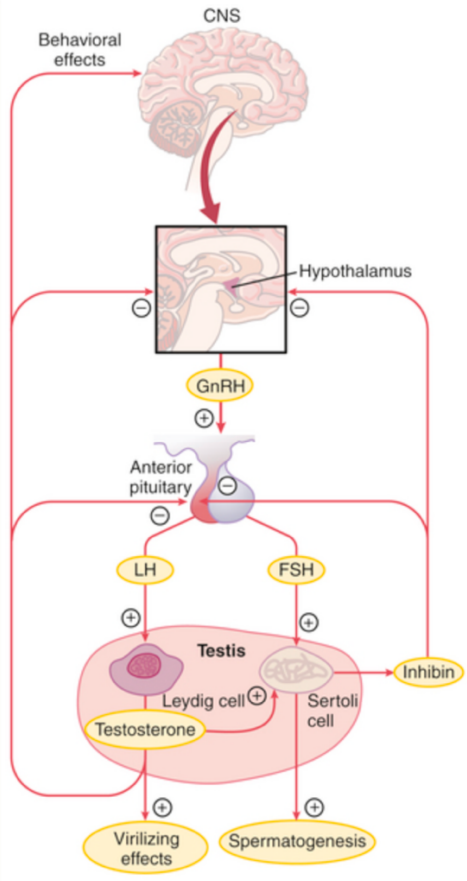


Figure 8. The hypothalamic-pituitary-gonadal axis. (https://basicmedicalkey.com/85-1)

2.3 Epididymal Maturation

Once spermatogenesis is complete, sperm cells undergo further maturation during their path to, and throughout, the epididymis. Human epididymis measures 5-6 cm and is divided in caput, the proximal portion, corpus and cauda, continuous with the ductus deferens. The transit of human sperm in the epididymis takes 2-4 days.

The epididymal luminal environment is immunoprotected, highly specialized and able to support sperm maturation process through the absorptive and secretory activities of its epithelium (Sullivan *et al*., 2016; Zhou *et al*., 2018).

As sperm cells are first expelled from the rete testis into the epididymis, nearly 90% of the fluid is rapidly absorbed by epithelial cells, resulting in a higher sperm concentration that allows to maximize the chances of fertilization. The high metabolic activity of epididymal epithelial cells produce an increase in reactive oxygen species (ROS), which can be detrimental for sperm health (El-Taieb *et al.*, 2009;): antioxidant enzymes are released in the epididymal lumen to neutralize ROS activity and protect sperm cells (Wu *et al.*, 2020).

During the epididymal transit spermatozoa acquire progressive motility and undergo to different morphological, biochemical, molecular and physiological changes. Progressive motility is one of the most important abilities that sperm acquire thorough the epididymis, since allows them to swim through the female reproductive tract once ejaculation occurs (Tourzani *et al.*, 2021). Morphologically, the most obvious change is the migration of the cytoplasmic droplet (CD) from the sperm neck toward the annulus; clear cells normally take up the contents of cytoplasmic droplets. In the epididymis spermatozoa develop the capacity to undergo acrosomal reaction, to recognize and bind the zona pellucida and to fuse with the oocyte membrane. Many potential mediators of sperm–egg communication are acquired or modified in the epididymis thanks to continuous re-coating and de-coating events occurring on the sperm head surface (James *et al*., 2020). Epididymal luminal fluid contains high concentrations of soluble glycohydrolases and glycosyltransferases, which are involved in the changes in composition of the sperm glycocalix during epididymal maturation (Tecle *et al.*, 2015).

Epididymal sperm maturation is associated also with modifications of lipid homeostasis: lipid peroxidation is physiologically important for several steps of the fertilization process such as sperm hyperactivation, capacitation, and acrosome reaction. While passing through the epididymis, sperm membrane changes in

composition and, at the final stage of epididymal maturation, reaches its final characteristics, with the 70% of phospholipids, 25% of neutral lipids (mainly cholesterol) and 5% of glycolipids (Laenzi *et al.*, 2000).

Moreover, during epididymal transit, sperm undergo changes in their protein, lipid, and sugar content. Spermatozoa are translationally silent: proteins, appearing in them after their maturation in the epididymis, are thought to be synthesized by the epididymal epithelium and then incorporated into the sperm cells. This large pool of proteins and molecules likely acts as an additional fertilization-relevant components and are thought to be delivered on the sperm membrane surface by small vesicle-like structures called epididymosomes. Epididymosomes have been suggested to play a role also a mode of paternal epigenetic inheritance via small ncRNAs (Zhang *et al.*, 2018; Chan *et al.*, 2020). The epididymis has also another important role associated to sperm nuclear chromatin organization: during epididymal maturation, intense disulfide bridging occurs in the nucleus, allowing a higher level of chromatin condensation.

2.4 The mature spermatozoon

In the mature spermatozoon is possible to recognize a head and a tail, or flagellum. The head is around 4 μm long and contains the nucleus, in which the DNA is strongly condensed into a compact hydrodynamic shape, covered by the nuclear membrane. Sperm nucleus is protected by a series of membranes and proteins that compose the perinuclear matrix, divided in three segments: the acrosomal segment, the equatorial segment and the postacrosomal sheath (PAS) or postacrosomal region (PAR).

In the acrosomal segment, between the nuclear membrane and the inner acrosomal membrane, it is possible to identify the subacrosomal layer, which has the function of anchoring the acrosome. The acrosome is a Golgi-derived vesicle organized as a cap on the nucleus of the sperm and containing proteases and receptors required for the interaction between sperm and oocyte. In this region also the inner (IAM) and outer (OAM) acrosomal membranes are visible, enclosing the dense acrosomal matrix. The equatorial segment includes the sperm membrane, OAM and IAM, and carries receptor molecules important in the initial binding of the sperm head to the egg plasma membrane. The PAS is thought to harbor a number of signaling proteins

collectively called SOAF, or the sperm borne oocyte activating factors (Gupta, 2005) (Figure 9).

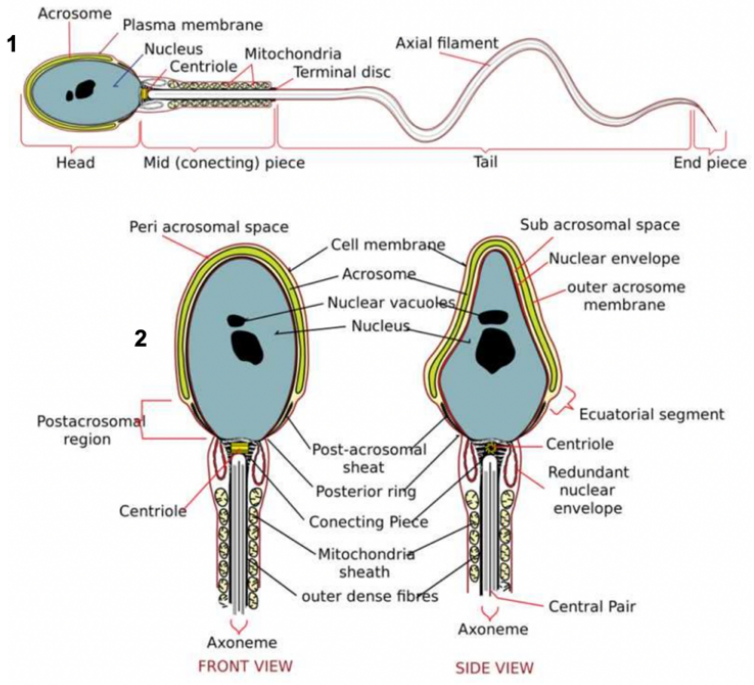


Figure 9. Mature sperm structure: 1. Main mature sperm structures; 2. Detail about head and connecting piece of a mature spermatozoon. (www.wikipedia.org)

The sperm tail or flagellum is around 65 μm long, provides motile force and is based upon a unique 9+2 arrangement of microtubules within the sperm flagellar axoneme. The 9+2 arrangement refers to nine peripheral, symmetrically arranged doublets of tubulin A and B microtubules connected doublet-to-doublet by dynein arms and to the sheath of central pair of microtubules by radial spokes. In the peripheral microtubule doublets, the subunit A is a complete microtubule made of 13 microfilaments, while the B subunit contains only 10 microtubules and completes its structure by the connection with the A subunit. The outer doublets, but not the central pair, are paralleled by nine outer dense fibers that provide flexible but firm support during flagellar movement. The sperm tail can be divided into four major segments, from proximal to distal end: the connecting piece, the midpiece, the principal piece and the end piece. With the exception of the end piece containing only a central couple of microtubules, the entire flagellum is organized in the characteristic cylindrical structure of the axoneme. Each microtubule doublet is externally anchored to 9 corresponding asymmetric outer dense fibers (ODF), which are surrounded by the fibrous sheath in the principal piece and by packed mitochondria in the middle piece of the flagellum (Luconi *et al.*, 2006).

The connecting piece is composed of nine segmented columns in direct continuation with the outer dense fibers of the other flagellar segments. These nine columns enclose the dense mass of the capitulum, which contains the proximal centriole. The basal plate provides the connection between proximal terminus of the connecting piece and the implantation fossa of the sperm head. The midpiece is covered by the mitochondrial sheath, composed of a helix of approximately 75-100 sperm mitochondria, which produce the energy for sperm flagellar motility. The principal piece is separated from the midpiece by the annulus or Jensen's ring, a traverse ring of dense material, and is covered by the fibrous sheath, a sheath made of two longitudinal columns running parallel to the outer dense fibers three and eight which provides support for the sperm axoneme. The end piece contains axonemal doublets and the ends of the outer dense fibers and fibrous sheath (Figure 10).

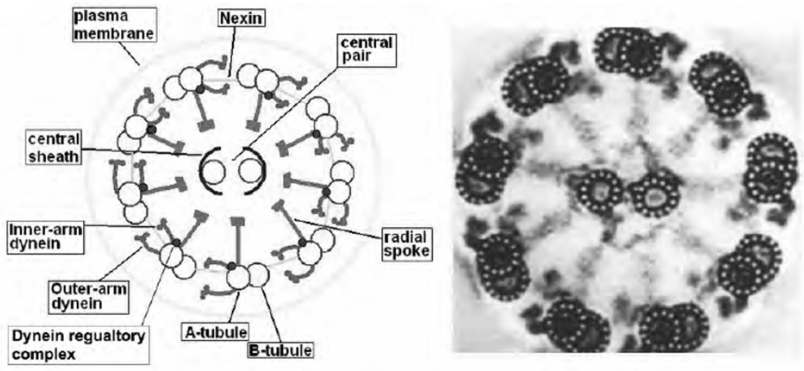


Figure 10. On the left, schematic representation of the axonemal structure. On the right, flagellar transversal section viewed by transmission electron microscopy. (Guan, J., 2009)

The complex organization of the axoneme permits sperm swimming, characterized by a rhythmic three-dimensional and asymmetric movement of the flagellum. The sliding of each couple of microtubules originating from the sequential anchoring of the dynein arms on the neighbor couple, and ATP-dependent generation of sliding force, results in bends of alternating direction and propagation of the oscillations along the tail. Oscillations can originate from different regions of the flagellum and the beat frequency is controlled by the basal region, which acts as a sort of pacemaker (Luconi *et al.*, 2006).

2.5 Sperm DNA integrity as a determinant of male infertility

The incidence of male infertility is increasing worldwide, with a wide range of causes and risk factors either hypothesized or proven to contribute to this trend. It is possible to categorize these potential factors into different broad causes: (1) biological, genetic and medical causes; (2) behavioural and lifestyle factors; (3) environmental and occupational factors.

Uro-genital infections, varicocele, cryptorchidism and hormonal abnormalities are the most common biological factors related to male infertility, but also testicular surgeries or trauma can lead to impaired spermatogenesis. As genetic factors is common to notice chromosomal abnormalities such as Klinefelter syndrome (additional X-chromosome) or chromosome Y microdeletions, leading to spermatogenic defects. Other genetic conditions, such as CFTR gene mutations or Kallmann syndrome, are strictly connected to male infertility and can be related to more severe conditions as the congenital bilateral absence of vas deferens (CABVD).

Various lifestyle factors are able to decrease male fertility, such as smoking, alcohol consumption, recreational drug use, poor diet or obesity (Agarwal *et al*., 2021). In addition, different medications affect male fertility: calcium-channel blockers, alpha-blockers, testosterone replacement therapy, cortico-steroid prolonged use and chemotherapeutic treatments (Fainberg *et al*., 2019).

To date, environmental exposure to different chemical compounds able to affect the normal hormonal regulation is widely investigated. These factors, termed endocrine disrupting chemicals (EDCs) are daily used and include parabens, phthalates, bisphenols, pesticides and chemicals present in cosmetic products, food, water and air. Moreover, occupational or frequent exposure to heat, radiation or electromagnetic fields and/or heavy metals contribute to male infertility issues.

Spermatozoa can report nucleus abnormalities which can be divided in sperm chromosomal abnormalities and sperm DNA abnormalities and represent another important factor affecting male fertility. Sperm DNA fragmentation (SDF) is included in the DNA abnormalities group.

Sperm DNA fragmentation is the accumulation of single- and double-strand DNA breaks in the genome and can be identified in ejaculated human sperm. Single-strand breaks (SSB) are related to fertilization rates and are an extensive damage

that appears throughout the entire genome mainly produced by reactive oxygen species (ROS) (Muriel *et al*, 2006; Avendaño *et al.,* 2010; Aitken *et al,* 2013). Double-strand breaks (DSB) in sperm DNA are associated with a higher risk of miscarriage and lower embryo kinetics (Ribas-Maynou *et al,* 2012; Casanovas *et al,* 2019) and are produced during meiosis in the seminiferous tubules to allow homologous recombination and DNA hypercondensation processes (Keeney S. *et al*, 2014). DSB can be also produced at post-testicular level following activation of sperm endonucleases (Ward *et al.,* 2004).

Sperm lack some of the key enzymes required for DNA repair, therefore is possible to detect the presence of sperm with DNA damage in the ejaculate (Dos Santos Hamilton *et al*., 2020). For this reason, after fertilization, the cooperation of the oocyte to repair paternal DNA damage is needed and, depending on the extent of SDF, the oocyte might not have sufficient DNA repair capacity. Unsuccessfully repaired SDF has been linked to infertility and poor embryo development. Moreover, high SDF levels are negatively correlated to fertilization rates, embryo development, implantation and pregnancy and live birth rates (Sedò *et al*., 2017; Borges *et al*., 2019; Ribas-Maynou *et al*., 2022).

Since high levels of sperm DNA fragmentation are frequently reported in infertile men, SDF takes an important role in unexplained infertility and it is estimated to cover almost the 40% of the cases (Mei *et al*., 2022).

According to the European Association of Urology (EAU), SDF can be induced by both extrinsic and intrinsic factors. Extrinsic risk factors include lifestyle habits as nutrition, smoking, prolonged abstinence periods, exposure to ionizing radiations, radiotherapy and chemotherapy (Kumar *et al*., 2013; Comar *et al*., 2017; Jurewics *et al*., 2018). Intrinsic factor inducing SDF includes genital tract infections, varicocele, advanced male age, defects in the replacement of histones by protamines (Yoshida *et al*., 2018), defective or sub-optimal chromatin packaging (Tarozzi *et al*., 2009), oxidative stress and abortive apoptotic-like changes (Shukla *et al*., 2012; Dorostghoal *et al*., 2017).

In summary, these factors can lead to SDF through three important conditions: (1) oxidative stress (2) defective spermatogenesis and (3) abortive apoptosis.

Oxidative stress is a condition of imbalance between the physiological antioxidant defence of the cell, such as superoxide dismutase and catalase enzymes, and reactive oxygen species and can originate from increased production of ROS or

diminished antioxidant reserves. A physiological amount of ROS is involved in many different functions: they are fundamental for sperm maturation, viability, motility, and for processes such as hyperactivation, capacitation, acrosome reaction and sperm-oocyte binding (Agarwal *et al.*, 2003). Oxidative stress, resulting from inflammation, infection or in various diagnoses of male infertility, accounts for almost 80% of DNA fragmentation (Lee *et al*., 2019).

Spermatozoa are susceptible to oxidative stress since their plasma membrane is rich in polyunsaturated fatty acids (PUFAs), which are essential to maintain fluidity but also provide numerous potential sites for oxidation by free radicals. The damage of sperm membrane leads to direct exposure of sperm DNA to ROS and, since sperm lack of DNA repair mechanisms and retain small amounts of intracellular antioxidant enzymes, this can lead to a wide range of DNA strand breaks.

Moreover, sperm mitochondria can be both the source and the target of free-radical oxidation. The energy-generating process of oxidative phosphorylation produce ROS as by-products and this production can be one of the features of apoptosis, together with the activation of caspases and the externalization of phosphatidylserine (PS) (Aitken *et al*., 2011). The separated locations of mitochondria, present in the neck of the spermatozoa and producing the endonucleases needed for cell death, and DNA, located in the head, can lead to a delay in cell death. Because of this delay, sperm with DNA damage are still present in the ejaculate and can fertilize the oocyte, resulting in miscarriages and morbidity in offspring (Aitken *et al*., 2011).

Another important source of DNA damage are the mechanisms of *defective spermatogenesis*, especially failure in the repair of sperm DNA or defects in chromatin packaging. DNA strand brakes are normally generated to decrease the torsional distress produced by the process of chromatin packaging and compaction during spermiogenesis. The enzyme DNA topoisomerase II has the role to repair these DNA damages, but repair mechanisms could fail and ejaculated spermatozoa could still present DNA breaks (Aitken *et al*., 2011). For these reasons, sperm DNA fragmentation may be the result of unresolved strand breaks due to overwhelmed DNA repair mechanisms.

