Histol Histopathol (2016) 31: 819-831

**http://www.hh.um.es**

**Histology and Histopathology**

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## *Review*

# Steroid receptors in human ejaculated sperm as molecular markers of the detrimental effects related to the pathophysiology of testicular varicocele

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**Summary.** The exact physiopathologic effect of testicular varicocele on male fertility is not defined yet. The detrimental role of the varicocele in fertility is supported by the presence of a higher frequency of affected men among the infertile population. However, the mechanism/s by which a varicocele impairs sperm production, structure and function, is not known. In spite of active interest, our understanding of the human male gamete ultrastructural molecular organisation is still incomplete and therefore our knowledge of the sperm molecular anatomy is very limited. The presence of steroid binding sites on human spermatozoa has been evidenced since the 1970s, and afterwards, spermatozoa physiology was linked to the action of different steroids. The presence of steroid/steroid receptor systems was demonstrated in mature spermatozoa as membrane but also as nuclear conventional receptors, suggesting that both systemic and local steroids, through sperm receptors, may influence male fertility.

From new data, it emerges that varicocele may induce damage in the male gamete at molecular level, opening a new chapter in the already multifactorial pathophysiology of the varicocele, complicating this issue. In sperm from varicocele, a decreased expression of steroid receptors and a consequent reduced responsiveness to steroids may represent a mechanism involved in the physiopathology of varicocele. Therefore, the modulation of these nuclear receptors pave the way for novel therapeutic opportunities in the treatment of the male pathologies related to human reproduction. The purpose of this review is to gain new insight into the physiopathology of varicocele and to study its impact on human sperm molecular anatomy.

**Key words:** Estrogen receptors, Progesterone receptors, Androgen receptors, Steroids, Varicocele

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DOI: 10.14670/HH-11-761

## Introduction

Testicular varicocele, a dilatation of the pampiniform venous plexus within the scrotum, is widely accepted as the most common cause of male infertility (Romeo and Santoro, 2009), however the scientific support is almost lacking. The detrimental role of varicocele in fertility is raised by the presence of a higher frequency of affected men among the infertile population (Practice Committee of American Society for Reproductive Medicine, 2008; World Health Organization, 2010). In fact, the prevalence of varicocele is reported to be as high as 10-15% in the general population, in particular the 30-35% of men with primary infertility and the 69-81% of men with secondary infertility are affected. The etiology and physiopathology of the varicocele is still unclear and controversial (Skoog et al., 1997; Hauser et al., 2001; Naughton et al., 2001) and the molecular mechanisms by which varicocele leads to testicular dysfunction and

infertility are not completely understood. Experimental data from clinical and animal models demonstrate an adverse effect of varicocele on spermatogenesis, since venous reflux causing testicular temperature elevation induces impaired spermatogenesis (Zorgniotti and Macleod, 1973; Gorelick and Goldstein, 1993). In both men and rats, varicocele causes endocrine and testicular paracrine imbalances, hyperthermia, retrograde flow of adrenal blood, hypoxia, oxidative stress, apoptosis and dysfunction of the epididymis (Marmar, 2001; Turner, 2001; Fretz and Sandlow, 2002). Subclinical varicoceles were not generally associated with significant changes in testicular volume (Kantartzi et al., 2007), although Zini et al. (1997) showed that they are associated with a decreased testicular volume.

Many efforts have been made to find semen indicators of varicocele, still several controversies persist among reproduction experts. The variability of the results could be due to a lack of uniformity in patient selection, follow-up, treatment techniques (Yavetz et al., 1992), statistical methods (Comhaire, 1983) as well as the evaluation of semen quality itself that is not always performed according to the same criteria. Indeed, the varicocele may be associated with a variety of spermatogenic conditions, ranging from completely normal seminal parameters to a moderate oligoasthenoteratozoospermia or azoospermia. Therefore, the impact of the varicocele on sperm functions is still under discussion. Several studies have shown significant association between varicocele and poor sperm quality, although few researchers have focused upon the effect of the varicocele on human sperm functions and structure (Rodriguez-Rigau et al., 1981; Naftulin et al., 1991; Zini et al., 2000; Hauser et al., 2001; Ziemba et al., 2002).

Steroid hormones influence spermatogenesis and fertility (Aquila and De Amicis, 2014). The existence of the steroid binding sites on spermatozoa and the steroid effects on sperm provided new cues to ameliorate fertility potential. In fact, the regulation of sperm fertilization ability by steroid hormones and their receptors has been the object of intense efforts, although controversial data are often reported. The main problem for the andrologists was to accept the presence of transcription factors, including the steroid receptors, in a cell like sperm that has been considered silent from a transcriptional point of view. Most of the studies on the steroid receptors in spermatozoa have supported the original hypothesis that they work as specific membrane receptors through which they induce rapid, non-genomic responses in target cells. Indeed, it is now clear that the classical steroids can also act in a rapid non-genomic fashion. Interestingly, in recent years, a new picture of the sperm cell is emerging: it expresses various receptor types, including nuclear receptors (Travis and Kopf, 2002; Aquila et al., 2005a,b, 2007), and it produces their ligands, suggesting that through an autocrine short loop, it may modulate its own functions independently by systemic regulation. Recently, it has also been demonstrated that spermatozoa are able to translate de novo by mitochondrial-type ribosomes and numerous data suggest that transcriptional activity also occurs in these aploid cells (Gur and Breitbart, 2006; Oliva, 2006). Despite this, while questions linger, different intriguing avenues remain to be extended on the biology of this cell.

