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# Naturally-occurring antisperm antibodies in men: interference with fertility and clinical implications. An update

**Felice Francavilla[1], Riccardo Santucci[1], Arcangelo Barbonetti[1], Sandro Francavilla[1]**

*[1]Department of Internal Medicine, Andrologic Unit, University of L'Aquila, Coppito, 67100 L'Aquila, Italy*

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## 1. ABSTRACT

Naturally-occurring anti-sperm antibodies (ASA) in men can affect fertility by various mechanisms. Some of them are mainly related to the extent of the sperm autoimmunization (e.g., sperm-agglutination and impaired cervical mucus penetration); others are also related to immunoglobulin (Ig)-isotype (e.g., complement-mediated sperm injury through the female genital tract), or to antigenic specificity of ASA (e.g., interference with gametes interaction). The unavailability of current diagnostic tests to determine the antigenic specificity of ASA, and the difficulty in quantifying the antibody density on the sperm surface make it hard to establish in each individual patient, whether, or to what extent, these interfering effects occur, apart from sperm-agglutination and the impairment of cervical mucus penetration. However, the rational use of current ASA-tests can be effective in screening and quantifying sperm autoimmunization relevant to infertility. The degree of sperm-autoimmunization is the only empirical (rather effective) criterion in choosing more or less invasive assisted reproductive techniques (ART) for couples, where male immunological infertility is diagnosed. A more rational treatment strategy would be possible with the development of tests detecting ASA directed against defined fertilization-related antigens. In this article, the growing knowledge in this field is reviewed.

## 2. INTRODUCTION: CONTROVERSIES ON THE SIGNIFICANCE OF ANTISPERM ANTIBODIES STILL REMAIN

Although the significance of naturally occurring antisperm-antibodies (ASA) in male infertility has been largely investigated since Rumke (1) and Wilson (2) reported the presence of serum sperm-agglutinating antibodies in infertile men in 1954, it is still a debated matter. In the context of this debate, the old assertion by Taylor and Collins (3) that "any link between sperm antibody presence and impaired conception must be considered hypothetical" has more recently been renewed by Helmerhorst et al. (4) as well as strongly criticized (5-7). While, on one hand, the routine use of current ASA testing has been questioned as an essential procedure in the fertility work-up, as any treatment on the basis of such tests would not be justified (4), on the other hand, intracytoplasmatic sperm injection (ICSI) has been advocated as the primary choice of treatment in the presence of sperm autoimmunization (8-10).

Actually, although a vast body of literature has provided evidence that ASA can affect sperm fertilizing ability at various levels, it is still hard to establish in each individual patient, whether, or to what extent, these interfering effects occur. A major reason is the inadequacy of current diagnostic tests. They include: 1) indirect tests

detecting biological activities of circulating ASA, i.e., sperm agglutination and complement-dependent cytotoxicity (11); 2) antiglobulins-based test, mainly used as a direct test to detect antibodies coated to the surface of ejaculated spermatozoa. The most widely used are the mixed antiglobulin reaction (MAR) test (12) and the immunobead binding test (IBT) (13). They reveal the percentage of antibody-coated spermatozoa, the Ig-isotype and grossly the regional specificity of ASA. The major problem is the inability of current diagnostic tests to quantify the antibody density on the sperm surface and to define the antigenic specificities of ASA, main determinants of their anti-fertility effect.

Furthermore, as current diagnostic tests detect ASA with different sensitivity and specificity, different approaches used for studying ASA and for the recognition of male immunological infertility have strongly contributed to generate confusion on the physiopathological and clinical significance of ASA.

This review critically updates current understanding of the mechanisms of the interference of ASA with the sperm fertilizing ability. Clinical implications are discussed later focusing on: 1) the effectiveness of a rational use of current ASA-tests in screening and quantifying sperm autoimmunization relevant to infertility, in spite of their claimed inadequacy; 2) the possibility to establish a rational strategy for the treatment of infertile male patients with ASA.

## 3. MECHANISMS OF FERTILITY IMPAIRMENT

Only ASA directed towards surface antigens have a physiopathological and clinical significance in the male immunological infertility, because sub-superficial antigens cannot be exposed to antibodies by living cells along the male genital tract.

### 3.1. Effect on semen quality

Sperm agglutination is the only well established semen alteration related to the presence of ASA. A significant increase in the proportion of motile sperm involved in agglutinations has been reported in the presence of ASA, whenever investigated (14-17). However, sperm agglutination, which is a time-dependent phenomenon, only rarely involves a large proportion of motile sperm soon after liquefaction, even when all ejaculated spermatozoa are antibody-coated. Therefore, sperm agglutination, although extremely suggestive of sperm autoimmunization, does not represent an important mechanism of antibody-interference with fertility in most cases.