Chromatin packaging occurs through protamination and the dysregulation of this process, resulting in chromatin condensation disorders, generates DNA double-stranded fragments and chromatin structure destruction (dos Santos Hamilton *et al*.,

2020). Indeed, incomplete protamination leads to spermatozoa that are more vulnerable to different agents as mutagens, nucleases and/or free radicals. In humans, protamines P1 and P2 are typically found in equal ratios (Sarasa *et al*., 2020). Altered P1:P2 ratios and elevated histone-to-protamine ratios has been linked to male infertility (Aoki *et al*., 2006; Rogenhofer *et al*., 2013) and are negatively correlated to IVF success and embryo quality (Rogenhofer *et al*., 2017; Amor *et al.,* 2019). Since protamination occurs also during epididymal maturation to maintain the integrity of the DNA in ejaculated spermatozoa, the presence of DNA breaks could be related to both defective chromatin remodelling during spermatogenesis or maturation failure (Panner Selvam *et al*., 2021).

In recent years, the connection between male infertility and apoptotic processes received special attentions. While apoptosis is a critical component of germ cell development in males, the mechanism does not always operate efficiently, resulting in *abortive apoptosis* (Champroux *et al*., 2016). Apoptosis, or programmed cell death, is a key physiological mechanism that maintains testicular homeostasis by eliminating abnormal spermatozoa. This process also prevent the participation of sperm with DNA damage to fertilization, primarily through the rapid loss of motility triggered at the onset of the apoptotic cascade (Aitken *et al*., 2011). During spermatogenesis, it is estimated that 25% to 75% of germ cells are removed through apoptosis due to developmental defects (Qiu *et al*., 2020). Germ cells destined for elimination typically express the surface marker Fas, which interacts with Fas ligand (FasL) on Sertoli cells, leading to their phagocytosis. This surveillance system is not fully efficient: failures in Fas–FasL signaling or insufficient FasL expression can allow defective germ cells to evade apoptosis (Aitken *et al*., 2011). As a result of abortive apoptosis, these sperm may proceed through maturation and be present in the ejaculate as defective spermatozoa. This process has been associated with male infertility and is particularly evident in individuals with abnormal semen parameters and high SDF levels (Sakkas *et al*., 1999). In semen samples form infertile males, is possible to detect sperm cells showing apoptotic features such as the disruption of the mitochondrial membrane potential (MMP), the activation of caspases, the externalization of phosphatidylserine (EPS) and increased DNA fragmentation (Dirican *et al*., 2008).

Phosphatidylserine is a phospholipid typically confined to the inner leaflet of the plasma membrane in vital cells with intact membranes (Vermes *et al*., 1995) and its

externalization is a well-recognized marker of apoptosis. Beyond its role in cell death, PS is involved in several physiological processes, including cell–cell interactions, sperm–oocyte fusion, acrosome reaction and fertilization (Norozi-Hafshejani *et al*., 2022). Modifications in the sperm phospholipid bilayer are also part of the normal physiology of sperm capacitation. Thus, the extent and localization of PS externalization can help distinguish between apoptosis and capacitation (Said *et al*., 2008).

Under physiological conditions, apoptotic sperm are identified and removed in the female reproductive tract by phagocytes via “silent phagocytosis,” a non-inflammatory process that prevents fertilization by spermatozoa with compromised DNA integrity (Aitken *et al*., 2011). However, this natural selection mechanism is bypassed when conventional sperm-selection methods are used in assisted reproductive technology (Pacheco *et al*., 2020). To overcome this limitation, novel sperm-selection techniques have been developed, many of which are based on functional and physiological events that occur during capacitation: magnetic-activated cell sorting (MACS) and microfluidic sperm sorting are an example of this kind of methods.

To date, sperm DNA fragmentation is strongly recognized as able to have important repercussions across the whole reproductive process. Several studies linked high SDF to poor embryo quality and altered morphokinetics parameters as delayed cleavage timing abnormal division patterns (Nikolova *et al*., 2020; Borges Junior *et al*., 2021; Setti *et al*., 2021). Research has associated high SDF to higher incidences of miscarriages in natural pregnancies (Robinson *et al*., 2012) and fertilization failure following IVF and ICSI (Velez de la Calle *et al.*, 2008). Some studies associated mild to low SDF with embryonic arrest after embryonic genome activation (Simon *et al*., 2014). Additionally, many studies support a correlation between SDF and detrimental effects on offspring health (Aitken *et al*., 2020).

Given the strong link between sperm DNA fragmentation, male infertility and reproductive outcomes, research has focused on strategies to reduce SDF or select spermatozoa with high chromatin integrity for ART. Moreover, SDF testing is recommended case of recurrent miscarriages following natural conception, unexplained infertility and intrauterine insemination (IUI) or IVF/ICSI failure, but also in pathological conditions such as varicocele or in eventual exposure to risk factors (Esteves *et al*., 2021).

The European Association of Urology classified SDF tests as direct and indirect methods, based on their ability to measure the maturity and integrity of sperm chromatin. Direct methods are able to determine the extent of DNA damage, as TUNEL assay, while indirect methods allow the determination of susceptibility of DNA to protein denaturation, giving an idea about the status of the nucleus in terms of integrity and compaction of chromatin: they include COMET assay, SCSA and SCD assay (Figure 11).

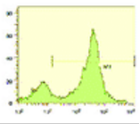
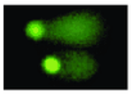
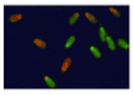
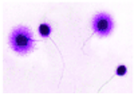
TEST	PRINCIPLE	ADVANTAGE	DISADVANTAGE
TUNEL 	Quantifies the enzymatic incorporation of dUTP into DNA breaks. Uses optical microscopy, fluorescent microscopy and flow cytometry.	Sensitive, reliable with minimal inter-observer variability. Can be performed on a few sperm.	Requires standardization between laboratories.
COMET ASSAY (SCGE) 	Electrophoretic assessment of DNA fragments of lysed DNA. Uses fluorescent microscopy.	Can be done in very low sperm count. It is sensitive and reproducible.	Requires an experienced observer. Inter-observer variability.
SCSA 	Measures the susceptibility of sperm DNA to denaturation. Uses flow cytometry.	Reliable estimate of the percentage of DNA-damaged sperm.	Requires the presence of expensive instrumentation (flow cytometer) and highly skilled technicians
HALO TEST (SCD) 	Assess dispersion of DNA fragments after denaturation. Uses oprical or fluorescent microscopy.	Simple test	Inter-observer variability

Figure 11. Details of different SDF test. (Adapted from Agarwal *et al.,* 2016)

2.6 Semen processing in ART

Male infertility is continually growing and represents a widespread phenomenon in the population, with high heterogenicity in the causes. Male infertility conditions may be related to pre-testicular (alterations of the hypothalamic-pituitary axis), testicular or post-testicular factors (urogenital obstructions, vasectomy or dysfunction of accessory glands). Assisted reproductive technology has improved and in vitro fertilization and its variants are largely used to treat almost all causes of male infertility (Calvert *et al*., 2022; Conforti *et al.*, 2022). Intrauterine insemination (IUI) and embryo transfer following IVF or intracytoplasmic sperm injection (ICSI) are three treatment techniques: the treatment approach for each couple is personalized according to the type of infertility encountered. In case of substantial or severe male factor infertility, a typical cycle of ART includes gonadotropin stimulation in the woman, followed by insemination of oocytes aspirated from multiple ovarian follicles, and selection of the best spermatozoa to ensure fertilization with IVF or ICSI (Carson *et al*., 2021). Sperm are retrieved through ejaculation or, in case of obstructive or non-obstructive azoospermia, through surgical methods. Surgical methods include microsurgical epididymal sperm aspiration (MESA), percutaneous epididymal sperm aspiration (PESA), testicular sperm aspiration (TESA) and testicular sperm extraction (TESE). An innovative tool for micro-TESE is Raman spectroscopy, which is able to non-invasively differentiate seminiferous tubules with complete and incomplete spermatogenesis (Liu *et al*., 2014). The success of an ART treatment is not related only to the diagnosis, sperm retrieval and insemination technique alone, but also to sperm preparation and selection procedures.

In nature, sperm cells go through a complex selection process that occurs in the female genital tract, starting with the ejaculation in the upper part of the vagina and ending with only one sperm fertilizing of the oocyte in the ampulla of one fallopian tube. After ejaculation, sperm move out of the seminal plasma, penetrate the cervical mucus and swim toward the upper part of the female genital tract. Within this environment, sperm undergo capacitation, a process that induces biochemical and structural changes required for fertilization. Capacitation involves alterations in motility, leading to the hyperactivation of the sperm tail, as well as tyrosine phosphorylation, which prepares the cell for the acrosome reaction. Cumulus cells release progesterone, which acts as chemoattractant for spermatozoa, prepare for acrosome reaction and facilitates receptor-mediated interactions with the oocyte.

The fertilization of the oocyte occurs in the ampulla, reached by a small fraction of sperm. This natural selection process ensures that fertilization occurs by a functionally competent sperm cell, mature and with high DNA integrity, thereby increasing the likelihood of forming a high-quality embryo.

With the introduction of IVF, the need led to develop a wide range of sperm selection techniques, in order to mimic in vitro the selection that the male gamete undergoes during the transit through the female reproductive tract. Effective semen preparation methods aim to isolate the healthiest sperm based on their motility, morphology and genetic integrity to increase fertilization rates and optimize embryo development. Moreover, an optimal method should ensure efficient separation of spermatozoa from seminal plasma and other factors that are detrimental for fertilization, like decapacitation factors, dead and immature sperm cells, leucocytes, bacteria and other inclusions that might increase reactive oxygen species (ROS).

The ideal techniques should be quick, easy and cost-effective and should isolate as much motile sperm as possible without causing damages. Since none of the available methods meet all these requirements, the choice of the sperm preparation technique depends on the nature of the semen sample and on its purpose.

Next to conventional sperm selection methods, recent ART improvements have introduced novel advanced sperm selection techniques. The development of new techniques capable of selecting a good population of spermatozoa is essential for artificial insemination, IUI, IVF, ICSI, and sperm cryopreservation. The objective is to discard low-quality spermatozoa, in terms of motility, morphology and DNA fragmentation, with the aim of increasing ART cycles success rates.

2.7 Conventional sperm selection techniques

The most daily used conventional sperm processing techniques are swim-up (SU) and density gradient centrifugation (DGC), based respectively on motility and density characteristics.

Swim-up is the oldest one of the easiest and most cost-effective sperm selection methods: it is widely used due to the simplicity of the approach and it is very effective in selecting motile sperm. This technique is based on the ability of spermatozoa with good motility to spontaneously migrate from a semen sample (direct swim-up) or a sperm pellet (pellet swim-up), placed on the bottom of a centrifuge tube, up into an overlayed washing medium.

Preparing a swim-up is relatively simple. After ensuring that the semen sample is well mixed, 1 mL is placed at the bottom of a sterile conical centrifuge tube and is gently overlayed with 1 mL of sperm washing medium. The tube is then inclined at an angle of approximately 45° to increase the surface area at the interface between the semen sample and the semen washing medium, and is placed into an incubator at 37°C for 45-60 min. During this period, viable motile spermatozoa swim from the bottom into the upper culture medium, while immotile spermatozoa remain in the semen at the bottom of the tube (Figure 12). Taking care not to disturb the semen sample, the supernatant is then removed from the tube and placed into a clean tube and, in case of direct swim-up, is washed with 1-2 mL of fresh medium and centrifuged at 300-500X*g* for 5 min. The supernatant may then be removed, leaving the final sperm preparation in approximately 0.5 of medium at the bottom of the tube. In case of a swim-up from pellet, the retrieved sample from the upper layer of the tube is ready to be used without further centrifugation steps.

Since motility is a criterion for natural selection, swim-up enriches the sample with active motile sperm, which correlates with an enhanced fertilizing potential. While effective, the swim-up method possesses some drawback and limitations in cases of sperm with poor motility, as in severe male factor infertility. It is advisable to use the pellet SU in cases of oligoasthenoteratozoospermia since, compared to direct SU, it enables a more concentrated final suspension of spermatozoa to be obtained. Concluding, SU method provides a relatively simple and economical means of sperm preparation for IUI and IVF, though is less effective for men with severe oligoasthenozoospermia due to its low recovery rate.

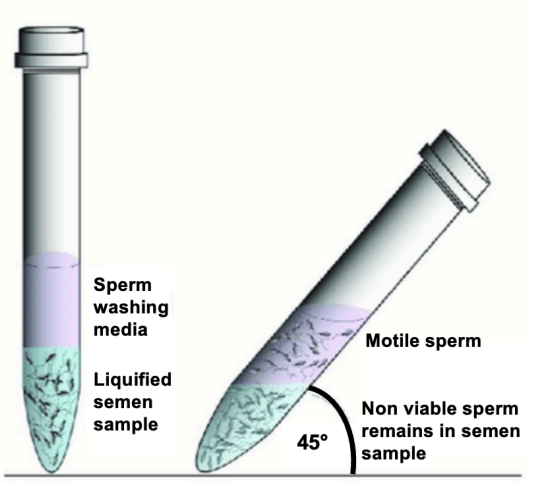


Figure 12. Schematic representation of swim up. (Adapted from Baldini *et al.,* 2021)

Another broadly used technique is discontinuous density gradient centrifugation, developed to mimic, in vitro, the natural selection of morphologically normal, viable spermatozoa by cervical mucus in vivo. As its name implies, sperm separation using DGC essentially relies upon the greater density of morphologically normal spermatozoa, which possess a dense and homogeneous nucleus, compared to those with morphological anomalies of the sperm head, tending to be less dense. Therefore, DGC selects spermatozoa according to their density, size and shape, and allows the selection of motile spermatozoa with good morphology. In discontinuous DGC, the main choice as gradient material is colloidal silica coated with silane or polyvinylpyrrolidone. Indeed, colloidal silica particles help to cushion spermatozoa during centrifugation, thereby minimising potential disruption of the sperm-nuclear chromatin. The desired concentration of gradient material can be purchased or prepared in advance of the use. Typically, 45% and 90% solutions of silane-coated colloidal silica are employed for standard semen preparation. To prepare a discontinuous gradient, 1 mL of the 90% gradient is placed into a sterile conical centrifuge tube and is gently overlaid with 1 mL of the 45% gradient, taking care to avoid mixing them. Then, the discontinuous gradient is gently overlaid with 1 mL of the premixed semen sample. The tube is centrifuged at 300x *g* for 20 min to separate the spermatozoa from the seminal plasma and other constituents of the semen sample. Taking care not to disturb the sperm pellet, the supernatant is removed, leaving approximately 0.5 mL of 90% gradient containing the sperm pellet at the bottom of the tube. The bottom 0.25 mL containing most of the spermatozoa is then transferred to a clean tube containing 5 mL of fresh sperm preparation

medium where it is mixed and then centrifuged at 300x *g* for 5 min; this step is then repeated to ensure that the sperm pellet has been washed twice. Selected spermatozoa will be found at the bottom of the tube, while other constituents of semen such as seminal plasma, bacteria, debris, leucocytes, non-gametic cells and immature sperm cells either fail to penetrate the colloidal silica or migrate to different densities of the discontinuous gradient (Figure 13). Hence, DGC tends to be the sperm preparation method of choice for handling semen samples from patients with known infectious diseases. DGC is particularly suitable for sperm separation even in men with oligozoospermia. In men with severe oligoasthenozoospermia is possible to apply mini gradients, using lower volumes than standard.

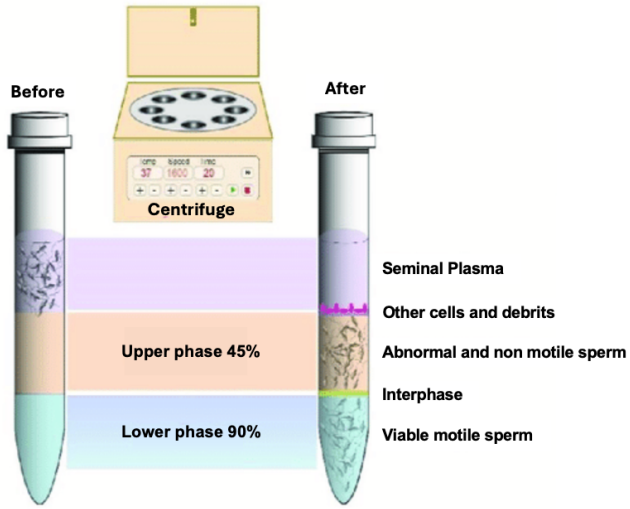


Figure 13. Schematic representation of density gradient centrifugation. (Adapted from Baldini *et al.,* 2021)

Swim-Up and DGC represent two efficient options for motility and morphology-based sperm selection. SU produces a higher percentage of progressively motile spermatozoa than DGC but is less effective in selecting against spermatozoa containing single-stranded DNA damage (Enciso *et al*., 2011). Moreover, sperm preparated by DGC were found to be more capacitated than SU sperm, while SU-obtained spermatozoa possess fewer vacuoles in their head compared to DGC (Hernandez-Silva *et al*., 2021; Raad *et al*., 2021). Comparing these two techniques, no significant differences in fertilization, good-quality embryo or blastocyst formation rates were found in IVF/ICSI cycles (Rao *et al*., 2022). Indeed, the choice of the sperm selection technique to use is mainly determined by the nature of the semen sample.

2.8 Advanced sperm selection techniques

As an alternative to the conventional techniques as swim-up and discontinuous density gradient centrifugation, advanced sperm selection techniques have been developed. Advanced methods have emerged as more precise tools for selecting sperm with reduced apoptosis and increased sperm DNA integrity, although they face challenges in terms of their standardization, cost and clinical adoption. Magnetic-activated cell sorting (MACS) and microfluidic are some of the recently developed methods for sperm separation with appropriate parameters and low SDF in ART for infertile men.