Although significant strides have been made on sperm biology and physiology, sperm cellular structure at molecular level is yet to be defined. During the past 40 years, studies on spermatozoa were prevalently focalized on biochemical changes related to the capacitation process as well as on the signalling events that regulate functions rather than on the sperm anatomic molecular composition. Our current knowledge on spermatozoa is marked by many breaks since the study of this cell was not updated according the advances in technology and molecular biology. Ejaculated mammalian spermatozoa are highly attractive cells showing intriguing features and, curiously, they go through two different physiological conditions; they acquire anatomical and morphological maturation in the male genital tract while their functional maturation, known as capacitation, occurs in the female genital tract (Yanagimachi, 1994; Rathi et al., 2001; Suarez, 2008). The highly polarized sperm body includes head-region which carries tightly packed genomic material and a modified lysosome, called ‘acrosome’ and the tail-region or flagellum, which includes a microtubular core surrounded by dense fibre and mitochondrial sheaths. Sperm anatomical maturation is under the influence of androgens in the male genital tract, while sperm functional maturation is under the influence of progesterone and estradiol in the female genital tract.

Some studies using light microscopy have shown that ejaculated spermatozoa from men with varicocele show an altered morphology (Portuondo et al., 1983), others not. By light microscopy it was shown in ejaculated spermatozoa, from men with varicocele, an increased number of elongated tapered sperm heads (Portuondo et al., 1983). The transmission electron microscopy (TEM) is clearly a better tool to evaluate sperm structure and sperm morphology alterations caused by varicocele (Baccetti et al, 1991, 1993; Reichart et al, 2000). Specifically, Roosen-Runge and Holstein (1978) showed that varicocele affects sperm morphology by an impairment of differentiation in the early spermatid stage. Reichart et al., (2000) suggested that varicocele may induce deleterious alterations in early spermatid head differentiation, causing sperm acrosome and nucleus malformations. Furthermore, it has been demonstrated that the sperm population in patients with testicular varicocele shows a combination of different and unrelated cellular characteristics typical of immature gametes, including uncondensed nuclei, small and badly localized acrosomes, large cytoplasmic residues, badly assembled mitochondria, and rolled up axonemes (Baccetti et al., 2002). TEM approach goes beyond a generic sperm morphology since it gives information at ultrastructural level, playing an important

role in studying sperm pathology, the discipline that characterizes the structural and functional deficiencies of altered sperm. In sperm pathology, two main forms of ultrastructural sperm anomalies can be distinguished: non-systematic or nonspecific sperm defects, and systematic sperm defects (Baccetti et al., 2001; Chemes and Rawe, 2003). The first and most frequent type includes a heterogeneous combination of alterations randomly affecting the head and the tail organelles in a different percentage of ejaculated sperm. These alterations might be related to andrological pathologies (i.e. infections, varicocele) or to other factors (Chandley, 1998; Singh et al., 2005), and are present in fertile individuals but, in higher percentages in infertile men. Recently, it was demonstrated that varicocele has a negative effect on sperm structure at molecular level, going beyond the abnormal sperm morphology described to date. It was shown that varicocele may lead to male factor infertility by a mechanism involving a reduced expression of some steroid receptors in human spermatozoa. This review focalizes on the current knowledge about the negative effect of varicocele on sperm anatomy at molecular level.

## Estrogen receptor (ER) α and ERβ

In mammals, estrogens have for a long time been considered as female hormones, however their important role in male reproductive physiology emerged about 15 years ago. The effects of estradiol on human sperm have been associated with an enhanced motility, oocyte, penetration, longevity, oxygen intake, lactate production and metabolization of several exogenous substrates (Idaomar et al., 1989). In addition, estradiol leads to a rapid increase of intracellular cAMP, calcium concentrations and to an enhanced tyrosine phosphorylation of proteins in sperm (Luconi et al., 1999; Aquila et al., 2004). The physiological responses to estrogen are known to be mediated by at least two distinct receptor subtypes, estrogen receptor (ER) α and ERβ, playing a crucial role in reproduction and normal physiology. The development of male transgenic mice lacking estrogen receptors (ERs) genes has demonstrated that estrogens play a key role in the human male reproductive system (Eddy et al., 1996).

In human testis, conflicting data are present regarding the distribution of the different ER subtypes; both receptors are expressed in germ cells at various stages of development from spermatogonia to elongated spermatids (Mäkinen et al., 2001). In human seminiferous tubules there are controversial data indicating either the absence (Pelletier and El-Alfy, 2000) or the presence of ERα and ERβ (Pentikäinen et al., 2000; Saunders et al., 2001, 2002). ERα has been detected in the nuclei of spermatogonia, spermatocytes and early developing spermatids. Elongating spermatids, mature sperm, Sertoli and Leydig cells were negative for ERα, indicating that ERβ is likely to be the isoform mediating estrogen effects in human testis (Mäkinen et al., 2001). Nevertheless, other studies have presented conflicting data (Pelletier and El-Alfy, 2000; Pelletier et al., 2000). Cheng et al. (1981) showed a specific binding of estradiol to human sperm and it was only in 1998 that (Durkee et al., 1998) demonstrated the presence of ERα in human spermatozoa. Classical ERα and ERβ were detected in germ cells from spermatogonia to spermatozoa (Hess et al., 1995; Aquila et al., 2004; Lambard and Carreau, 2005) both at protein and mRNA level. Even though spermatozoa are considered to be genetically inert cells, the presence of mRNAs (Dadoune, 2009) and their potential roles in spermatozoa were shown (Miller et al., 2005; Galeraud-Denis et al., 2007). ERs' function was related to sperm survival and evidence was provided that estrogens through both receptors activate the PI3K/Akt pathway: ERα and ERß seem to influence this pathway at different levels and may co-work in controlling sperm survival (Aquila et al., 2004).

Data from immunolocalization experiments have demonstrated the presence of ERα and ERß in human and rat sperm. In human sperm, by immunohystochemistry, ERα was prevalently located in the midpiece region whereas ERß was uniformly distributed along the tail (Durkee et al., 1998; Aquila et al., 2004). An overlap between the ERs occurs in the proximal region of the tail (midpiece) and the ERß is the main ER isoform expressed in all the male genital tract.