With some exceptions (18,19), most epidemiologic studies did not find any significant difference in the principal semen parameters (sperm count, motility and morphology) among infertile patients with and without ASA (14-16,20). Furthermore, normal sperm motility parameters were reported using computer aided semen analysis (CASA) even in the presence of strong sperm autoimmunization (21,22). In any case, there is little evidence that suggests a cause/effect relationship between ASA and abnormality of semen parameters, apart from sperm-agglutination. An antibody effect on semen quality should involve a complement mediated sperm cytotoxicity occurring within the male genital tract. However, anti-complementary activity has been reported in human semen (23,24), and it was recovered in the low molecular weight of the seminal plasma (20-60,000 Daltons) using gel filtration chromatography (23). This fraction inhibited total complement activity as well as the activity of the early C components C1 and C3. Afterwards, a potent inhibitor of C5b-7 complexes was identified in human seminal plasma, where it was found in 5- to 10 -fold higher concentrations than in serum. A sulphated glycoprotein termed clusterin was also found on ram sperm (25), and purified human seminal clusterin was shown to inhibit C5b-6 mediated hemolysis (26). Finally, D'Cruz and Haas (27) demonstrated the lack of a detectable product of C activation (SC5b-9) in the seminal plasma of men with sperm bound antibodies (IgG were present in most cases). Taken together, these findings suggest that human seminal plasma contains inhibitors for both the initial and the terminal portions of the C cascade, thereby protecting spermatozoa from C-mediated injury in the male reproductive tract.

As an increased sperm count in some oligozoospermic patients with ASA was reported in response to corticosteroid therapy, it was suggested that in those cases a cell-mediated immune reaction at the level of rete testis and/or epidydimis responsive to the anti-inflammatory effect of corticosteroids might underlie the low sperm count (28). However, clinical and pathological evidence of immune orchitis in men exhibiting natural autoimmunity to sperm has never been provided.

### 3.2. Interference with cervical mucus penetration

The impairment of sperm penetration through the cervical mucus represents the best known and most well-established mechanism of antibody interference with fertility (29). Definitive clinical demonstration of this impairment has been produced analysing the outcome of *"in vivo"* as well as *"in vitro"* tests of sperm cervical mucus interaction.

Several studies have shown a significant association between a poor *post coital* test (PCT) outcome and sperm autoimmunization (e.g., 30,31). It is worth noting that the degree of the impairment of PCT outcome was found to correlate with the proportion of sperm exhibiting surface-bound antibodies (32), as well as with the titre of circulating ASA (31).

The outcome of the *in vitro* cervical mucus penetration test comparing men with and without ASA has largely confirmed this impairment (18,33,34). Finally, the demonstration of the actual responsibility of ASA in impairing cervical mucus penetration was produced by matching donor sperm suspensions exposed to sera containing ASA against the same sperm suspensions exposed to control sera without ASA (35-37).

Although some reports suggested a prominent role for IgA-ASA in impairing sperm penetration of cervical mucus (38-40), other findings indicate that an abnormal interaction between the Fc portion of both IgA and IgG bound to the sperm surface and constituents of the cervical mucus is responsible, at least in part, for the characteristic shaking phenomenon and the impairment of mucus penetration. Comparing the swimming ability of antibody-coated sperm within cervical mucus with that of sperm exposed only to the Fab' fragments of the same antibodies, mucus penetration was abolished by complete antibody, whereas it was only reduced but not abolished by Fab' fragments (41). Bronson *et al.* (42) found an improvement in the ability of antibody-bound sperm to penetrate human cervical mucus *in vitro*, after exposure to an IgA1 protease, which was expected to liberate Fc fragments of IgA1 antibodies bound to the sperm surface. This improvement varied inversely with the amount of remaining IgGs, not degraded by protease, indicating a role for both IgG and IgA sperm-bound antibodies in impairing cervical mucus penetration.

A 15 kD protein with the N-terminus identical to that of secretory leukocyte protease inhibitor and capable of binding all classes of immunoglobulins was found in human cervical mucus (43). It could represent the putative Fc-receptor involved in the trapping of ASA-coated spermatozoa in the cervical mucus.