2.8.1 Magnetic-activated cell sorting (MACS)

Cell sorting is a process that consists of selecting cells based on characteristics such as size, morphology or membrane markers, allowing the analysis of defined cell populations. The procedure, firstly reported in 1977 by Monday *et al*., was implemented developing separation columns with magnetized matrices capable of retaining cells bound to iron microspheres (Miltenyil *et al*., 1990). The method employs the use of magnetic particles conjugated to proteins or antibodies to tag cells of interest and was patented and marketed under the name of MACS (Magnetic Activated Cell Sorting, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). More recently, MACS shown to be efficient to separate apoptotic from non-apoptotic spermatozoa on a molecular level, starting from semen samples (Grunewald *et al*., 2001). The procedure was based on the phenomenon of externalization of phosphatidylserine on the outer cell surface during the apoptotic process. Phosphatidylserine has a high and selective affinity for annexin V, a 35kDa calcium-dependent phospholipid binding protein. Since annexin V Is able to bind phosphatidylserine only when is externalized in the outer layer of the plasma membrane, the binding of this protein on sperm plasma membrane point out the apoptotic state of the sperm (Figure 14).

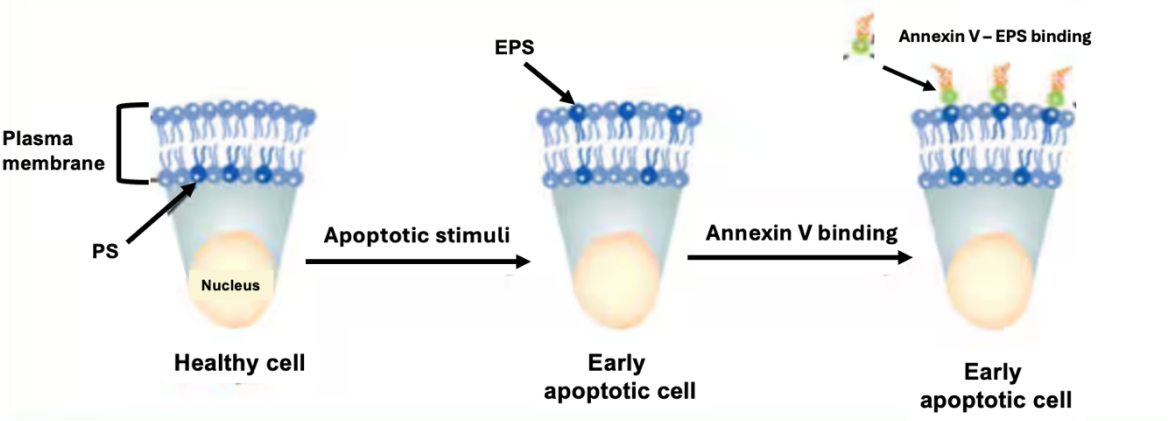


Figure 14. Schematic representation of Annexin V-Externalized PS binding in early apoptotic cells. PS: Phosphatidylserine, EPS: Externalized Phosphatidylserine. (www.novusbio.com)

In MACS sperm selection procedure, the semen sample is mixed with colloidal superparamagnetic microbeads conjugated with specific antibodies to annexin V (MACS Annexin V microbeads or AN-MB): annexin V will recognize and bind sperm cells which expose phosphatidylserine as a result of the beginning of the apoptotic process. The mixture is then loaded on a specific MACS separation column placed into a magnetic field of a MACS separator. Non-apoptotic sperm will not bind to the microbeads and will pass through the column, while apoptotic sperm bounded to the annexin V microbeads will be magnetically retained inside the column (Figure 15). The magnetically retained spermatozoa can be eluted after the removal of the magnetic field (Rosen *et al*., 2019). At the end, MACS sperm selection procedure releases an annexin V-negative sperm fraction (AN-MB-, intact membranes, non-apoptotic sperm) and an annexin V-positive sperm fraction (AN-MB+, externalized PS, apoptotic sperm). Since the selection of non-apoptotic spermatozoa is an important step to reach a successful fertilization and apoptosis is closely associated with sperm DNA fragmentation, the use of MACS should increase the percentage of DNA-intact spermatozoa within the final sperm preparation used for IUI, IVF or ICSI. In the sixth edition of the WHO Manual for Human Semen Analysis (2021) MACS is reported as a sperm preparation technique.

MACS is an innovative technique in sperm selection with a high specificity and sensitivity, but is expensive if compared to other techniques, not widely available and can be time-consuming in the ART laboratory daily routine. Moreover, there are still conflicting results regarding the benefits of this technique in ART outcomes.

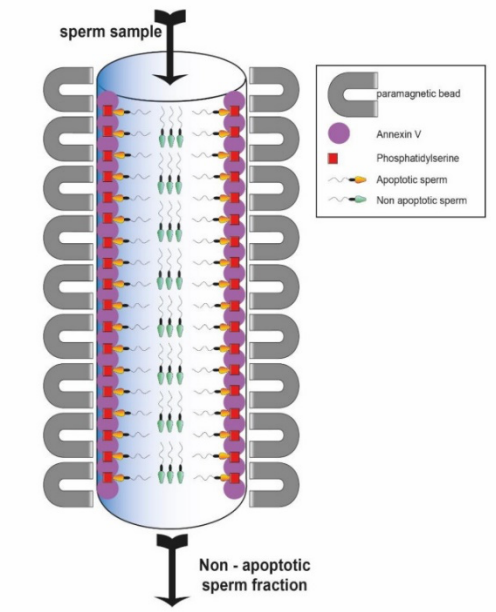


Figure 15. Schematic representation of sperm selection through the MACS column. (Adapted form https://encyclopedia.pub)

There is conflicting evidence regarding MACS. Some authors reported an improvement in ART outcomes by enhancing fertilization rates and achieving cost-effective treatments and a better embryo quality (Pacheco *et al*., 2020), while other studies underline that MACS shows minimal statistically significant changes in clinical pregnancy rates and live birth rates (Ziarati *et al*., 2019; Mei *et al*., 2022). After MACS procedure, some articles reported spermatozoa with less DNA fragmentation and higher morphological parameters (Hoogendijk *et al*., 2009; Rawe *et al*., 2009) and other authors reported abnormalities in sperm tail (Stimpfel *et al*., 2018) and impaired motility compared to fresh samples (Cakar *et al*., 2016). Moreover, MACS shown to be able to improve sperm motility and cryo-survival rates after sperm cryopreservation (Said *et al*., 2005) and resulted also more efficient than PICSI in the selection of DNA-intact spermatozoa in idiopathic infertility cases (Ahmadi *et al*., 2022).

To remove from the ejaculate detrimental components such as plasma, debris and leukocytes, MACS needs to be combined with other conventional sperm preparation techniques. Recent studies have investigated the combination of MACS with DGC to further improve the selection efficacy of DGC while MACS ensure that selected sperm have an intact DNA. The best results are obtained when MACS is performed after DGC instead of before DGC. This sperm preparation method demonstrated to be optimal in providing spermatozoa with highest quality in terms of motility, viability, oocyte penetration potential and apoptosis markers (Said *et al*., 2008). Some

authors demonstrated that DGC-MACS, compared to DGC alone, SU, and DGC followed by SU, provides a higher percentage of viable spermatozoa with normal morphology and intact DNA in teratozoospermic patients, increasing the success rates of ART (Bibi *et al*., 2023). In addition, DGC followed by MACS strongly reduces the rate of spontaneous abortion in patients with a DNA fragmentation level higher than 30% in ICSI cycles (Sànchez-Martìn *et al*., 2017). However, further investigations are required to confirm the effect of this two-step procedure.

2.8.2 Microfluidic sperm sorting

Microfluidics is the science and technology of accurate manipulation of fluids at sub-millimetre scale and has been implemented for many years in different disciplines including nanotechnology, microsystems engineering and biomedical applications (Feng *et al*., 2019). Recently, this technology has been applied in IVF laboratories as a novel sperm selection method. Microfluidic sperm selection methods attempt to mimic the natural progression of sperm through the female reproductive tract, including physical aspects of the fallopian tubes (Leung *et al*., 2022; Vasilescu *et al*., 2023). This sperm selection methods select sperm according to size, motility and other characteristics, such as DNA integrity, without the need for centrifugation (Quinn *et al*., 2018; Bastuba *et al*., 2020). Not involving washing and centrifugation steps, microfluidic is less damaging for sperm DNA and decrease ROS production when compared to other conventional sperm selection methods. Indeed, microfluidic sorting has been shown to be helpful in cases of male infertility with high levels of DNA fragmentation since it enriches samples with sperm possessing lower SDF and a better morphology (Wu *et al*., 2023).

Researchers have reported promising results from the microfluidics use in ART procedures such as better fertilization rates, improved embryo development, superior live birth outcomes, and an increased number of high-quality euploid blastocysts (Agarwal *et al*., 2022; Parrella *et al*., 2019; Yetkinel *et a.l*, 2018).

Microfluidic-based sperm selection devices are proposed as a simple, reliable and standardized methods with a minimal hands-on time and increased automation.

To date, there are several different commercially available sperm selection methods based on microfluidic, such as SwimCount™ Harvester (MotilityCount ApS, Denmark), SpermGuide (NeoGenix Biosciences, Australia) and ZyMōt® (ZyMōt® Fertility, DxNow Inc., USA); the results reported in this thesis are based on the use

of the device ZyMōt® (Figure 16). ZyMōt® is a single-use, flow-free dual-chambered device; the lower chamber contains a sample inlet and fluid channel separated from the upper collection chamber by a microporous membrane with 8 μm pores, demonstrated as the optimal size for selection of sperm with higher motility and higher morphology (Ashgar *et al*., 2014; Chinnasamy *et al*., 2016). The design of the sperm sorting chip utilizes sperm forward motility to sort healthy motile sperm from compromised, poorly motile sperm present in the raw semen sample. After sperm enter the lower channel through the inlet, the more motile sperm swim through the microchannel and up through the filter pores within the membrane to reach the outlet. The device produces a 500 µL sample that can be used for ICSI, IVF, or intrauterine insemination (IUI). ZyMōt® device demonstrates to select motile sperm with low values of DNA damage (both SSB and DSB) and to improve reproductive outcomes after ICSI in patients with high DSB values (Quinn *et al*., 2018; Pujol Masana *et al*., 2022; Lara-Cerrillo *et al*., 2023).

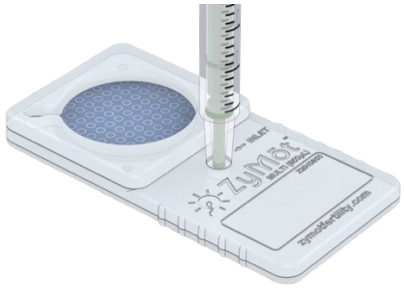


Figure 16. Imagine of ZyMōt® device. (https://www.zymotfertility.com)

3. Research study purpose

In the last years, several novel sperm selection methods have been introduced in human IVF to isolate spermatozoa with optimal parameters and intact DNA. Nevertheless, no consensus has yet been reached regarding the most effective approach. Since DNA integrity plays a crucial role in determining sperm fertilizing potential and can significantly influence fertilization, embryo development, implantation and pregnancy rates, the present thesis aims to investigate the role of MACS and ZyMōt® as alternative sperm selection methods in IVF treatments. In particular, this work evaluates their potential benefits on semen preparation and ICSI outcomes, including fertilization, cleavage and pregnancy rates. Both retrospective and prospective data were collected from homologous and oocyte donation cycles in which MACS or ZyMōt® were used as sperm selection methods. For the same couples, data from previous cycles using conventional sperm preparation technique, such as swim-up and density gradient centrifugation, were also analysed. The effects of MACS or ZyMōt® were then evaluated by comparing outcome obtained with those innovative approaches to those achieved with standard selection methods. The collected data are expected to inform clinical practice in ART by assessing the effectiveness of these sperm selection methods. The findings may support the development of evidence-based guidelines and help clinicians identify couples who could benefit most from MACS or ZyMōt®, ultimately improving reproductive outcomes.

4. Materials and methods

4.1 Study design

The data of this thesis are from cycles performed at the Assisted Reproductive Technologies (ART) laboratory of the Chianciano Salute S.p.A. Centre. Both retrospective and prospective data were collected from ICSI cycles, including homologous (H) and heterologous (Oocyte Donation cycles, OD) treatments. Sperm selection was performed using DGC followed by MACS or ZyMōt® device. These were classified as "MACS group" and "ZyMōt® group", respectively. For each couple, data from previous failed cycles using conventional sperm preparation technique (swim-up or DGC alone) were also retrieved and used as internal control. These were defined as "Control w/o MACS group" and the "Control w/o ZyMōt® group". The graphical representation of the study cohort is shown in Figure 17.

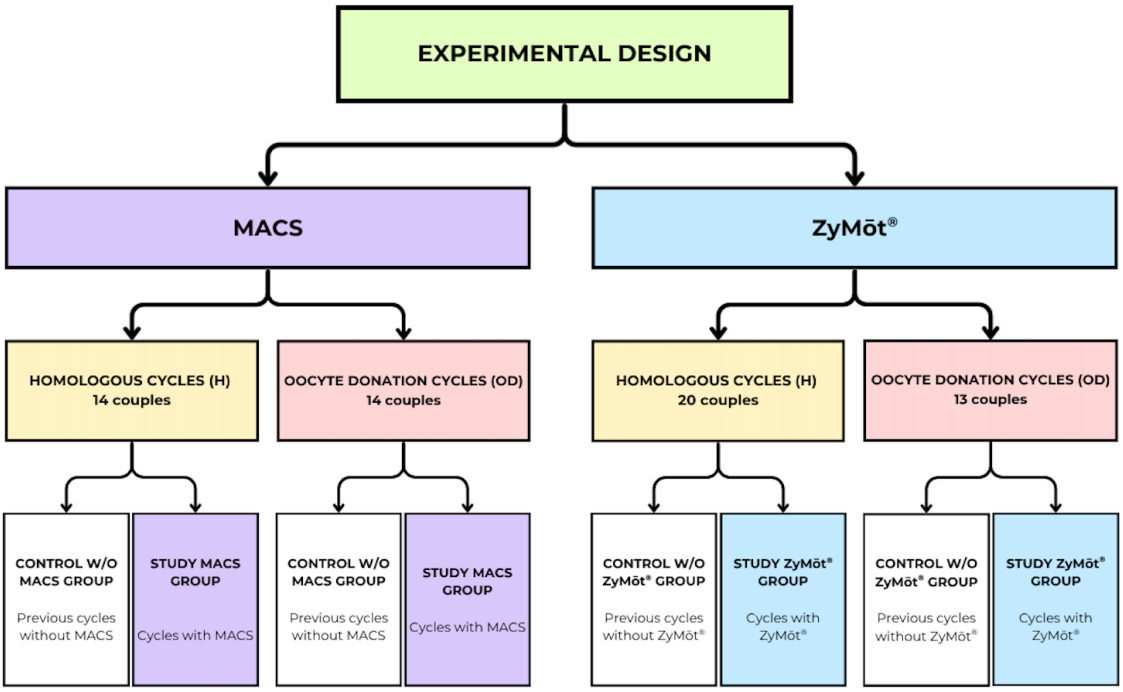


Figure 17. Study groups scheme

The workflow of all ART procedures included in this study followed a standardized protocol, summarized in Figure 18, which illustrates the overall design and flow of the experimental groups. Each cycle included semen and oocytes collection, their laboratory preparation, ICSI, embryo culture, and finally fresh or frozen embryo transfer.

For all these, the following parameters were evaluated: sperm progressive motility (type A), fertilization rate and cleavage rate. When the embryo transfer was performed, β-hCG positivity and clinical pregnancy were also assessed. The results were compared between control groups and study groups, for both MACS and ZyMōt®, as well as between the MACS and the ZyMōt® study groups.

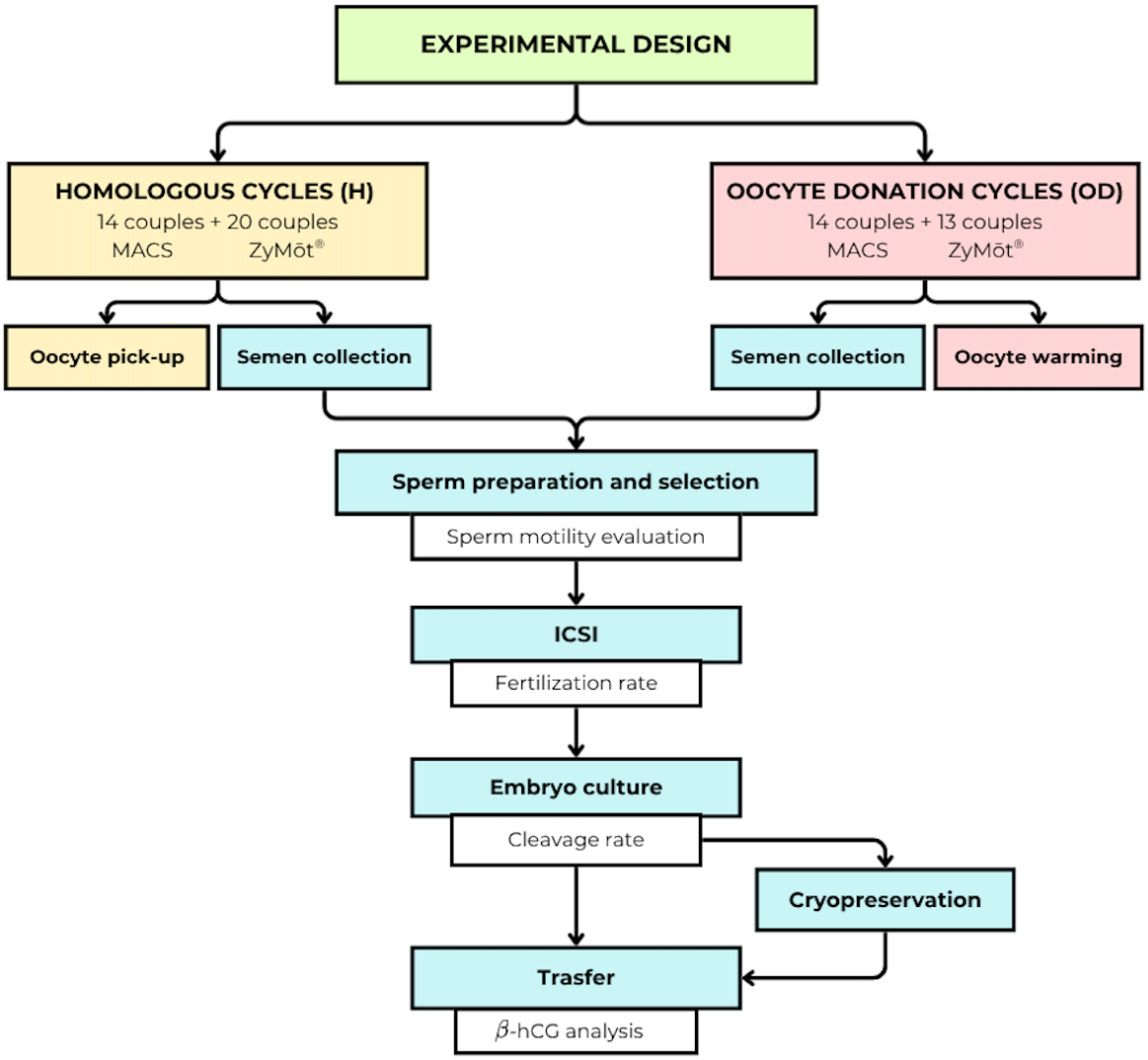


Figure 18. Flow diagram of the experimental design.