The midpiece contains mitochondria arranged in a spiral helix that completes 12-13 turns around the axoneme. The disorganization of the midpiece-principal piece junction has been reported to be associated with asthenozoospermia (Lhuillier et al., 2009). Recently, a study from Pelliccione et al. demonstrated that structural defects in mitochondrial membranes represent a main feature of severe, unexplained asthenozoospermia, although the pathogenetic mechanism has yet to be explored (Pelliccione et al., 2011). A perfect assembly of sperm flagellum is the prelude for the normal and efficacious motility needed to reach and fertilize the oocyte. Most patients with severe asthenozoospermia have an increased number of sperm with non specific flagellar anomalies compared to fertile men. Non specific defects of the sperm flagellum randomly affect axonemal or periaxonemal structures in a varying percentage of ejaculated spermatozoa. Their incidence changes during the clinical evolution of the pathology and it differs among patients. However, these altered flagella appear generally well shaped at the light microscopy level, therefore an ultrastructural analysis performed by TEM is relevant to detect structural defects of the sperm tail leading to more or less severe asthenozoospermia. Axonemal anomalies mainly consist of alterations concerning the number of microtubules or their position in a 9+2 organization. A frequent finding, in examining the ejaculates from infertile patients, is the presence of a variable percentage of coiled tails which indicate that axonemal and periaxonemal structures are generally severely compromised. These defects are

frequent in immature spermatozoa detected in the presence of varicocele (Baccetti et al., 2006) or cryptorchidism (Moretti et al., 2007).

Recently, a further deepening by TEM with immunogold analysis clearly evidenced the ERs location at ultrastructural level as well as their amount. In this regard, both immunopositivity for ERα and ERß have been described in the healthy sperm in restricted and definite areas. While the presence of ERα has been reported only in the midpiece of healthy sperm (Fig. 1), ERß exhibits wider distribution patterns and extends through the midpiece and the principal piece of the flagellum, including axonemal (central bridge, projections, radial spokes, peripheral links) and periaxonemal cytoskeletal structures (fibrous sheath, accessory fibres, striated columns) (Fig. 2). The sperm heads appear to be totally devoid of both these proteins. The expression of ERα and ERß becomes significantly lower in the sperm samples of patients with varicocele (Figs. 1, 2).

This pattern of ERs expression in varicocele may be considered a molecular marker of the damaging effects of the pathology since different amounts in the ERs expression between normal and varicocele sperm were observed. Interestingly, the reduced expression of both ERs in ''varicocele'' sperm correlated with a reduced responsiveness to E2 on capacitation, acrosin activity, motility and metabolism.

### Progesterone receptors (PRs): PR-A and PR-B

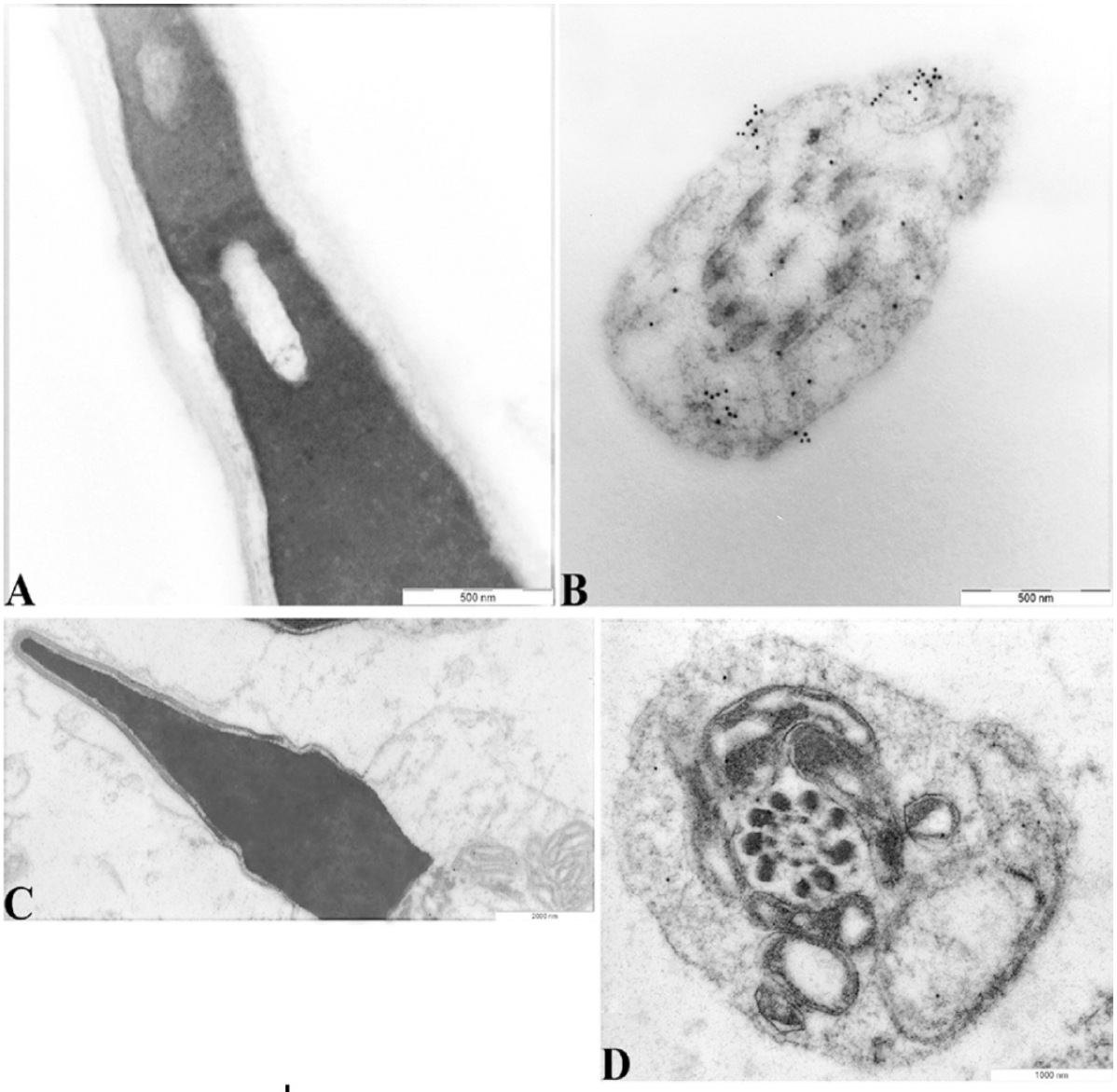
Progesterone (Pg) is an essential regulator of several female reproductive events such as ovulation, regulation of the menstrual cycle, implantation and maintenance of pregnancy (Rothchild and Gibori, 1975; Graham and Clarke, 1997). However, in contrast to the established roles of Pg in the female reproductive tract, limited data are reported on Pg effects in male reproductive events. When sperm travels in the female reproductive tract it is exposed to Pg and several positive effects of this steroid on human sperm were demonstrated. It was reported that Pg induces hyperactive motility and acrosome reaction of mammalian spermatozoa during the transit along the female reproductive tract (Kay et al., 1994; Gadkar-Sable et al., 2005). Pg was shown to activate several signaling pathways involved in the regulation of human sperm physiology, such as generation of cAMP, an increase of intracellular calcium ($Ca^{2+}$), promotion of tyrosine phosphorylation of proteins, and activation of phospholipases (Thomas and Meizel, 1989; Blackmore et al., 1990). Studies have also been carried out to determine whether an assessment of a sperm's responsiveness to progesterone may predict its fertilizing ability *in vitro* (Fuse et al., 1993; Tesarik and Mendoza, 1993; Calvo et al., 1994; Luconi et al., 1996; Patrat et al., 2000). A significant correlation has been found between the outcome of IVF and progesterone stimulated Calcium influx (Gadkar et al., 2002). Nevertheless, although a physiological role for progesterone in the acquisition of sperm fertilizing ability has been reported, the mechanism(s) through which Pg acts in this context and the role of Pg in male reproductive events are not defined yet.