4.2 Study population

The study population includes a total of 34 couples who underwent homologous cycles and 27 couples who underwent oocyte donation cycles. In particular, for MACS were considered 14 couples for homologous cycles and 14 couples for oocyte donation cycles, while 20 couples for homologous cycles and 13 couples for oocyte donation cycles were evaluated for ZyMōt®. The population was described by number of previous failed ART cycles, female age, male age and male body mass index (BMI). The male population was described also by some semen parameters: sperm concentration (10^6/ml), total sperm motility (%), normal sperm morphology (%), and sperm DNA fragmentation index (SDFI).

The *number of previous failed cycles* was retrieved from the centre database. The majority of the couples reported a clinical history with one to three failed cycles (Table 1).

The mean female age for the homologous cycles group was 37 years while for the oocyte donation cycles group was 43 years. For males, the mean age for the homologous cycles group was 42 years while for the oocyte donation cycles group was 44 years (Table 1).

Moreover, *body mass index* (BMI) expressed in Kg/m2 was retrieved from the centre database (Table 1).

VARIABLE	HOMOLOGOUS CYCLES 34 couples	OOCYTE DONATION CYCLES 27 couples
Number of previous failed cycles (number) mean ± s.d.	2 ± 1	2 ± 1
Female age (years) mean ± s.d.	37 ± 4	43 ± 5
Male age (years) mean ± s.d.	42 ± 10	44 ± 5
Male BMI (Kg/m^2) mean ± s.d.	22 ± 2	24 ± 2

Table 1. General characteristics of the studied population: number of previous failed ART cycles, female age, male age and male body mass index in homologous and oocyte donation cycles.

4.3 Semen collection and analysis

Semen samples from male partners were collected into sterile plastic containers by masturbation in a dedicated room of the centre after an abstinence period of 2-7 days. After liquefaction at room temperature within 30 minutes from the ejaculation, the liquefied semen analysis was performed according to the World Health Organization guidelines (WHO, 2021). Sperm concentration was assessed using a Neubauer Improved Chamber: 10 μl of semen sample was incubated in the chamber to record the number of sperm ($x10^6$) per mL. Total sperm motility was evaluated with the same chamber and expresses the percentage of motile spermatozoa, considering three types of motility: A, B, C. Spermatozoa were considered with A motility when they swim fast and progressively forward, type B motility refers to slow progressive motility and the type C is described as non-progressive motility. According to WHO the reference limit for total sperm motility is 42% and the reference limit for progressive motility (A+B) is fixed at 32%. Sperm morphology was assessed at the optical microscope at 400X magnification, incubating 10 μl of semen sample on pre-colored Test-Simplets glass slides, reporting abnormalities of head, neck and tail of spermatozoa and evaluating the presence of cytoplasmic residues. The reference limit fixed by WHO is 4% of morphologically normal spermatozoa (WHO laboratory manual, 2021).

4.4 Sperm DNA fragmentation analysis

The evaluation of *sperm DNA fragmentation* was performed using the sperm chromatin dispersion test (SCD) or Halo test, performed using Halosperm® kits (Halotech DNA SL, Madrid, Spain). First of all, the sample is subjected to acid denaturation which denatures DNA molecules in sperm cells with fragmented DNA. Then the lysis solution removes the nuclear proteins and, in absence of massive DNA breakage, produces nucleoids with large halos of spreading DNA loops which emerge from a central core. Dehydration, staining and microscopic evaluation conclude the process (Figure 19). The DNA fragmentation index (SDFI) is represented by the ratio of number of spermatozoa with fragmented DNA (without halo). It was considered normal an SDFI <20%, borderline SDFI between >20% and <30%, altered SDFI >30%. This last value is the cut-off above which the fertilization and the embryo development are heavily compromised.

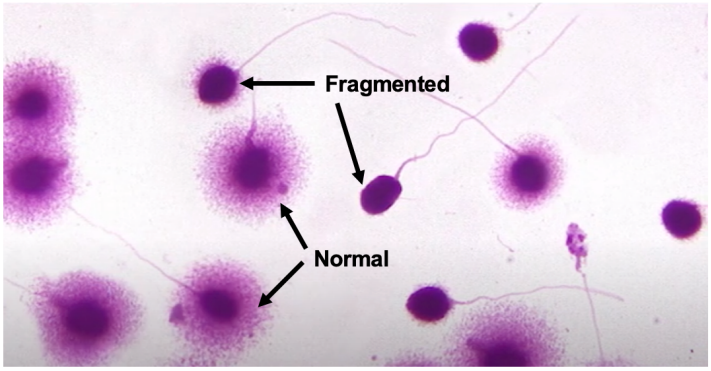


Figure 19. Picture of sperm chromatin dispersion test under microscope. Normal sperm without SDF show large or moderate sized halo around a compact core; fragmented sperm show small or absence of halo. (Adapted from https://www.givelegacy.com/guides)

4.5 Semen selection procedures

4.5.1 Swim-Up

Swim-up was performed for patients with normal semen parameters according to WHO and negative infectious exams. After the sample was gently mixed, an aliquot was washed with SpermRinse (SpermRinse, Vitrolife, Sweden) medium in a conical tube and subsequently centrifuged at 1600 rpm for 10 minutes. The supernatant was discarded, and the pellet resuspended in the same medium for a second wash. Subsequently, two drops of SpermRinse were gently layered on 0,3 ml of sperm suspension. The tube was inclined at an angle of 45° and incubated at room temperature for 45 minutes. Ate the end, motile spermatozoa were recovered from the upper interface and a small aliquot was evaluated.

4.5.2 Density gradient centrifugation (DGC)

Discontinuous gradients were prepared in conical tubes using SpermGrad solutions (SpermGrad, Vitrolife, Sweden). After removing gelatinous bodies and mixing the sample, an aliquot was gently stratified on the gradient and centrifuged at 1600 rpm for 15 minutes. After centrifugation, the supernatant was discarded, and the pellet was resuspended in SpermRinse for two washing steps and the final semen evaluation.

4.5.3 Magnetic activated cell sorting

Semen samples underwent firstly to DGC and then to MACS, using the Annexin V MicroBead Kit from Miltenyil Biotec.

The system is based on the externalization of phosphatidylserine which is one of the earliest signs of apoptosis. Thanks to the high binding affinity of Annexin V to phosphatidylserine, apoptotic sperm are labelled with Annexin V MicroBeads and isolated.

The first step is the column washing with 1000 µL of MACS ART Binding Buffer. Then, 0,5 ml of sperm fractions were added with 1,5 ml of the same Buffer and, after the resuspension of the pellet, a centrifugation at 1600 rpm for 4 minutes was performed. The supernatant was discarded, and the pellet was added with 100 µL of MACS ART Annexin V Reagent. It was made up to the mark of 500 µL with the Buffer, mixed well and incubated at room temperature for 15 minutes. The mixture was then loaded in the magnetic MACS® MS Column located in a magnetic field of a MiniMACS Separator, which is basically a magnet causing the retention of magnetically labelled cells (Figure 20). As a result, viable spermatozoa, which were not magnetically labelled with annexin V-conjugated magnetic microbeads, passed through the column and were collected. On the other hand, the labelled annexin V-positive apoptotic sperm were selectively retained in the column. Finally, the column was washed once with 500 µL of the Binding Buffer to collect all unlabelled spermatozoa. The eluted fraction was washed with sperm preparation medium and evaluated.

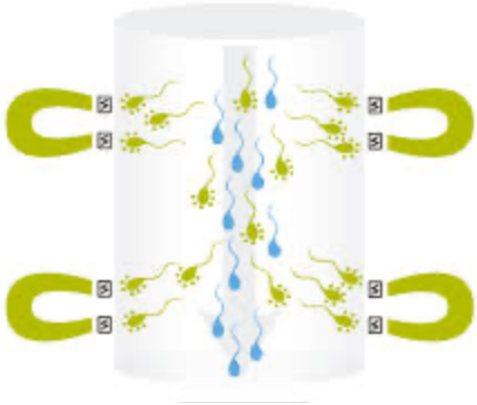


Figure 20. Schematic representation of magnetic sorting of spermatozoa. (Adapted from www.fertilitycenter.com)

4.5.4 ZyMōt®

When ZyMōt® was applied, 850 μL of semen sample was slowly drawn up with a syringe and injected into the inlet port of the sperm separation device and 750 μL of pre-warmed sperm washing medium (MOPS Plus, Vitrolife) was drawn up a fresh syringe and added to the upper collection chamber in order to cover the membrane surface. The ZyMōt® sperm separation device was then incubated at 37°C for 30 min. Following this time, 500 μL of processed sample was slowly aspirated with a syringe from the outlet port and transferred into a sterile 5 mL round bottom tube (Figure 21). An aliquot of the obtained sample was examined.

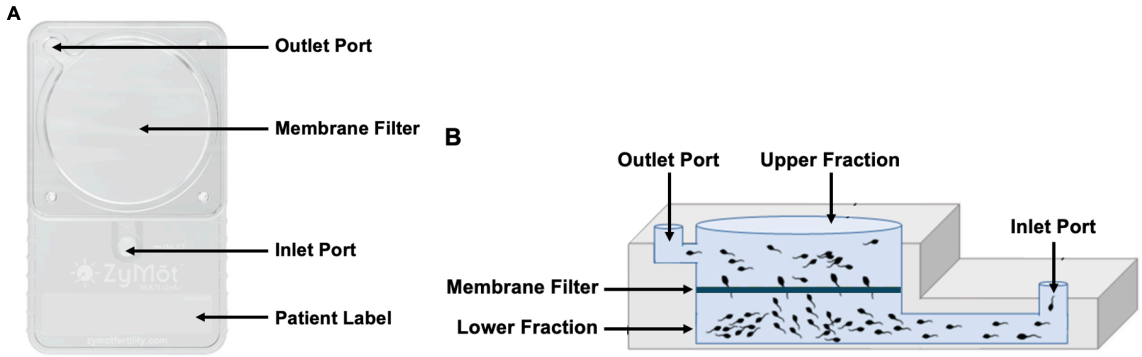


Figure 21 A. Representation of main component of the ZyMōt® device. (Adapted from www.coopersurgical.com) **B.** Schematic representation of ZyMōt® device, lateral view. (Adapted from Serrano-Albal *et al*., 2024)

4.6 Oocyte collection and ICSI

In homologous cycles (H), oocytes were collected through pick up procedure after controlled ovarian stimulation. The controlled ovarian stimulation was performed with gonadotrophins and the daily dose of FSH was adjusted according to the single patient ovarian response, based on serum estradiol levels and number and size of ovarian follicles monitored by transvaginal ultrasonography. A single injection of hCG was administered to induce final follicular maturation. Oocyte retrieval was performed by transvaginal ultrasound-guided follicle aspiration 36 hours after hCG injection and was performed under total anaesthesia. The retrieved follicular fluid was placed into a petri dish and scanned for the presence of oocytes. Collected oocytes were transferred in a culture dish inside the incubator. Before ICSI, oocyte-

cumulus complexes were denuded using hyaluronidase: denudation allows detailed assessment of oocytes maturity and their grading.

In oocyte donation cycles (OD, heterologous cycles), cryopreserved oocytes from a foreign donor bank were used. On the ICSI day, the oocytes were warmed and transferred into a pre-equilibrated IVF-Plus (IVF-Plus, Vitrolife, Sweden) culture dish inside the incubator. Oocyte warming was performed using Kitazato Warming kit (Kitazato Corporation, Japan).

ICSI was performed under inverted microscope equipped with micromanipulators and microinjections while on heated stage following the procedure described by Palermo (Palermo *et al*., 1992). The chosen sperm was mechanically constricted with the injection pipette at the bottom of the dish to induce the rupture of its plasma membrane and its subsequent immobilization. The immobilized sperm was aspirated tail-first into the injection pipette and carefully transferred to the oocyte. The oocyte was hold with the holding pipette and oriented with the polar body at 12 or 6 o'clock position. The tip of the injection with the immobilized sperm was introduced into the oocytes at the 3 o'clock position, the zona pellucida and the oolemma were penetrated and a little amount of the ooplasm was aspirated before the release of the sperm (Figure 22). Once ICSI was completed, all injected oocytes were moved into a new pre-equilibrated 4 well IVF-Plus culture dish inside the incubator.

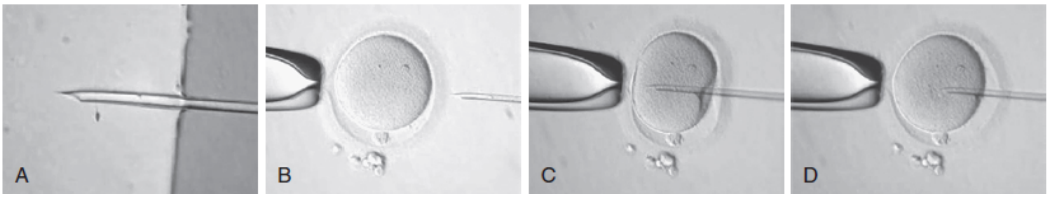


Figure 22. Microscopic images of various ICSI steps: **A.** Sperm immobilization; **B.** Positioning of the gametes with holding and injection pipette; **C.** Injection of the oocyte; **D**. Release of the sperm into the ooplasm. (Rosen et al., 2019).

4.7 Embryo culture and transfer

Successful fertilization was assessed at 16–18 hours post-ICSI and confirmed by the presence of two pronuclei and two polar bodies. Before fertilization check, inseminated oocytes were transferred into a pre-equilibrated multi-droplet G-TL (G-TL, Vitrolife, Sweden) culture dish. Fertilization rate was expressed in percentage and calculated by dividing the number of zygotes showing 2PN after ICSI by the

total number of inseminated MII oocytes multiplied by 100. Embryos were observed every day in order to monitor their development (Figure 23) and scored in relation to their kinetics of division, morphology, number and size of blastomeres and presence of fragmentation. Blastocysts were scored based on their degree of expansion, inner cell mass (ICM) and trophoblast characteristics.

The cleavage rate was expressed in percentage and defined by dividing the number of embryos reaching the third day of development by the number of zygotes multiplied by 100.

Depending on medical indications, transfer was performed on day 3, 4, 5 or 6 or embryos were cryopreserved. Embryo cryopreservation and warming were performed using Kitazato Vitrification kit and Kitazato Warming kit (Kitazato Corporation, Japan), respectively.

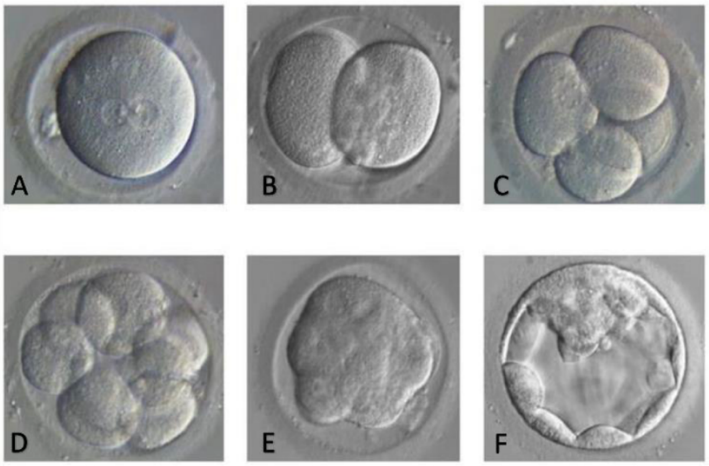


Figure 23: Different stages of embryo development. **A**. Zygote with pronuclei; **B**. Two blastomeres embryo; **C**. Four blastomeres embryo; **D**. Eight blastomeres embryo; **E**. Morula; **F**. Blastocyst. (Magli *et al*., 2012)

Transfer could involve one or two embryos depending on patient history, cycle characteristic and medical indications. Before the transfer, embryos were transferred into a centre-well culture dish and transfer was performed using a Wallace® Sure-Pro transfer catheter (CooperSurgical Inc., U.S.A) under ultrasound guidance. Fourteen days after embryo transfer, *beta human chorionic gonadotropin (β-hCG)* level was measured by blood test. The hormone is measured in milli-international units per millilitre of blood (mIU/mL) and, according to the American Pregnancy Association, it is considered negative for pregnancy when its levels are less than 5 mIU/mL, while it is considered positive for pregnancy any value above 25 mIU/mL (Figure 24).