The effects of Pg generally act via the progesterone receptor (PR) of which two isoforms, called A (PR-A) and B (PR-B) were discovered. They are intracellular receptors belonging to the superfamily of transcription factors (Horwitz and Alexander, 1983; Kastner et al., 1990). Gene targeting strategies showed reproductive abnormalities in PR null female mice (Lydon et al., 1995; Conneely and Lydon, 2000). No detailed evidence of the reproductive phenotype when PR is disrupted in male animals are described. At the moment, it has been demonstrated that mice null for steroid receptor coactivator-1 (SRC-1), a PR coactivator, show a reduction in testicular growth and a lowered fertility compared with their wild-type littermates (Xu et al., 1998).

Specific Pg sperm-binding sites were found to be located on the plasma membrane of the spermatozoon (Blackmore et al., 1994). Cheng et al. (1998a,b) reported the existence of a sperm plasma membrane PR in stallion spermatozoa. Later, a nongenomic plasma membrane PR (Contreras and Llanos, 2001) was found in the acrosomal region (Gadkar et al., 2002; Wu et al., 2005). Recently, the expression of the conventional intracellular PRs was demonstrated and their functional role has been related to capacitation, Akt and p60c-src (src) activities, acrosome reaction, lipid and glucose metabolism (De Amicis et al, 2011).

TEM with immunogold examination revealed largely diffuse immunoreactivity of conventional PRs (Fig. 3). PRs localize not only to membranous compartments but also in the entire sperm body as a component of the nucleus, the midpiece and the flagellum between the ribs of the fibrous sheath, the outer dense fibres and the axoneme. Particularly, the label decorated mostly the head (Fig. 3A) and the midpiece with the mitochondria also showing an appreciable presence of gold particles (Fig. 3B). PR expression was progressively reduced from the principal piece of the flagellum up to the end piece (Fig. 3C). The presence of varicocele is associated with a reduction of PRs expression from the head, along the midpiece and through the tail (Fig. 3D-F). Immunosignal seems to progressively decrease toward the distal end of the principal piece.

The presence of PRs along all the sperm body supports earlier evidence showing that the responses to progestins in mammals involve different signalling pathways related to the different sperm activities (Kay et al., 1994; Cheng et al., 1998b; Shah et al., 2005). By the time of ovulation, Pg is almost everywhere in the egg microenvironment affecting the competence of the spermatozoa to fertilize. The reduced PRs expression in varicocele sperm was found to be associated with a lowered responsiveness to Pg and this negatively influenced the sperm function. The altered PRs



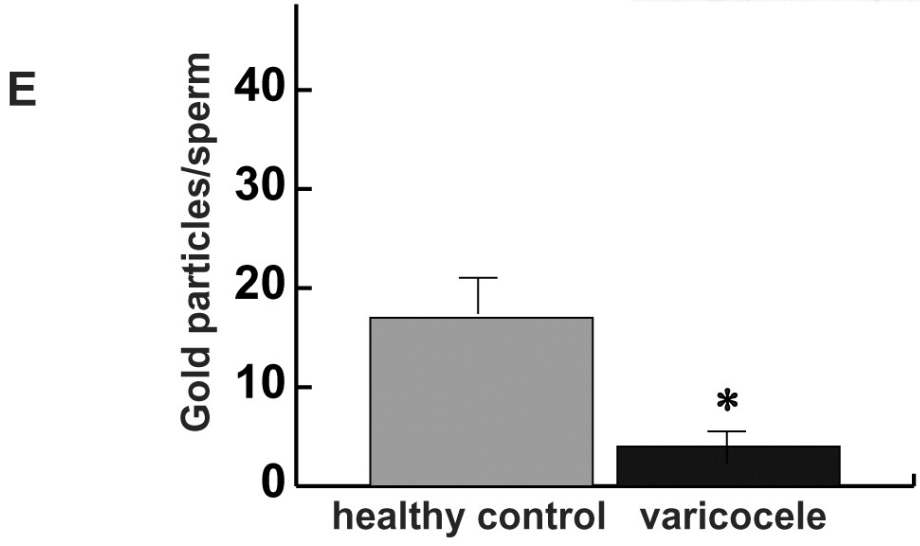




**Fig. 1.** Distribution of ERα in the ejaculated sperm of healthy or varicocele-affected subjects. Micrographs of sections from ejaculated sperm probed with mouse monoclonal Ab to human ERα. Longitudinal section through the head and cross section of the midpiece in the healthy controls (**A and B** respectively). Corresponding sperm regions in "varicocele" samples (**C and D** respectively). Statistical evaluation of immunogold labeling experiments **(E)**. *P<0.001

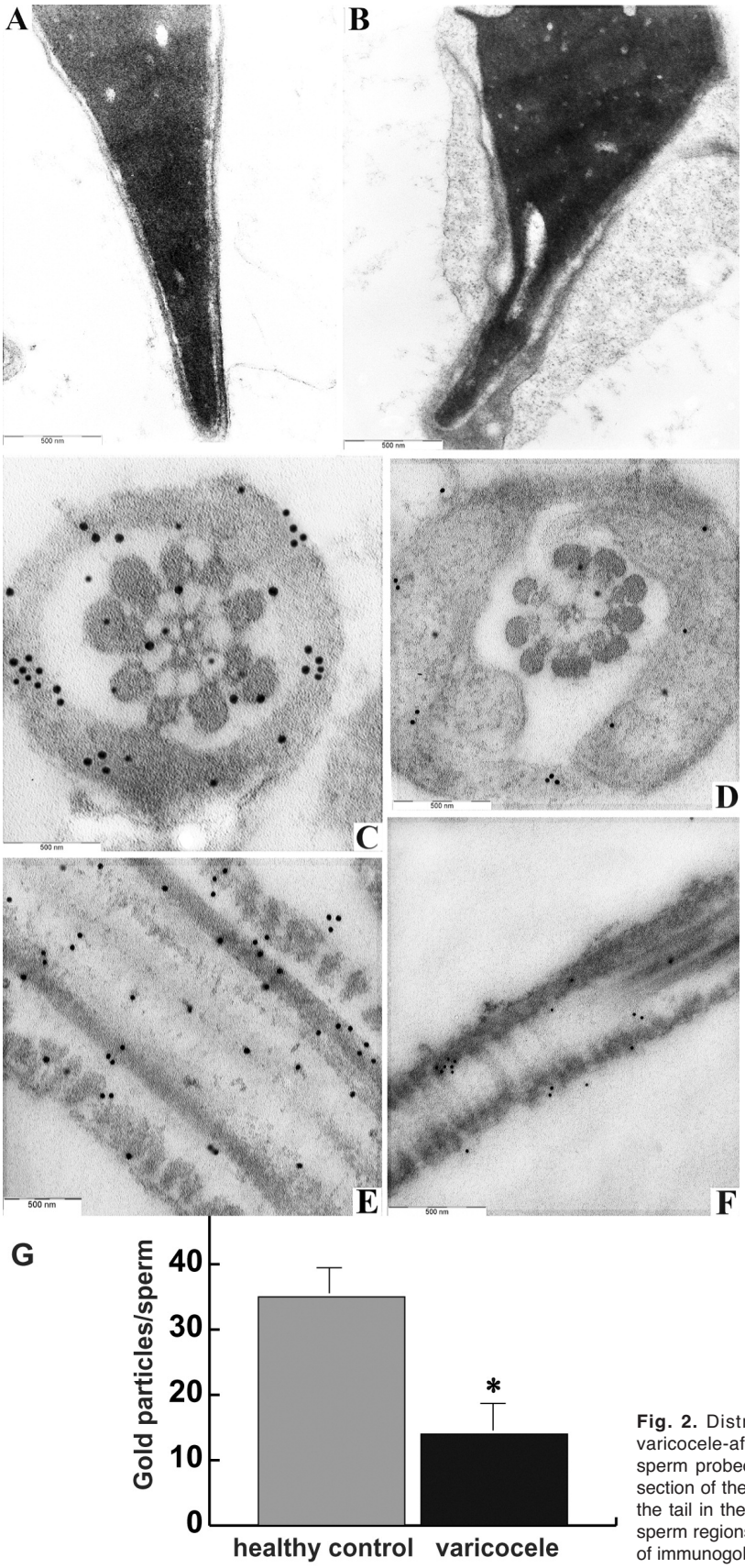



**Fig. 2.** Distribution of ERβ in the ejaculated sperm of healthy or varicocele-affected subjects. Micrographs of sections from ejaculated sperm probed with mouse monoclonal Ab to human ERβ. Longitudinal section of the head, cross section of the midpiece, longitudinal section of the tail in the healthy controls (**A**, **C and E** respectively). Corresponding sperm regions in "varicocele" samples (**B**, **D and F**). Statistical evaluation of immunogold labeling experiments **(G)**. *P<0.001

expression might be a cause of a broad range of sperm functional anomalies with the consequent reduction of fertilizing ability.