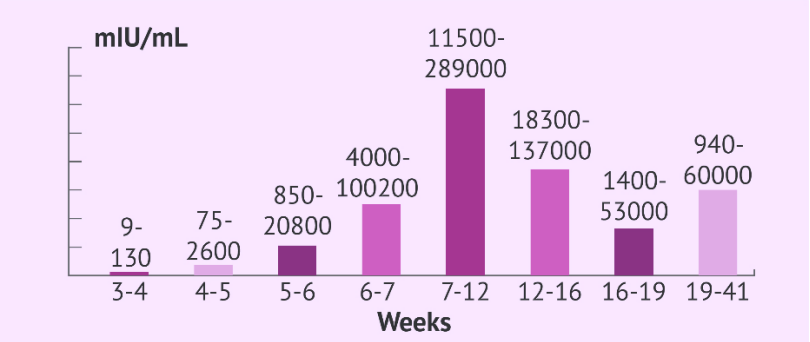


Figure 24. Reference intervals of β-hCG hormone value in blood according to the weeks of pregnancy (9-130 mIU/ml: 3-4 weeks pregnant; 75-2600 mIU/ml: 4-5 weeks pregnant; 850-20800 mIU/ml: 5-6 weeks pregnant; 4000-100200 mIU/ml: 6-7 weeks pregnant; 11500-289000 mIU/ml: 7-12 weeks pregnant; 18300-137000 mIU/ml: 12-16 weeks pregnant; 1400-53000 mIU/ml: 16-19 weeks of pregnancy (2nd trimester); 940-60000 mIU/ml: 19-41 weeks of pregnancy (3rd trimester) (www.invitra.com/en/beta-hcg-levels-during-pregnancy/)

4.8 Statistical Analysis

All the analysed parameters were reported in the database of the centre. They were extrapolated and resumed in an Excel database, which specifically contain information on the couples and cycle characteristics as well as their outcomes.

Statistical analysis was performed using the GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Variables were tested for normality and the Student T-test was used to compare parameters. The statistical significance was set at $p \leq 0.05$.

5. Results and discussion

It is widely known that ejaculates with normal parameters may hide molecular abnormalities, such as DNA fragmentation, which can affect the success of ART treatments. Such DNA damage can be pre-existent because of pathological conditions or may be introduced or amplified during handling steps in IVF procedures. Based on this considerations, novel sperm selection techniques have been developed with the goal of improving ART outcomes by minimizing DNA fragmentation while preserving or enhancing the functional competence of spermatozoa. Among these, this study evaluates the use of MACS, which selects non-apoptotic sperm using annexin V binding, and ZyMōt®, a microfluidic sperm selection device able to mimic a more physiological sperm selection route avoiding centrifugation steps. The impact of these two alternative sperm selection methods on ART cycles is evaluated with different parameters, including sperm motility type A, fertilization rate, cleavage rate and pregnancy rate. Are included in the study couples who performed both a previous IVF cycle using a conventional sperm selection procedure (Control w/o MACS group and Control w/o ZyMōt® group) and, after its failure, another cycle in which MACS or ZyMōt® are used (Study MACS group and Study ZyMōt® group). For both techniques, this comparison was performed in couples who underwent homologous (H) and oocyte donation cycles (OD).

The purpose was to investigate the potential beneficial effect on ART cycles when spermatozoa are selected with MACS or ZyMōt® instead of a classical sperm preparation procedure.

5.1 Male population

For each patient main sperm parameter were measured, including concentration, motility, morphology and sperm DNA fragmentation index (SDFI). As reported in Table 2, the male population showed normal semen parameters in term of sperm concentration, total sperm motility and morphology, while SDFI values were 35% in male partners of couples who underwent homologous cycles and 30% in male partners of couples who underwent an oocyte donation cycle. Overall, semen samples of the studied population showed a borderline or altered SDFI value.

VARIABLE	HOMOLOGOUS CYCLES 34 couples	OOCYTE DONATION CYCLES 27 couples	WHO 2021
Sperm concentration (10^6/ml)	38 ± 44	40 ± 24	≥ 16
Total sperm motility (%)	40 ± 24	43 ± 16	≥ 42
Normal sperm morfology (%)	4 ± 3	5 ± 3	≥ 4
Sperm SDFI (%) mean ± s.d.	35 ± 9	30 ± 8	< 30

Table 2. Semen samples characteristics of the studied male population: sperm concentration, total sperm motility, sperm morphology and DNA fragmentation index (SDFI).

Since the correlation between sperm SDF and paternal age is frequently reported, sperm DNA fragmentation was evaluated also dividing the population in two age groups: under 40 years and over 40 years. The cut-off of 40 years was selected in accordance with previous literature where this threshold is applied to define the possible association between advanced paternal age and higher SDF levels (Rosiak-Gill *et al.,*2019; Gonzalez *et al.,* 2022; Deenadayal Mettler *et al.,* 2020).
The means of sperm DNA fragmentation index were calculated in both groups and statistical analysis was performed. The average SDFI in males with <40 years and that in males ≥40 years was exactly the same (32%) (Figure 25). For this reason, the study was performed without a subdivision based on the SDF level.

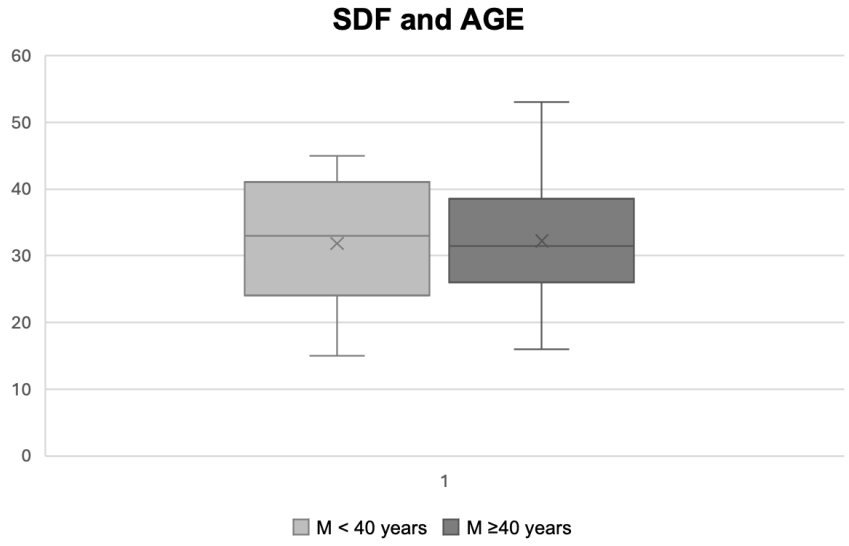


Figure 25. Sperm DNA fragmentation index (SDFI) in two male age groups: <40 years and ≥40 years.

5.2 Sperm motility type A

The mean motility among both “MACS study group”, “ZyMōt® study group” and control groups was calculated. The potential statistical difference between study groups and control groups was calculated by Student T-test. In addition, to evaluate the potential relationship between the male age and the sperm motility type A, male partners were divided in two groups based on their age: “Male < 40 years old” and “Male ≥ 40 years old”. The mean sperm motility type A was calculated in the following groups: “Study MACS - Male < 40 years old”, “Study MACS - Male ≥ 40 years old”, “Study ZyMōt® - Male < 40 years old”, “Study ZyMōt® - Male ≥ 40 years old”, “Control w/o MACS - Male < 40 years old”, “Control w/o MACS - Male ≥ 40 years old”, “Control w/o ZyMōt® - Male < 40 years old” and “Control w/o ZyMōt®- Male ≥ 40 years old”. The potential statistical difference between “Male < 40 years old” and “Male ≥ 40 years old” groups among all the groups was calculated by Student T-test.

5.2.1 Influence of MACS on sperm motility type A

After the initial analysis, for all the 28 couples were evaluated the effects of MACS as sperm selection method on sperm motility type A, and results were compared to those obtained by a conventional selection method. The study MACS group reported an increase, even if not significant, of sperm progressive motility compared to the Control w/o MACS group (26% vs 16%) (Figure 26).

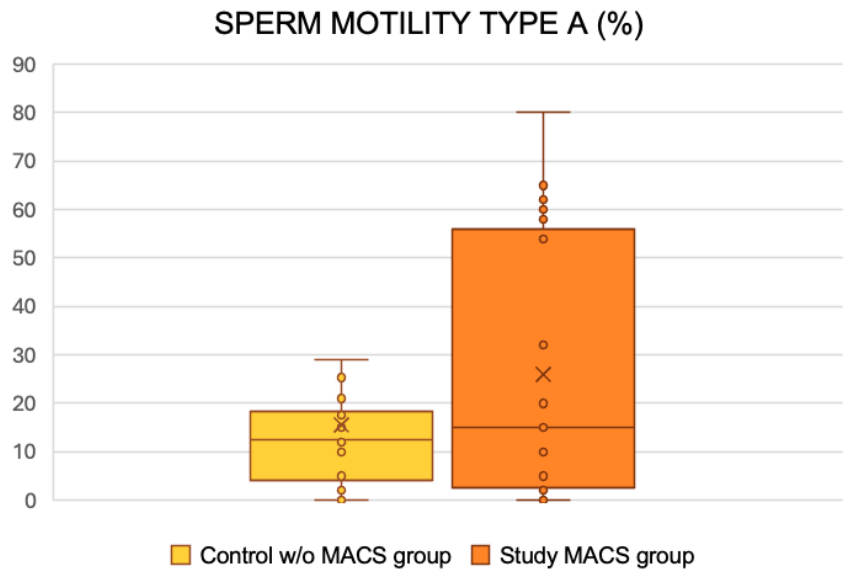


Figure 26. Box and Whisker Plot. Comparison of the percentage of sperm motility type A in the Control w/o MACS group and in the Study MACS group.

These results are aligned to different studies which report that fertilization potential in term of sperm motility is significantly higher when MACS is used instead of DGC (Said *et al*., 2005; de Vantéry Arrighi *et al*., 2009). In contrast, the study of Nadalini *et al.* (2014) concluded that MACS did not improve fertilization potential to the same extent as the traditional swim-up procedure. This conclusion was reached by comparing progressive motility of sperm fractions subjected to different sperm preparation procedure: DGC, DGC and magnetic sorting and DGC prior to traditional swim-up. They observed that DGC + MACS procedure and DGC + SU resulted in a significantly higher percentage of sperm progressive motility to the one registered in fraction after DGC only (+7.65 %, p <0.05 and +18.23 %, p <0.05, respectively) and conclude that the combination of DGC + SU was superior to MACS.

Since strong scientific evidences support that male fertility is strongly impacted by male aging, which also affects sperm motility (Collodel *et al.,* 2021; Colasante *et al.,* 2019), it was decided to investigate the impact of conventional sperm preparation techniques (Control w/o MACS group) and magnetic activated cell sorting (Study MACS group) on sperm progressive motility in two age groups: younger (<40 years old) and older (≥ 40 years).

In both groups was reported a positive trend in terms of sperm motility type A: from 14% to 17,8% in the <40 years group and from 12% to 23% in the ≥ 40 years group

(Figure 27). For both groups the difference was not significant, but the observed results probably suggest that spermatozoa of elderly men better react to capacitation procedures.

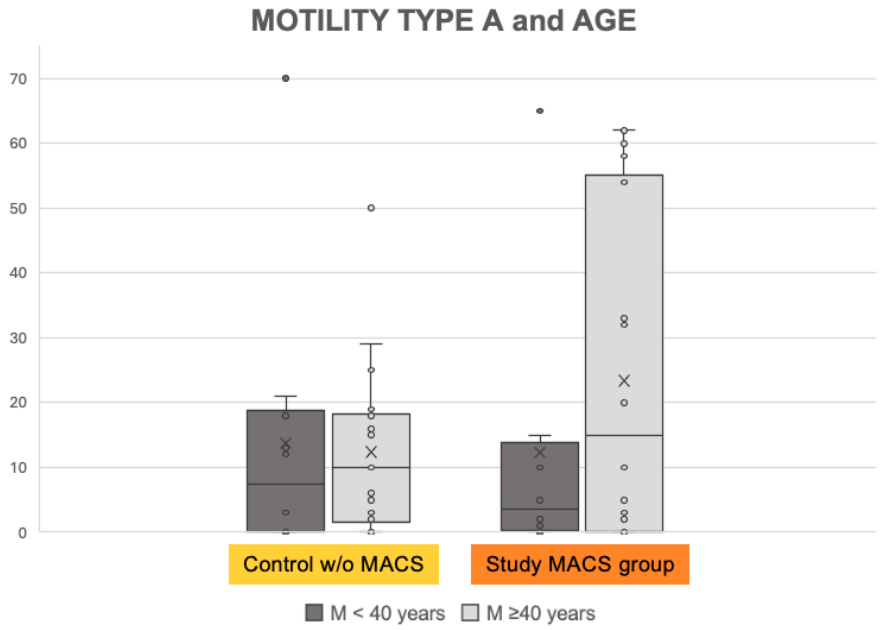


Figure 27. Box and Whisker Plot. Comparison of the percentage of sperm motility type A in both Control w/o MACS group and Study MACS group according to the male age.

It is important to consider that the small size of the study cohort is a limitation on the results of this thesis and a higher sample size and more precise definition of the study groups could further support the beneficial effect of MACS on sperm progressive motility.

5.2.2 Influence of ZyMōt® on sperm motility type A

The effects of ZyMōt®® on sperm motility type A was evaluated in all the 33 couples. Results showed a decrease, even if not statistically significant, in the percentage of progressive motile sperm selected with ZyMōt®® compared to control (16,25% in the Control w/o ZyMōt® group VS 12,28% in the Study ZyMōt® group) (Figure 28).

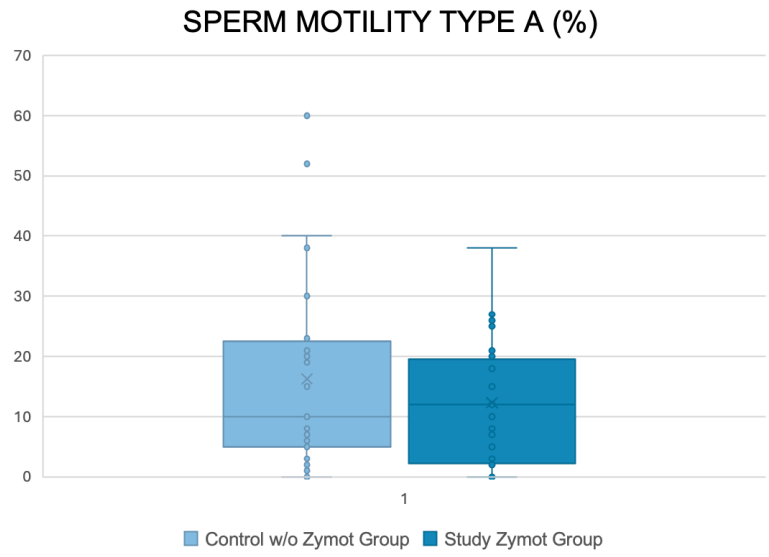


Figure 28. Box and Whisker Plot. Comparison of the percentage of sperm motility type A in the Control w/o ZyMōt® group and in the Study ZyMōt® group.

As for MACS, the parameter was then evaluated dividing the considered male population in two age groups: younger (<40 years old) and older (≥ 40 years). Both age groups showed a decline in sperm motility type A, with the younger men reporting a more considerable decrease compared to the older men (younger 16,9% VS 11%, older 15,8% VS 13,2%), even if not statistically significant (Figure 29).

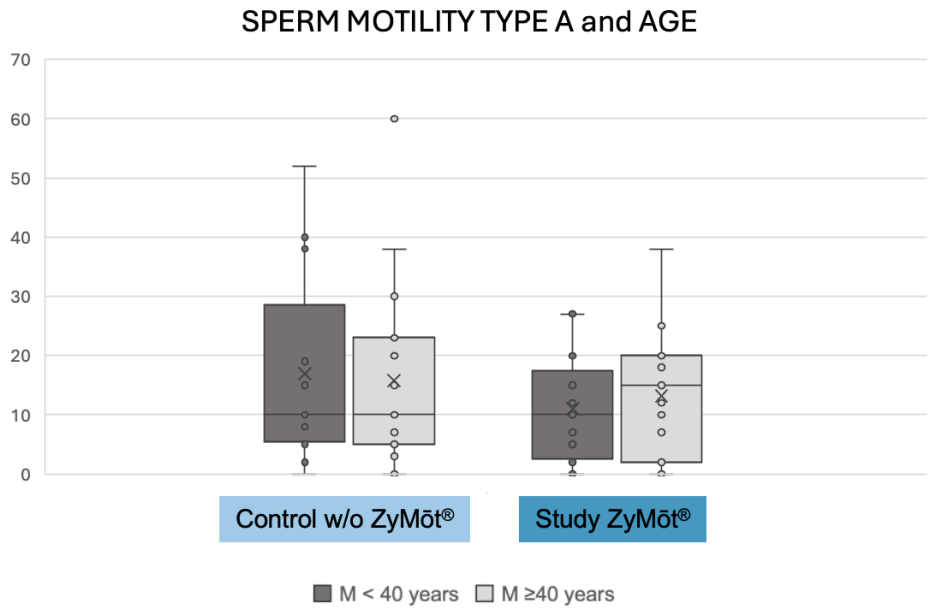


Figure 29. Box and Whisker Plot. Comparison of the percentage of sperm motility type A in both Control w/o ZyMōt® group and Study ZyMōt® group according to the male age.