### Androgen receptor (AR): AR-B and AR-A

Androgens have been shown to play critical roles in testis function (Collins et al., 2003). Although it is well known that androgens are involved in the development of male sexual characteristics as well as in spermatogenesis, their action in male infertility is not fully clarified. Androgens' role is mediated by androgen receptor (AR), a member of the nuclear receptor superfamily, which functions as a ligand-inducible transcription factor (Wang et al., 2009). To date, multiple isoforms of the AR have been described and among them two proteins are well characterized: AR-B and AR-A (Wilson and McPhaul, 1996). A functional androgen receptor (AR) is required for male embryonic sexual differentiation and pubertal development. The role of AR during spermatogenesis has been the subject of intense interest for many years and studies of androgen

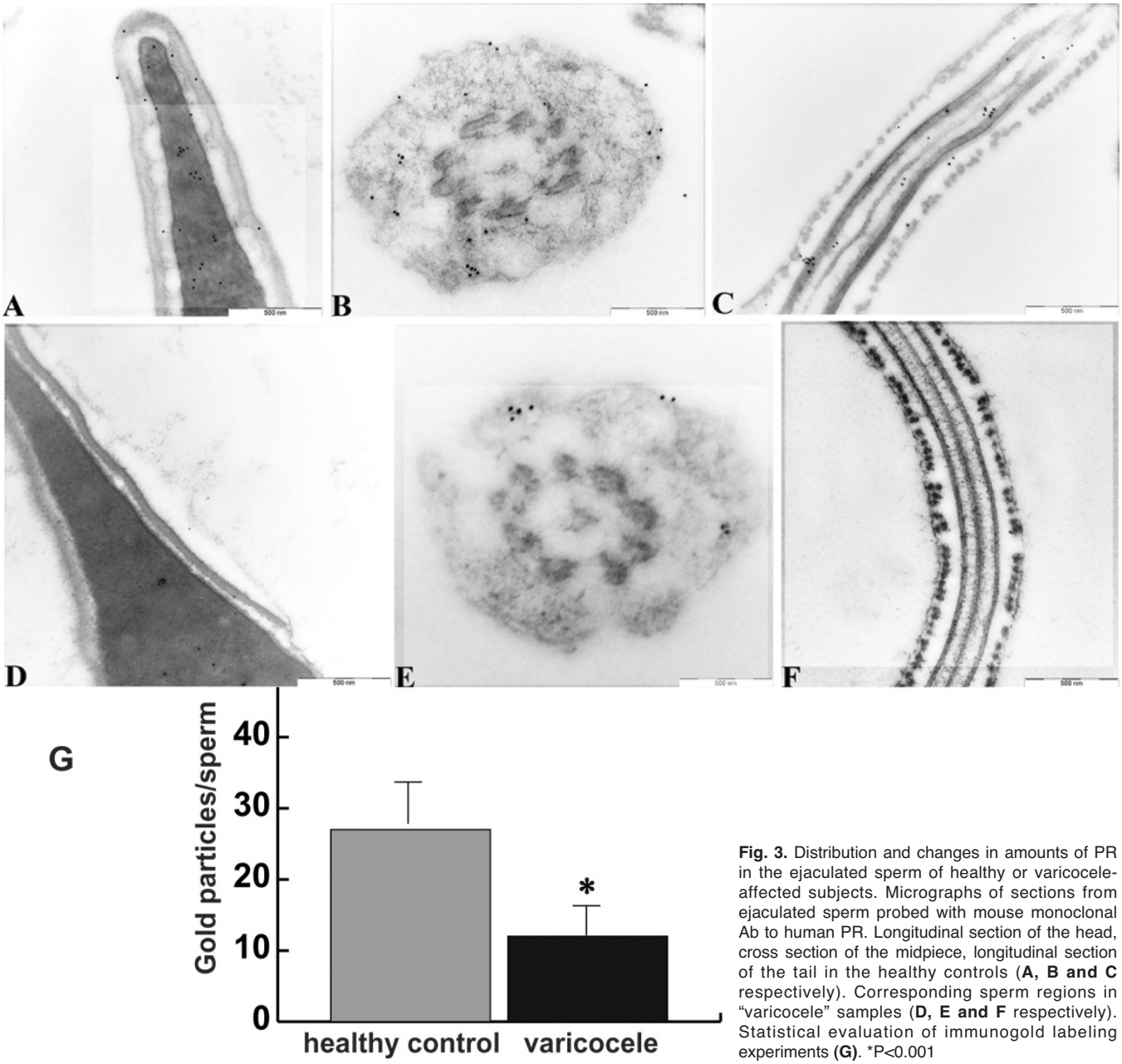



**Fig. 3.** Distribution and changes in amounts of PR in the ejaculated sperm of healthy or varicocele-affected subjects. Micrographs of sections from ejaculated sperm probed with mouse monoclonal Ab to human PR. Longitudinal section of the head, cross section of the midpiece, longitudinal section of the tail in the healthy controls (**A, B and C** respectively). Corresponding sperm regions in "varicocele" samples (**D, E and F** respectively). Statistical evaluation of immunogold labeling experiments **(G)**. *P<0.001

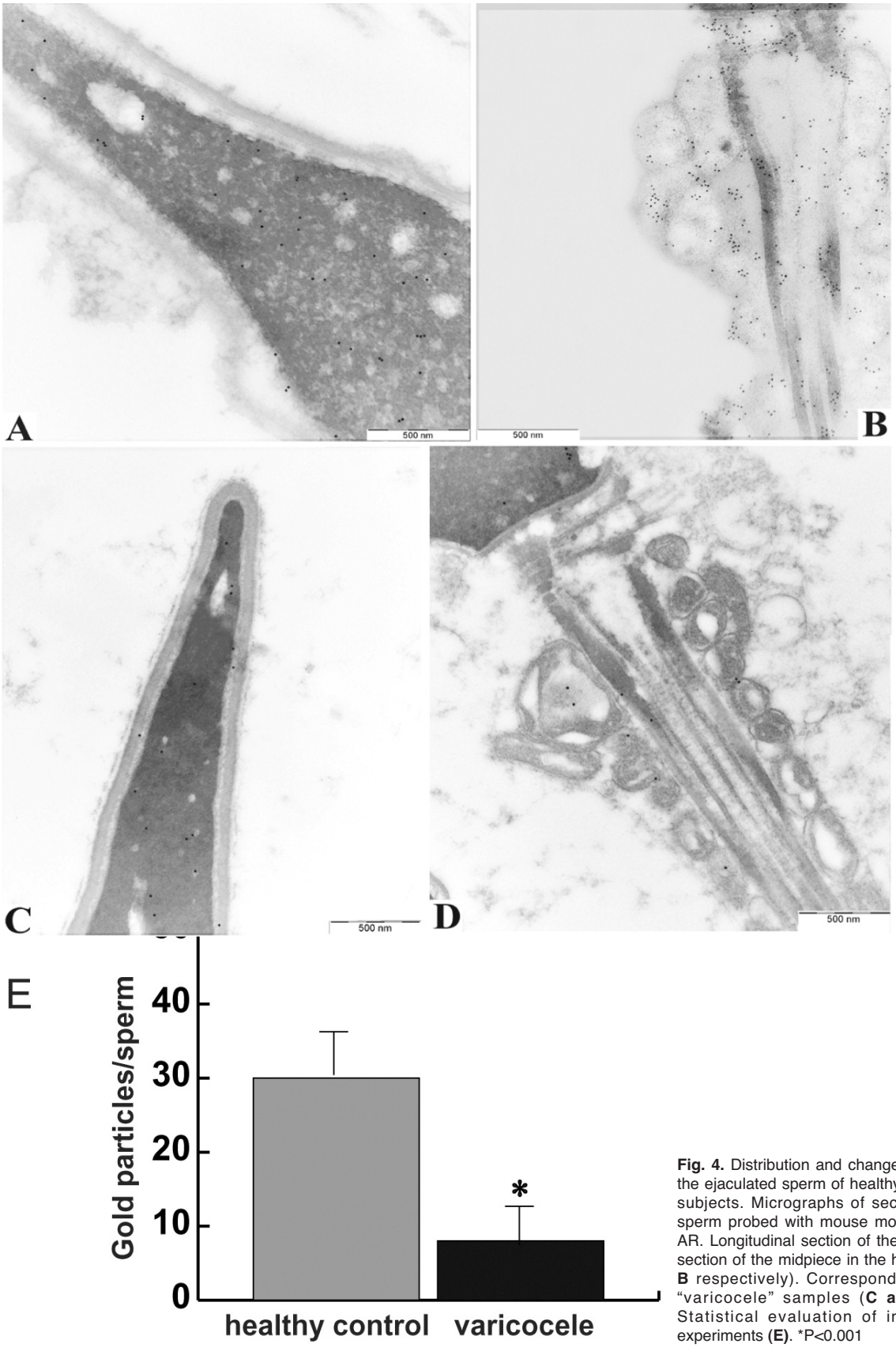



**Fig. 4.** Distribution and changes in amounts of AR in the ejaculated sperm of healthy or varicocele-affected subjects. Micrographs of sections from ejaculated sperm probed with mouse monoclonal Ab to human AR. Longitudinal section of the head and longitudinal section of the midpiece in the healthy controls (**A and B** respectively). Corresponding sperm regions in "varicocele" samples (**C and D** respectively). Statistical evaluation of immunogold labeling experiments **(E)**. *P<0.001

withdrawal and disruption of AR, either by surgical, chemical or genetic means, have demonstrated that spermatogenesis rarely proceeds beyond meiosis (Collins et al., 2003). In all of these model systems, very few round and even fewer elongated spermatids are observed (Yeh et al., 2002). Nevertheless, the mechanisms by which androgens regulate male fertility are not fully understood and the sites of androgen action within the male reproductive system are not resolved yet.

AR has been detected in Sertoli, Leydig, peritubular myoid and spermatid cells (round and elongated) (Vornberger et al., 1994; Kimura et al., 1998; Suarez-Quian et al., 1999). The prevailing view is that sperm does not contain AR and this stems from previous studies reporting that no AR immunostaining of germ cells was observed both in rat and in human testis (Suarez-Quian et al., 1999). Whereas few studies have raised the intriguing possibility that some germ cells may exhibit AR (Vornberger et al., 1994), other reports point to Sertoli cells or Leydig cells or peritubular/myoid cells as the exclusive androgen target cells in the testis (Ruizeveld de Winter et al., 1991; Goyal et al., 1994; Iwamura et al., 1994; Suarez-Quian et al., 1999). Besides, some authors reported the expression of AR as exclusively restricted in spermatogonia, suggesting a direct effect of androgens on germ cells during the stages of pre-spermatogenesis and early spermatogenesis (Zhou et al., 1994; Arenas et al., 2001). With the onset of chromatin condensation, no AR can be observed in these cells (Vornberger et al., 1994; Holdcraft and Braun, 2004).

However, several studies reported that in spermatozoa, the binding capacity of androgens was greater than that of estrogens or progesterone (Hyne and Boettcher, 1977; Cheng et al., 1981), and recently the classical AR was shown to be present in sperm by western blot and immunofluorescence assays and located in the midpiece (Solakidi et al., 2005). Further, both AR-B and AR-A isoforms in human sperm were demonstrated to be present by western blot and immunofluorescence in the head (Aquila et al., 2007). The discrepancy may be due to the different antibodies and/or the methods used to process samples. Few reports studied the role of androgens in ejaculated sperm physiology and in this context AR functionality was related to the modulation of the PI3K/AKT pathway, survival and energy metabolism management (Aquila et al., 2007; Zalata et al., 2013; Guido et al., 2014).