These data appear to be in contrast with other published results: a recent meta-analysis of 39 studies reported that microfluidic sperm selection (including ZyMōt®) significantly increases progressive motility and total motility compared to swim-up or density-gradient centrifugation (Gisbert Iranzo *et al.,* 2025). Moreover, Adolfsson *et al.* (2025) in a clinical validation study published that while ZyMōt® yields a much higher fraction of progressively motile sperm (97% vs. 83% with DGC), the absolute number of progressively motile sperm retrieved was significantly lower with ZyMōt®. These apparently discordant findings can be reconciled by recognising that ZyMōt® mechanism (passive microfluidic migration through a membrane) preferentially selects a smaller subpopulation of sperm with lower DNA fragmentation and higher individual motility, at the cost of reduced processed sample volume and lower total motile count compared with DGC or swim-up. Methodological differences between studies, including initial semen quality, device model/processing volume, sample dilution, incubation time, and the use of CASA settings for progressive motility thresholds are plausible drivers of inter-study variability and may explain why some datasets observe lower progressive motility after ZyMōt®.

5.3 Fertilization rate

After the insemination, data of couples who underwent homologous cycles and oocyte donation cycles were analysed. For oocyte donation cycles as well as for homologous cycles, the means of “Study MACS group” and “Control w/o MACS group” and “Study ZyMōt® group” and “Control w/o ZyMōt® group” were calculated and the potential statistical difference between study and control groups was obtained by Student T-test.

To better investigate the potential improvements in term of fertilization rates, for each couple was performed a calculation of the gaps between “Study MACS group” value and “Control w/o MACS group” value or “Study ZyMōt® group” value and “Control w/o MACS group”. Gaps were classified to facilitate the study (Figure 30). The score A defined the couples in which the difference between the fertilization rate of the Study Group and that of the Control Group was >25%; the score B has been attributed when the difference was from 6 to 25%; the score C has been assigned when the difference was from -5% to +5% and the score D when it was <-5%. Finally, the percentage of couples showing fertilization rate scores A, B, C, D both in the group of heterologous cycles and in that of homologous cycles was calculated. The results were reported in a Pie Chart.

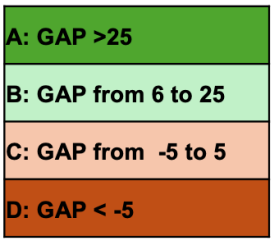


Figure 30. Score classification for fertilization rate.

5.3.1 Influence of MACS on fertilization rate

Since high levels of sperm DNA fragmentation are largely correlated to fertilization failure, the selection of spermatozoa using MACS could have a beneficial effect on fertilization rate in IVF cycles. In couples who underwent homologous cycles, the mean value of fertilization rate in the Study MACS group was considerably higher than that observed for Control w/o MACS group (64% vs 51%). The same trend was observed in oocyte donation cycles, in which the fertilization rate was 67% in the Study MACS group and 55% in the Control w/o MACS group (Figure 31). There

were no statistically significant differences between the H Study MACS group and OD Study MACS group and between the H Control w/o MACS group and OD Control w/o MACS group.

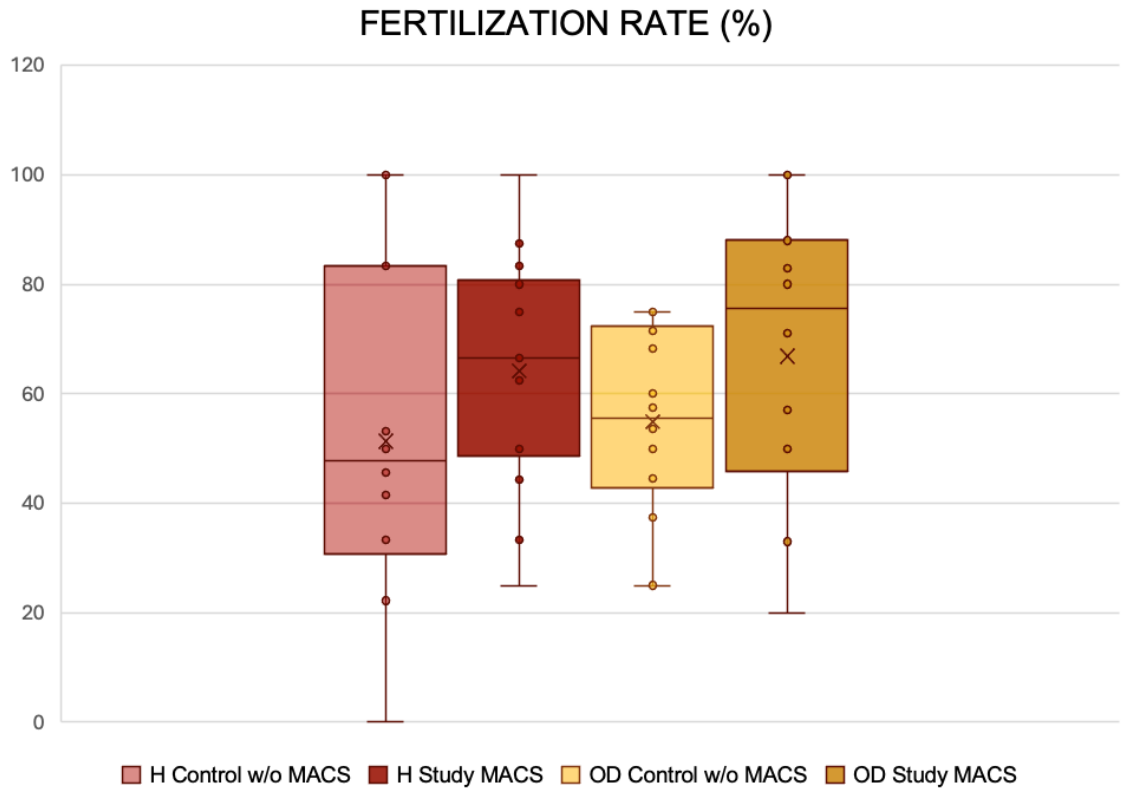


Figure 31. Box and Whisker Plot. Comparison of the fertilization rate of homologous (H) and oocyte donation (OD) cycles in both Control w/o MACS group and Study MACS group.

Other studies reported similar results, showing a trend of improvement in fertilization rate when MACS was used, without reaching a statistically significant difference even when the studies include a higher number of cases compared to this thesis (Merino-Ruiz *et al.*, 2019; Pacheco *et al.,* 2020).

Moreover, the average fertilization rate in the Study MACS group of oocyte donation cycles was higher of that of homologous cycles (67% vs 64%), as well as the average of this parameter in the Control w/o MACS groups of oocyte donation cycles and homologous cycles (55% vs 51%). This trend is probably accountable to female factors: the donors are young women selected and screened for numerous diseases, while female partners are older and often diagnosed with infertility conditions. Since is widely known that increased female age is linked to a worse oocyte quality, a lower fertilization rate in homologous cycles could be considered normal (Moghadam *et al.*, 2022).

These results were also individually analysed by the gap analysis in order to investigate the individual improvements in each couple. The results illustrating the percentage of couples showing fertilization rate scores A, B, C, D were reported in the pie charts Figure 32 for homologous cycles (H) (left panel) and for OD (right panel). Both in H and OD cycles, the 57% of couples showed an increase at least 6% of the fertilization rate in the Study MACS group compared to their previous cycles Control w/o MACS group (score A + score B). In particular, the 36% of couples who performed homologous cycles and the 43% of couples who underwent oocyte donation cycles, revealed an improvement in term of fertilization rate using MACS higher than 25% (score A – dark green). In contrary, among the homologous cycles, the 14% of couples showed small difference of fertilization rate between the Study MACS group and the Control w/o MACS group, ranging from -5 and +5% (score C – light red). Moreover, the 29% of couples revealed a considerable worsening (score D – dark red) of this parameter in ICSI-MACS cycles (Study MACS group). In the oocyte donation cycles, although only the 7% of couples showed the score C (light red), there was a consistent representation of the worst scenario with the 36% of couples who revealed a gap less than -5% (score D – dark red) suggesting that the previous cycles with classical sperm preparation techniques (Control w/o MACS group) had a better reproductive outcome than the cycles with MACS separation (Study MACS group). When these contradictory results are evaluated, it is important to consider male age and its influence on sperm fertilization potential: the MACS cycle occurs after one or more failed cycle, which means that MACS cycle can be performed months or years after the previous one. In the Study MACS group of oocyte donation cycles the average age of the male population was 43 years and this is aligned to studies reporting that a paternal age of ≥40 years can be considered as a relevant risk factor for infertility and frequent ART cycles failure (De la Rochebrochard & Thonneau, 2003).

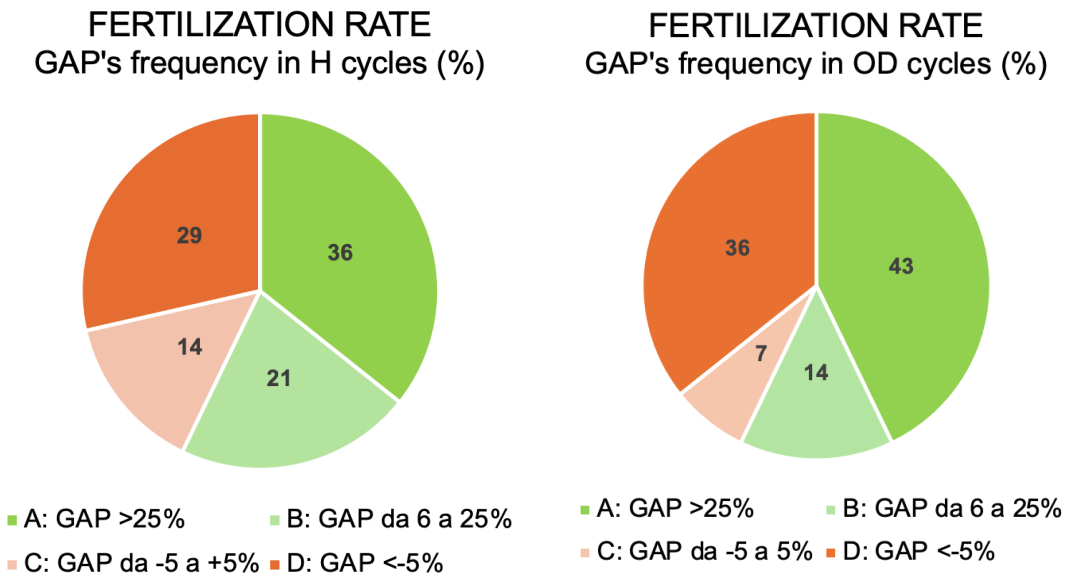


Figure 32. (left panel) Percentage of couples who performed homologous cycles (H) and (right panel) Percentage of couples who performed oocyte donation cycles (OD) showing fertilization rate scores A, B, C, D.

5.3.2 Influence of ZyMōt® on fertilization rate

In homologous cycles, the use of ZyMōt® showed a statistically significant improvement of fertilization rate when compared to control (81% in the Study ZyMōt® group VS 59% in the Control w/o ZyMōt® group, *p*= 0,035). In oocyte donation cycles no statistically significant difference between the Control w/o ZyMōt® group and the Study ZyMōt® group was observed (69% VS 72%) (Figure 33).

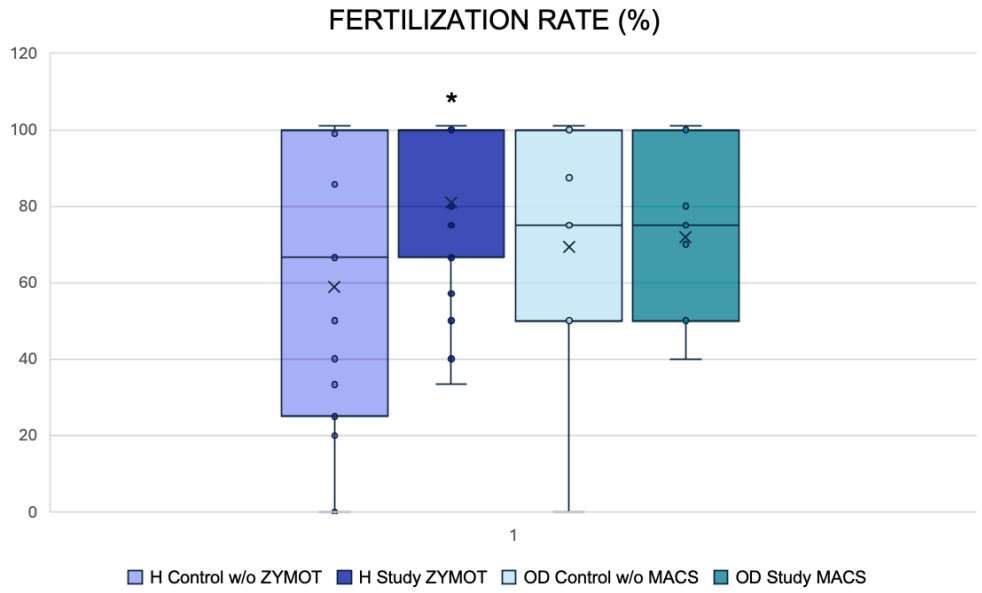


Figure 33. Box and Whisker Plot. Comparison of the fertilization rate of homologous (H) and oocyte donation (OD) cycles in both Control w/o ZyMōt® group and Study ZyMōt® group.

These findings are aligned with the mixed evidence in the published literature: some clinical studies and trials reported higher fertilization after microfluidic selection, suggesting that this novel method can enrich the treated sample with spermatozoa having better functional competence (Banti *et al.,* 2024). However, other clinical validations and reviews reported no consistent improvement in fertilization rate or a slight increase with no statistically significant difference (Lara-Cerrillo *et al.,* 2024; Zaha *et al.,* 2023).

As for MACS, these results were also individually analysed by the gap analysis to investigate the individual improvements in each couple. The results illustrating the percentage of couples showing fertilization rate scores A, B, C, D were reported in the pie charts Figure 34 for homologous cycles (H) (left panel) and for OD (right panel). In homologous cycles, the majority of the couples (58%) reported an

increase in fertilization rate of at least 6% (score A + score B); in particular, the 42% showed an increase of more than 25% (score A – dark green). The 26% of the couples reported slight or no differences in terms of fertilization rate (score C – light red) and the 16% of the couples reported a decrease of more of the 5% (score D – dark red). In oocyte donation cycles, the 38% of the couples reported slight or no differences in fertilization rate when ZyMōt® was used instead of conventional methods (score C – light red) and an equal percentage of couples reported an increase of more of the 25% and a decrease of more of the 5% (31% for both score A and score D).

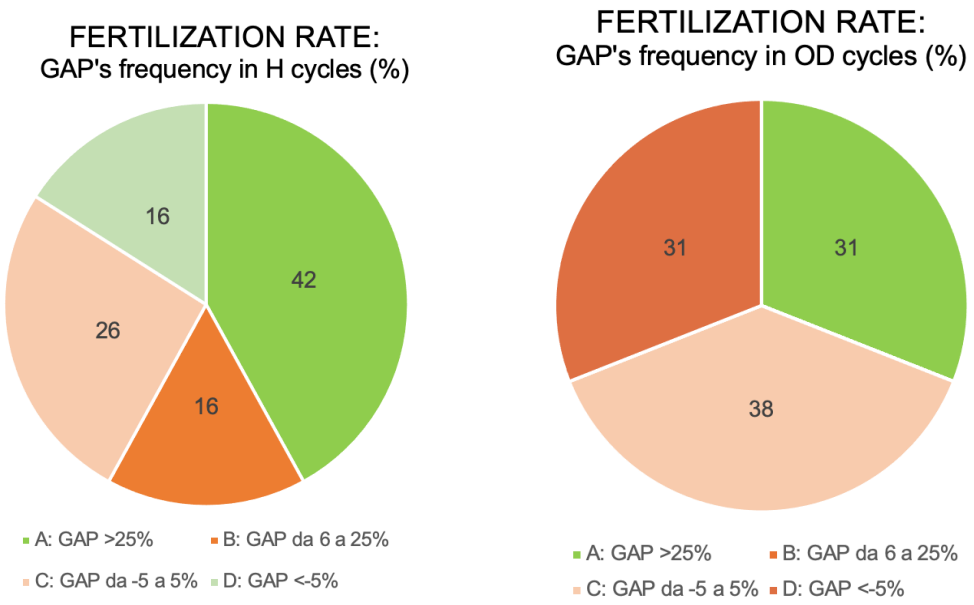


Figure 34. (left panel) Percentage of couples who performed homologous cycles (H) and (right panel) Percentage of couples who performed oocyte donation cycles (OD) showing fertilization rate scores A, B, C, D.

A plausible explanation of these differences in the results of homologous cycles compared to those of oocyte donation cycles could be related to differences in oocyte quality: donor oocytes are typically of high-quality, so any incremental sperm benefit from ZyMōt® produces only a small or non-detectable change in fertilization rate. By contrast, in autologous cycles, where oocyte quality and sperm function are more variable, the selective enrichment achieved by microfluidics may translate into a measurable increase in fertilization rate.

5.4 Embryo development

Recent findings have increasingly highlighted how anomalies in chromatin structure, particularly sperm DNA fragmentation, may be implicated in compromised fertilization outcomes, defective embryogenesis, and impaired embryonic development. Different studies reported the association between elevated levels of sperm DNA damage and suboptimal embryonic progression, particularly reduced cleavage rates and developmental arrest beyond the second or third cell division (Zini *et al*., 2011; Colaco & Sakkas, 2018).

The review by Colaco & Sakkas, in particular, described a statistically significant reduction in the development of high-quality embryos by day 3 in individuals exhibiting SDF levels equal to or exceeding 21%, in comparison to cohorts with lower sperm chromatin dispersion (SCD) positivity. This phenomenon is biologically plausible considering that embryonic genome activation (EGA) typically starts after the second cell division. Consequently, DNA integrity anomalies in the paternal genome are likely to manifest their deleterious effects during these later stages of preimplantation development (Braude *et al*., 1988).

Despite these insights, identifying a reliable biomarker for embryo quality remains one of the most significant challenges in assisted reproductive technologies. In this context, enhancing the selection of non-apoptotic, mature spermatozoa with high genomic integrity represents a promising strategy for optimizing embryonic development and clinical outcomes.

Cleavage rates and means of homologous cycles and oocyte donation cycles in MACS and ZyMōt® study groups and control groups were calculated and Student T-test was performed in order to investigate the eventual significant differences between study and control groups.

As for fertilization rate, gap analysis was performed to evaluate the potential improvement of the cycle success in each studied couples and gaps were classified. The score A defined the couples in which the difference between the cleavage rate of the study group and that of the control group was >25%; the score B has been attributed when the difference was from 6 to 25%; the score C has been assigned when it was from -5% to +5% and the score D when it was <-5%.

The percentage of couples showing each cleavage rate score was calculated in both oocyte donation cycles and homologous cycles and results were illustrated by a Pie Chart.

5.4.1 Influence of MACS on embryo development

Since some publications report MACS as a useful method to reduce apoptotic sperm and improve overall embryo quality (Colaco & Sakkas, 2018), the potential positive effect of MACS on clinical outcomes was investigated through cleavage rate.

The results of homologous cycles report no statistically significant differences between the Control w/o MACS group and the Study MACS group even if the cleavage rate in the Study MACS group was slightly higher than in the Control w/o MACS group (84% vs 81%). The same trend was observed in oocyte donation cycles, with an average cleavage rate of 78% in the Study MACS group compared to a 72% of the Control w/o MACS group, without a statistically significant difference (Figure 35).

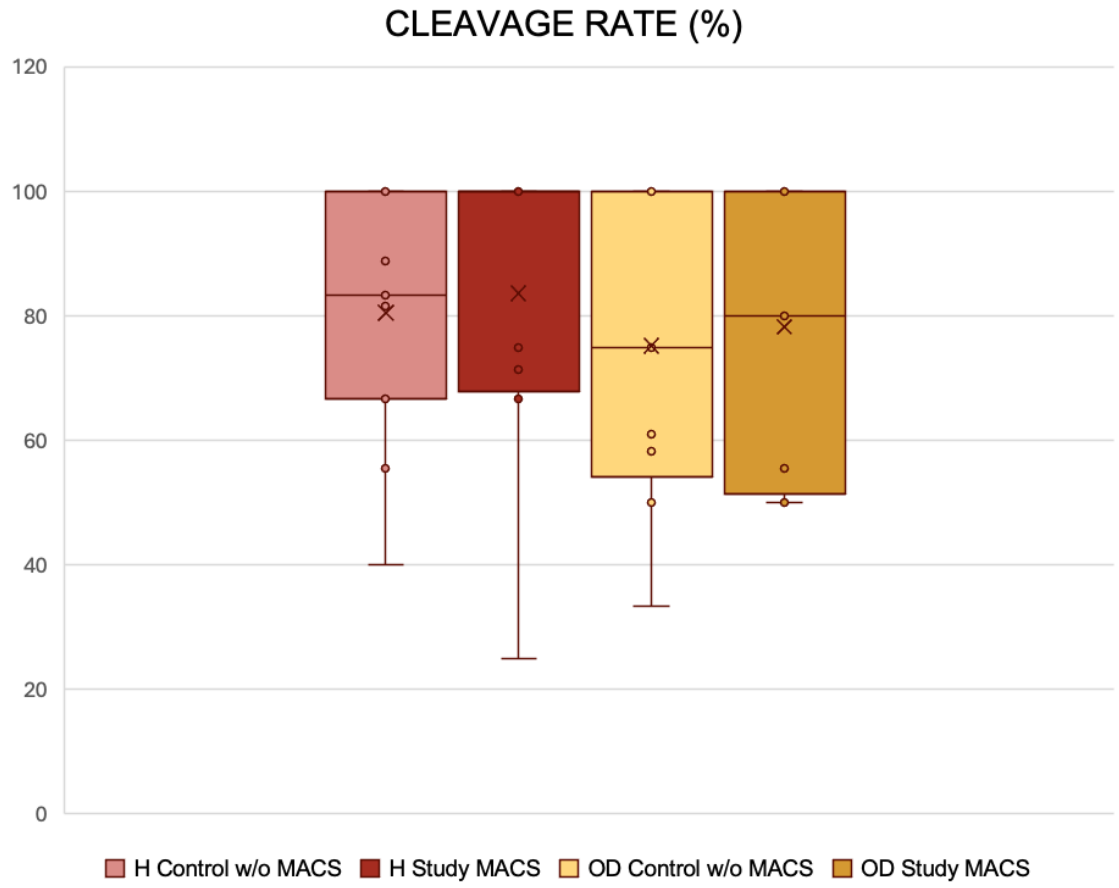


Figure 35. Box and Whisker Plot. Comparison of the cleavage rate of homologous (H) and oocyte donation (OD) cycles in both Control w/o MACS group and Study MACS group.

Different authors reported similar results, with an increased cleavage rate and higher embryo quality when MACS was employed (Dirican *et al.,* 2008; Stimpfel *et al.,* 2018; Ziarati *et al.,* 2018) and an increased embryonic segmentation compared

to cycles in which DGC was used (Baldini *et al.,* 2021). On the other hand, some studies noted no significant differences in cleavage rate between MACS groups and Control groups in oocyte donation cycles (Mei *et al.,* 2022) and the absence of improvement in terms of percentage of day 2 and day 3 good-quality embryos when MACS was used in oocyte donation cycles (Romany *et al*., 2014).

Moreover, by comparing the average cleavage rates between homologous Study MACS group and oocyte donation cycle Study MACS group no statistically significant differences were observed (84% vs 78%), as well as for the homologous Control w/o MACS group and oocyte donation Control w/o MACS group (81% vs 72%).

Although the statistical analysis did not reveal any statistical differences, the gap analysis revealed a strong beneficial effect of MACS technique in the cleavage of embryos (Study MACS group) when compared with the cycles involving classical sperm preparation techniques (Control w/o MACS group): the 43% of couples in both homologous and oocyte donation cycles showed an improvement of the cleavage rate higher than the 25% (Score A – dark green) while the 21% in homologous cycles and the 14% in oocyte donation cycles showed a smaller improvement, ranging from 6 and 25%, using MACS (score B – light green) (Figure 36). The 7% of couples who performed homologous cycles and the 21% of those performing oocyte donation cycles revealed a similar cleavage rate both in the Control w/o MACS and in the MACS cycle (Score C – light red). Finally, the percentage of couples showing a worsening in the cleavage rate (score D – dark red) was higher among the homologous cycles group (29% of couples) than in the oocyte donation cycles group (21% of couples).

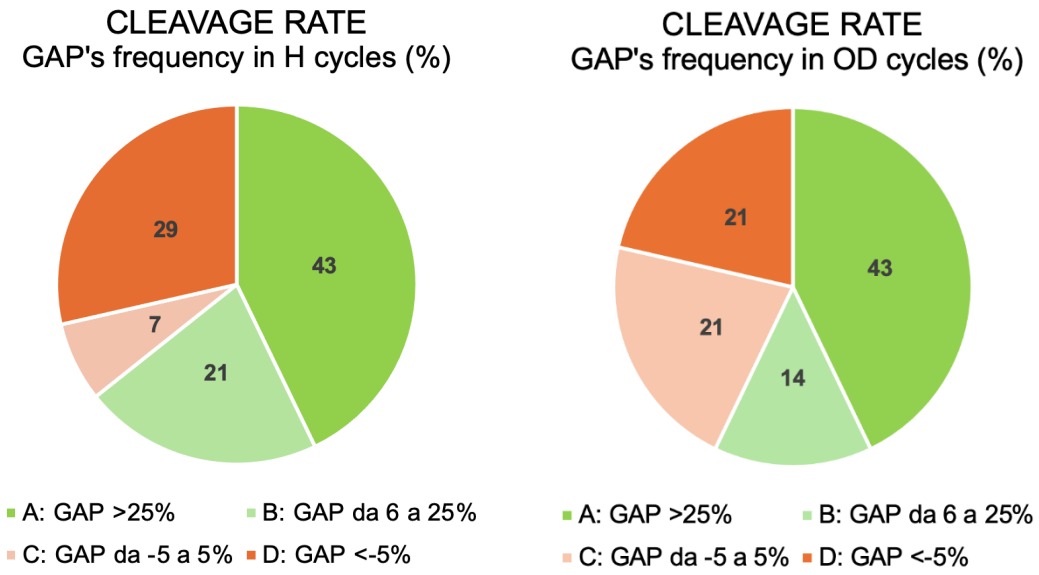


Figure 36. (left panel) Percentage of couples who performed homologous cycles (H) and (right panel) Percentage of couples who performed oocyte donation cycles (OD) showing cleavage rate scores A, B, C, D.

5.4.2 Influence of ZyMōt® on embryo development

For cleavage rate, the results of homologous cycles reported no statistically significant difference between the Control w/o ZyMōt® group (73,4%) and the Study ZyMōt® group (70,6%). In the oocyte donation cycles is possible to observe an increase, even if not statistically significant, of the cleavage rate in the Study ZyMōt® group when compared to the control (90,5% VS 75,4%) (Figure 37); the absence of a statistical relevance is probably related to the small number of couples of the oocyte donation group (13).

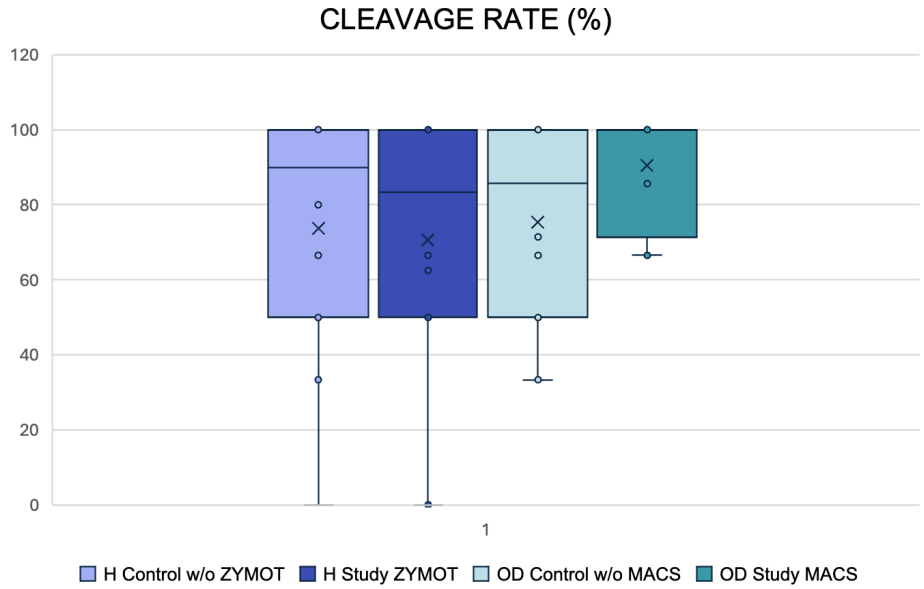


Figure 37. Box and Whisker Plot. Comparison of the cleavage rate of homologous (H) and oocyte donation (OD) cycles in both Control w/o ZyMōt® group and Study ZyMōt® group.

The gap analysis of homologous cycles showed that 25% of couples an improvement of cleavage rate of more of the 25% (score A – dark red), while the 50% of couples showed no difference (score C – dark red). In oocyte donation cycles, the 50% of couples reported an increase of at least 5% in cleavage rate (score A + score B), while 8% reported no differences (score C – light red) and the 42% showed a decrease in terms of cleavage rate of more than 5% (score D – dark red).

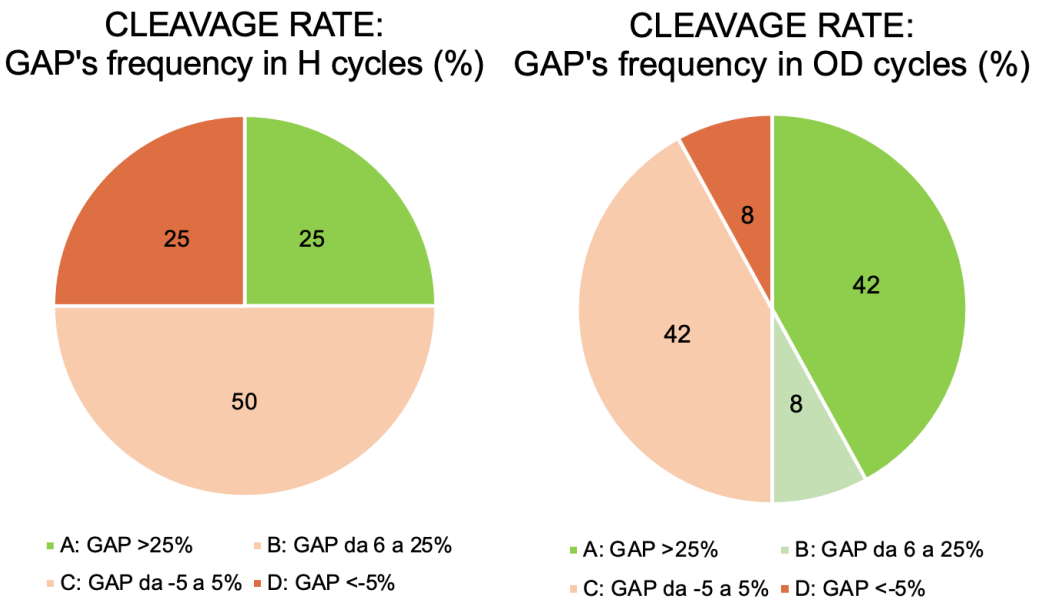


Figure 38. (left panel) Percentage of couples who performed homologous cycles (H) and (right panel) Percentage of couples who performed oocyte donation cycles (OD) showing cleavage rate scores A, B, C, D.

In this study, cleavage rate is comparable between conventional (SU/DGC) and ZyMōt® sperm selection method in homologous IVF cycles, while an increase, even if non-significant, was observed with ZyMōt® in oocyte-donation cycles. This aligns with previous findings showing no major differences in early embryo development after microfluidic selection (Yalcinkaya *et al.,* 2019; Parrella *et al.*, 2019). Moreover, Guler *et al.* (2021) reported no significant differences observing embryo development on day 3 in homologous ICSI cycles in which a novel microfluidic sperm sorting chip was used, suggesting that these results are comparable across different kind of microfluidic sperm sorting devices.

5.4 Pregnancy evaluation: β-hCG test

β-hCG test was performed 14 days after transfer. β-hCG hormone begin to be released by the developing embryo and its release increases consistently when the embryo performed the implant in the endometrium.

The percentage of positive β-hCG was calculated separately in the homologous cycles group (H) and in the oocyte donation cycles group (OD), comparing the values of the study groups and the control groups.

5.5.1 Pregnancy evaluation and MACS

In this study the evaluation of positive β-hCG test was performed in all cycles in which embryo transfer was performed. The 38,5% of couples of H Study MACS group revealed a positive β-hCG blood test after 14 days from embryo transfer, while in the OD Study MACS group the 35,7% exhibited this positivity. The same couples, when underwent previous cycles with the use of conventional sperm preparation techniques, revealed a positivity of 23,1% and 14,3% in homologous and oocyte donation cycles, respectively. The difference between the number of chemical pregnancies in the Study MACS group and that in the Control w/o MACS group was not statistically significant for both homologous and oocyte donation cycles (Figure 39).

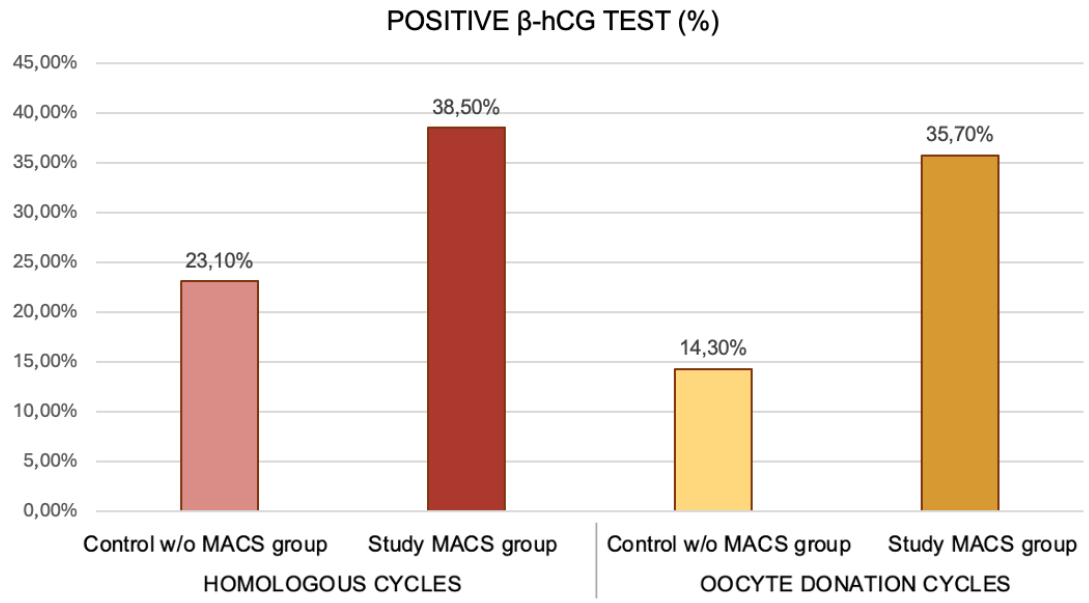


Figure 39. Grouped column chart. Percentages of couple who exhibited a positive β-hCG test in all cycles.