Recently, AR was studied with immunogold labelling by TEM analysis to characterize its pattern of distribution in human sperm under both healthy and pathologic states (Guido et al., 2014). AR has been reported to be present mostly within the head, which is a region that undergoes morphological and biochemical changes during capacitation (Fig. 4). Gold particles were clustered into discrete units in the region of the membranes of both the anterior and posterior head, the neck and the mitochondria-containing midpiece in healthy sperms. The inner acrosomal membrane frequently develops an intense immunolabelling while the membranes overlying the acrosome only exhibit scarce immunoreactive processes. Mild immunogold labeling was also documented throughout the fibrous sheath, over both the longitudinal columns and the circumferential ribs of the fibrous sheath itself.

It was reported that varicocele downregulates AR expression in the right and left testes (Soares et al., 2013). Because varicocele did not change the mRNA levels for AR, post-transcriptional mechanisms that regulate mRNA stability, protein translation, post-translation processing and/or protein stability may be involved in the regulation of AR (Jaworski, 2006; Anbalagan et al., 2012; Soares et al., 2013). However, whether these mechanisms are altered by varicocele remain to be explored. It was recently reported that AR expression was significantly decreased in infertile men with varicocele more than in infertile men without varicocele compared to fertile men (Zalata et al., 2013). A higher concentration of testosterone binding sites on the acrosomal and post-acrosomal regions of highly motile spermatozoa and their absence on the non-motile spermatozoa suggested that steroid binding ability may determine normalcy of spermatozoa.

Our data by TEM analysis appear to be in agreement with these studies since in sperm from varicocele AR expression, although with the same trend of distribution as in the normozoospermic sample, appears to be significantly lower in varicocele samples. This is associated with a diminished responsiveness to androgens at least on capacitation, glucose and lipid metabolism. The role of androgens in male reproduction has been object of intense effort, however studies on their action in human male gamete are limited. When we published our first finding on this issue, it was very difficult for the scientific community to accept that sperm possess ARs. However, with the support of all these new data, we think that now it might be logical to assume that sperm contain AR, since both sperm and AR are the main male-related features. Further work will be required to fully elucidate the role that AR plays in male fertility.

## Perspective

The enigma of varicocele has always attracted the researchers' attention as attested by the consistent literature on the topic, although conflicting data are reported. The controversy lies in the fact that the pathophysiologic mechanism by which varicocele can induce male infertility is not known with certainty, although several potential causes have been postulated. In both men and rats, varicocele causes endocrine and testicular paracrine imbalances, hyperthermia, retrograde flow of adrenal blood, hypoxia, oxidative stress and apoptosis, and dysfunction of the epididymis (Mohammed and Chinegwundoh, 2009).

Mammalian ejaculated spermatozoa have been

studied always with great interest because of their function in fertilization and their peculiarities as unique cellular type. Exciting progress has been made over the last years, but sperm molecular composition is not fully defined yet. It is necessary to investigate this issue in order to clarify clinical cases of unexplained male infertility. The gonadal steroids present in various concentrations in the genital tract secretions may be associated with sperm steroid binding proteins for acquisition of motility and fertilizing ability of spermatozoa during their transit through the genital tracts. It appears that an increase in binding sites on spermatozoa for different steroids are related to the maturation status of spermatozoa and it has been proposed that the steroid binding pattern on spermatozoa could be used as a parameter for objective evaluation of sperm function (Zalata et al., 2013). Recently, it has been demonstrated that sperm expresses different nuclear receptors, including steroid receptors, as well as their classical ligands. The presence of steroid/steroid receptor systems in ejaculated sperm, as membrane but also as nuclear conventional receptors, suggests that both systemic and local steroids, through sperm receptors, may influence male fertility.

In clinical practice, the traditional light microscopic method for evaluating semen quality have a central role in the assessment of male fertility potential. However, although ultrastructural analysis requires highly trained examiners and expensive equipment, this methodology is important to provide a more comprehensive approach to investigate sperm molecular composition and to identify sperm pathology. In sperm from varicocele patients, a decreased expression of steroid receptors and a consequent reduced responsiveness to steroids may represent a molecular mechanism involved in the physiopathology of varicocele. Analysis of patients with varicocele suggest that mild scrotal warming can be detrimental to sperm production, partly by the effects on the stem cell population and partly by the effects on later stages of spermatogenesis and sperm maturation. Therefore, the reduced expression of ERα, ERβ, PR and AR in 'varicocele' sperm, may occur during the spermatogenesis. Another hypothesis may take into account the transit of the sperm along the male reproductive tract where its surface undergoes major modifications in macromolecule composition.

From our data, it emerges that varicocele may induce damage in the gamete at molecular level, opening a new chapter in the already multifactorial pathophysiology of varicocele and further complicating this issue. The modulation of steroid receptors paves the way for novel therapeutic opportunities in the treatment of the male pathologies related to human reproduction.

## Statistical analysis

Immunogold labelling for ERα or ERβ or PR or AR was performed as previously reported (De Amicis et al., 2011; Guido et al., 2011, 2014). TEM images were also used to count and calculate the number of gold particles per cross or longitudinal-sectioned cell by three independent investigators. Per field, 80 cells were counted and four different fields were analyzed per sample. The histograms show quantification of gold particle labeling and represent mean ± SEM of the gold particles counted in 80 cells from four fields for each condition. Statistical evaluation of immunogold labeling experiments was performed using ANOVA followed by Newman-Keuls testing to determine the differences in the means. $p<0.05$ was considered statistically significant. All statistical analyses were performed using the SPSS software (v.17).

*Acknowledgements.* Our special thanks to Maria Clelia Gervasi and Serena Gervasi for the English language review of the manuscript.
*Conflict of interest:* No potential conflicts of interest were disclosed.

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Accepted March 30, 2016