Different authors reported conflicting results in term of implantation rate. A systematic review by Gil *et al*. (2013) found no significant differences in implantation rate when MACS was used and similar results are reported by different clinical reports (Romany *et al.,* 2014; Mei *et al.*, 2022), showing no statistically significant differences in implantation rate between MACS groups and control groups. On the other hand, a number of smaller or selected-population studies (for example, patients with high sperm DNA fragmentation) reported higher implantation rates when MACS was used (Norozi-Hafshejani *et al*., 2022). Taking together these data, MACS does not appear to confer a reproducible implantation benefit across all patients, but it remains a plausible targeted intervention for selected male-factor subgroups.

5.5.2 Pregnancy evaluation and ZyMōt®

At the evaluation of positive β-hCG test, the homologous cycles showed an improvement from 25% in the Control w/o ZyMōt® group to 35% in the Study ZyMōt® group, despite the difference was not statistically significant. The same trend was observed in oocyte donation cycles: the Control w/o ZyMōt® group reported 30% of positive β-hCG test while the Study ZyMōt® group showed the 38,5% of positive tests.

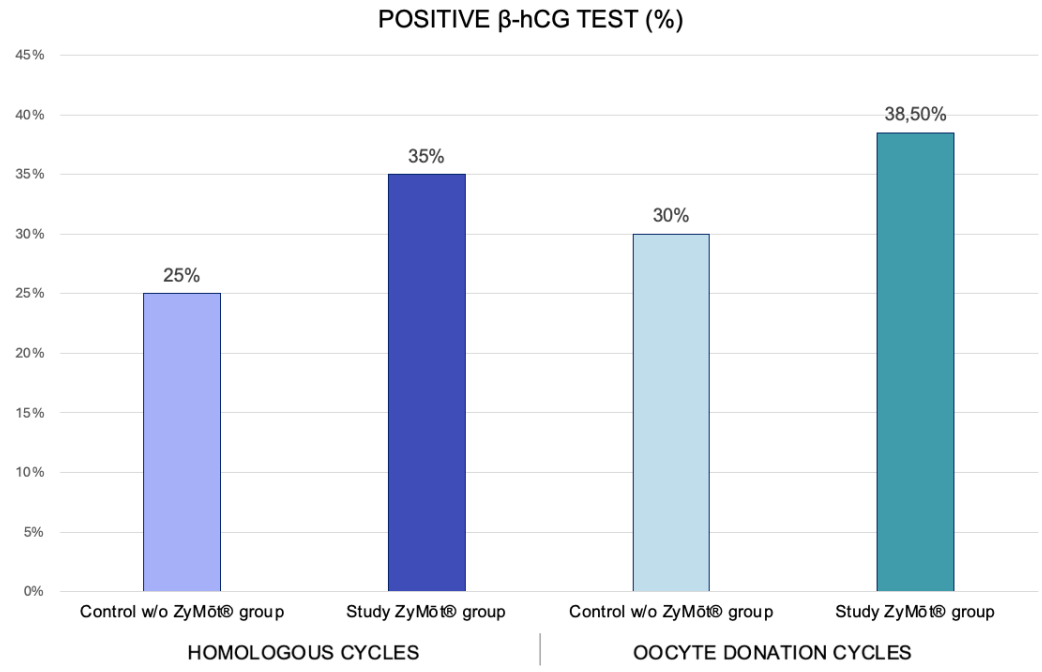


Figure 40. Grouped column chart. Percentages of couples who exhibited a positive β-hCG test in all cycles.

Different authors evaluated this parameter showing similar results: Gode *et al*. (2019) compared microfluidic to density gradient centrifugation and reported no statistically significant difference in terms of pregnancy rate (18,04% in the microfluidic group and 15,15% in the density gradient group) and Yetkinel *et al*. (2018) showed similar clinical pregnancy rates and live birth rates in 122 couples with unexplained infertility treated with SU or microfluidic.

In this thesis, the use of microfluidic sperm selection (ZyMōt®) was associated with a trend toward higher β-hCG positivity rates compared to conventional methods (SU/DGC) in homologous ART cycles, although the difference did not reach statistical significance. This tendency may suggest a potential benefit of microfluidic selection in improving early pregnancy outcomes, possibly due to enhanced sperm quality and reduced DNA fragmentation. Conversely, in oocyte donation cycles, where oocyte quality is not a limiting factor, the difference between methods was less pronounced, indicating that the impact of sperm selection on this parameter may be more evident when both gamete sources originate from the couple.

5.6 MACS VS ZyMōt®: a comparative overview

In the final part of this study, a direct comparison was performed between the two advanced sperm selection methods, MACS and ZyMōt®, to assess their relative effectiveness. Each parameter reported a different improvement or decline when compared to control: the percentage increase or decrease was calculated for each examined group and each parameter, in order to compare the results obtained with MACS to those obtained with ZyMōt®. The percentage difference for each parameter was calculated as follows:

$$\frac{\text{(Mean Value Control group)} - \text{(Mean Value Study Group)}}{\text{(Mean Value Control group)}} \times 100$$

Observing sperm motility type A, MACS and ZyMōt® reported opposite results: MACS showed a percentage increase of 62% while ZyMōt® a percentage decrease of 24,4% (Figure 41). As discussed before, these contrasting results are somewhat surprising given that many studies report that microfluidic selection tends to increase progressive motility compared to conventional methods (Vahidi *et al.,* 2025). On the other hand, evidence for MACS also supports improvements in sperm functionality: MACS is well established to reduce DNA fragmentation and select non-apoptotic sperm, and some works show it yields better motility or viability when combined with other methods (Gil *et al.,* 2013). The results of this thesis suggest that in our experimental / clinical setting MACS was more effective in boosting type-A motility, while ZyMōt® underperformed compared to control and it is important to consider also that method effectiveness can depend heavily on sperm parameters and laboratory protocols.

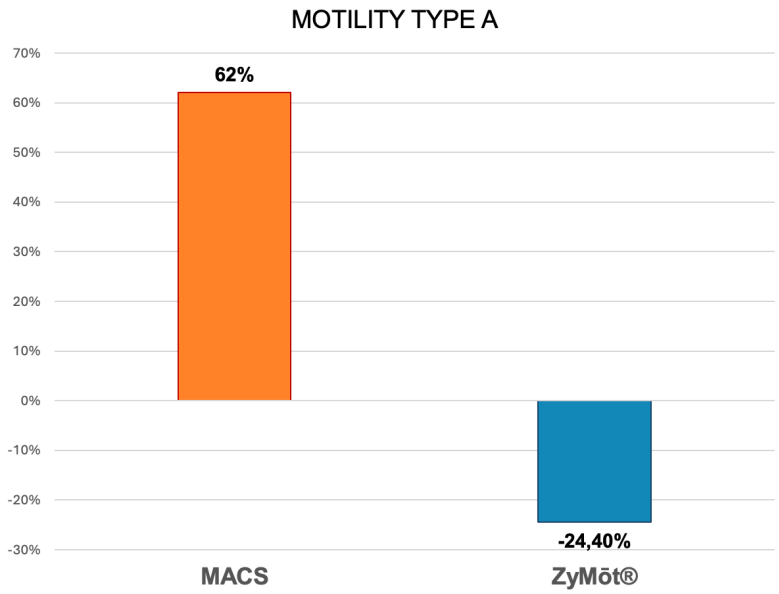


Figure 41. Comparison of the influence of MACS and ZyMōt® on sperm motility type A.

ZyMōt® demonstrated to be more effective in improving fertilization rate in homologous cycles: the percentage increase is of 37,7% when ZyMōt® was used and of 25,5% when MACS was used. In oocyte donation cycles, instead, MACS reported a percentage increase of 21,8% while ZyMōt® of 4,3% (Figure 42).

Both sperm selection techniques are described in literature as potentially able to rise fertilization rate due to their ability to select motile, morphologically normal and DNA fragmentation-free spermatozoa (Fleming *et al.,* 2024).

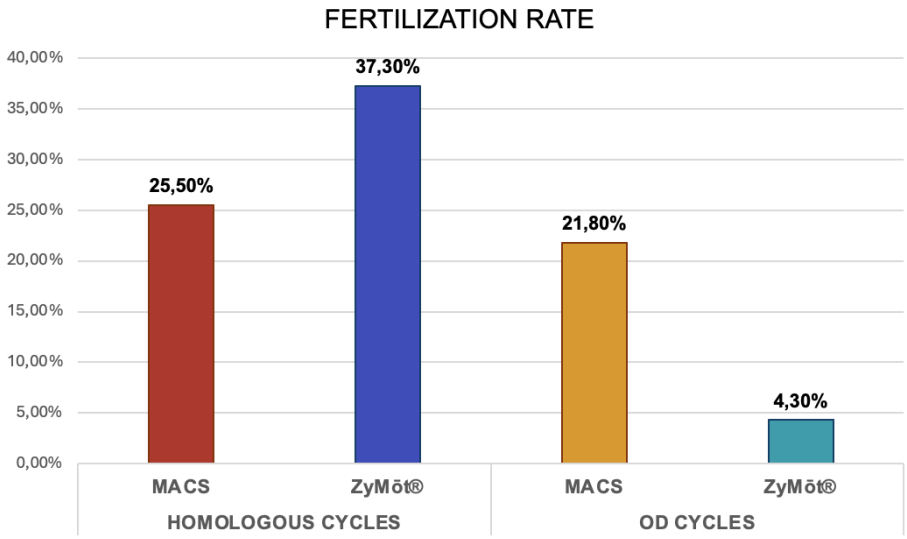


Figure 42. Comparison of the influence of MACS and ZyMōt® on fertilization rate.

In term of cleavage rate, MACS produced a slight increase in cleavage rate in homologous cycles (+3.7%), while ZyMōt® showed a slight decrease (−4.3%). In oocyte-donation cycles, both methods improved the cleavage rate, with a greater increase after ZyMōt® (+19.9%) compared to MACS (+8.3%), even if the difference was not statistically significant. These findings align with other studies showing that microfluidic selection and MACS can enhance early embryo development, particularly when high-quality oocytes are used, as in oocyte donation cycles (Hozyen *et al.,* 2022; Yetkinel *et al*., 2019).

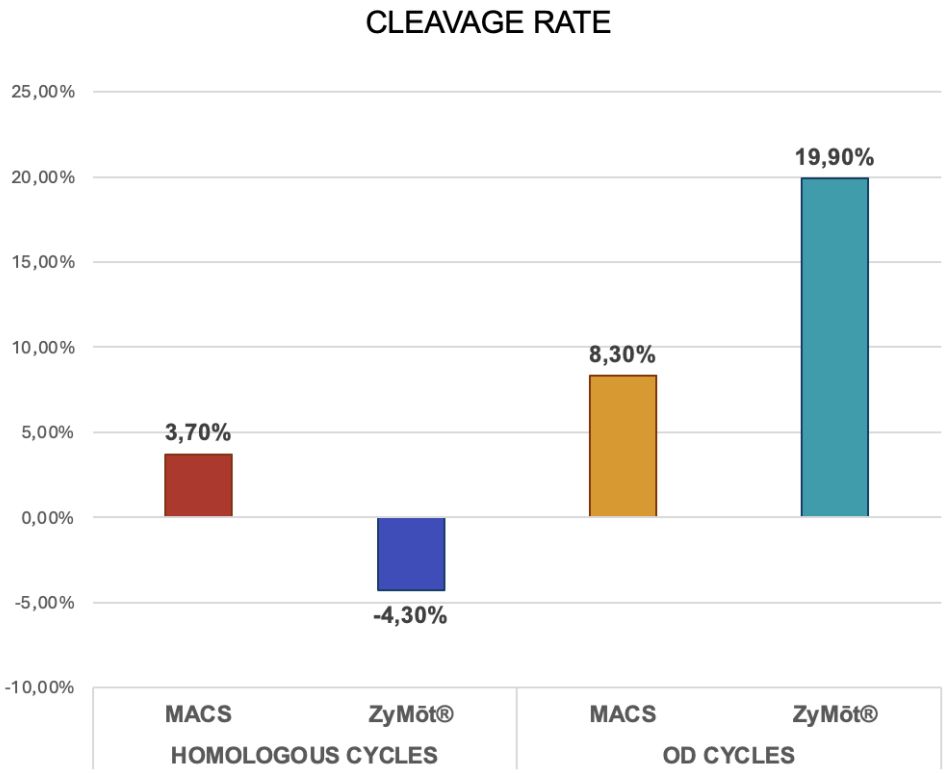


Figure 43. Comparison of the influence of MACS and ZyMōt® on cleavage rate.

The evaluation of β-hCG showed that both MACS and ZyMōt® can improve the rates of positive β-hCG, but the effect was more relevant for MACS (homologous +67% VS ZyMōt® +40%; oocyte-donation +149.6% VS +28.3%). This pattern is compatible with the heterogeneous results present in literature: meta-analyses and systematic reviews report that MACS can improve pregnancy outcomes compared with conventional methods in some cohorts (Gil *et al.,* 2021; Romany *et al.,* 2014). Microfluidic selection (ZyMōt® and similar chips) shows in several studies small improvements in clinical pregnancy, but results are variable across trials and patient populations (Adolfsson *et al.,* 2025; Aderaldo *et al.,* 2023).

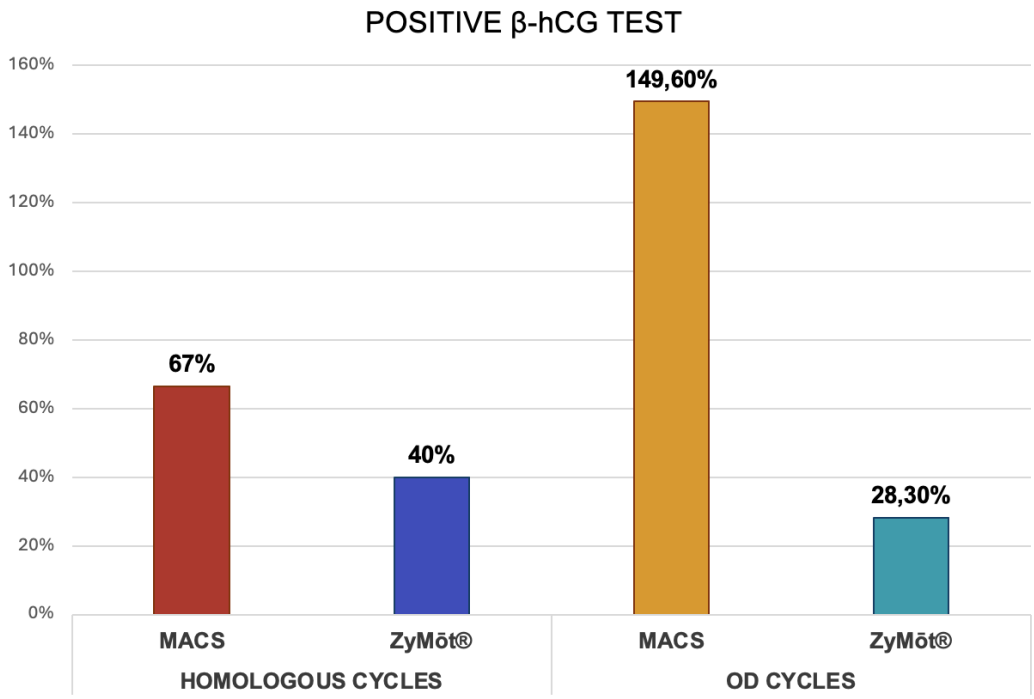


Figure 44. Comparison of the influence of MACS and ZyMōt® on β-hCG.

6. Conclusions

The ESHRE revised guidelines for good laboratory practice (ESHRE Guideline Group on Good Practice in IVF Labs, 2026) define that sperm selection prior to assisted reproduction aims to eliminate seminal plasma, debris and contaminants, to concentrate progressively motile sperm and select morphologically normal spermatozoa.

Traditional sperm selection methods such as swim-up and density gradient centrifugation, remain widely used and essential, but they are generally limited in targeting critical parameters such as DNA integrity, recently considered as a key factor influencing IVF success. In recent years, various innovative sperm selection methods have been proposed to reduce the impact of sperm DNA fragmentation. This study evaluated MACS and ZyMōt®, a microfluidic device, in homologous and oocyte donation IVF cycles.

The results of this study indicate that MACS led to an improvement in sperm progressive motility type A compared with conventional methods, whereas ZyMōt® showed a slight decrease in this parameter. Fertilization rates were positively influenced by both methods, with ZyMōt® achieving a statistically significant increase in homologous cycles. Regarding

cleavage rate, ZyMōt® showed a slight decrease in homologous cycles but an increase in oocyte donation cycles, while MACS demonstrated a tendency to improve cleavage in both cycle types. Furthermore, both techniques had a beneficial effect on pregnancy rate: MACS was particularly effective in oocyte donation cycles, while ZyMōt® showed a stronger effect in homologous cycles. A direct comparison suggested that had a more pronounced overall effect on pregnancy outcomes across both cycle types.

These results suggest that MACS and ZyMōt® valuable tools to enhance IVF outcomes, but their use should be tailored to specific infertility conditions and patient profiles. For instance, ZyMōt® may not be suitable for all oligozoospermic and asthenozoospermic patients, and MACS requires centrifugation steps that could negatively affect certain semen samples. This aligns with ESHRE recommendations, emphasizing that sperm selection should consider the characteristics of each semen sample. Although multiple studies have assessed these novel methods, the current body of evidence remains heterogeneous and

sometimes contradictory, likely due to differences methodology and the absence of standardized protocols.

Concluding, large, multi-centre, randomized controlled trials with pre-specified clinical endpoints, standardized sperm-processing protocols, and stratification (e.g. baseline SDF, male factor severity, cycle type) are required to define which patients are most likely to benefit from these techniques and to establish their cost-effectiveness.

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