### 3.3. Complement-mediated cytotoxicity and opsonizing effect through the female genital tract

One mechanism of ASA-interference with fertility may involve sperm injury potentially mediated by complement and/or phagocytic cells in the female genital tract. While complement-mediated cytotoxic effect by complement-fixing ASA is prevented in semen by its anti-complementary activity (see 3.1), when antibody-coated spermatozoa enter the female reproductive tract they might become liable to deleterious effects of complement activation, supposing that complement components are present in a sufficient amount through the female genital tract. In an elegant study, D'Cruz *et al.* (44) provided direct evidence for the involvement of complement-fixing ASA and complement activation in exerting sperm injury. Using flow cytometry to evaluate simultaneously the binding of antibody and complement to sperm cells, they demonstrated that incubation of donor sperm with sera containing IgG-ASA resulted in the activation of complement *in vitro* as assessed by the deposition of the initial (C3d) and the terminal (C5b-9) complement complex on the sperm surface. Antisperm antibodies and complement deposition resulted in a dramatic loss of sperm motility, as well as in activation and aggregation (rosetting) of polymorphonuclear leukocytes (PMN) to antibody- and complement-bound sperm. The inability of sera containing non-complement fixing IgG-ASA in promoting sperm binding to PMN suggested that IgG alone is insufficient to initiate the interaction, that is, it would preclude a direct interaction between the Fc portion of sperm-bound Ig and the Fc-receptor on PMN.

However, whether complement components are present through the female genital tract in a sufficient amount to exert these effects is still a debated matter (10,45,46). Price and Boettcher (47) documented full-complement component lytic activity in cervical mucus using a sensitive hemolysis assay. Although the level was 11.5% of the activity of serum complement, it was enough to cause complement-dependent immobilization of 50% of ASA-coated spermatozoa after 1 hour and of 70% after 3 hours.

Higher levels of complement activity were detected in human follicular fluid (one half of that in serum), and IgG-ASA were capable of activating follicular fluid complement as detected by their ability to deposit terminal complement complexes (MC5b-9) on human sperm (48). Due to the dilution on follicular fluid after ovulation, any sperm damage or dysfunction related "*in vivo*" to its complement activity is difficult to ascertain.

Nevertheless, an opsonizing effect exerted by IgG-ASA unrelated to complement activation was reported by London *et al.*(49), who demonstrated that the incubation of donor sperm with sera containing IgG-ASA enhanced sperm phagocytosis and lysis by peritoneal macrophages. This effect was hypothesized as mediated by Fc-receptor for IgG.

### 3.4. Interference with sperm/egg interaction

The actual role of naturally-occurring ASA in men in impairing sperm-egg interaction, as well as the level of this impairment, is not yet sufficiently known, because conflicting data have been produced. In this section we will attempt to analyze the reasons underlying these conflicting data, which could help to understand this debated matter.

#### 3.4.1. *In vitro* fertilization (IVF) as a model of study

Retrospective and prospective analyses of the *in vitro* fertilization and embryo transfer (IVF-ET) outcome provide a potential means of assessing possible effects of ASA on human gametes interaction.

Table 1 shows the fertilization rates reported in series including couples with sperm autoimmunization. In most reports the fertilization rate was significantly lower in the presence of sperm-bound antibodies than in the case of other indications for IVF (50-55). However, in some other reports no significant difference was found (56-59). Little evidence supports a role for the Ig-isotype. In an early report sperm head-directed IgA- more than IgG-antibodies seemed to be associated with a reduced fertilization rate (60), whereas, in subsequent reports a significant reduction of the fertilization rate was related to the degree of IgG plus IgA sperm autoimmunization (50,61,62).

However, the inference of the actual effect of ASA on sperm fertilizing ability from the analysis of IFV results is hindered by some serious causes. Firstly, non-immunological sperm abnormalities may bias the results. Only in some reports the conventional semen parameters were taken into account in the comparison between patients with and without ASA (52,53,55,63). In these series, an independent impairment by ASA was generally reported in the presence of normal semen parameters (52,55,63) as

**Table 1.** Fertilization rate in IVF-ET programs in the presence of antisperm antibodies (ASA) in the male

| ASA + [1] | ASA - [1] | | *References* |
|---|---|---|---|
| 65/131 (50%) | | IBT >20% | 128 |
| 18/66 (27%) | | IgA >80% | |
| 47/65 (72%) | | IgA <80% | |
| 8/14 (57%) | 118/180 (65%) | IBT >20% | 125 |
| 37/70 (53%) | 156/201 (78%) | IBT >20% | 119 |
| 15/39 (38%) | | IgG+IgA | |
| 22/31(71%) | | IgG or IgA | |
| 70/175 (40%) | | IBT >10% | 130 |
| 6/43 (14%) | | >70%(IgG+IgA) | |
| 31/52 (60%) | | <70% | |
| 132/273 (48%) | | MAR test >10% | 132 |
| 33/80 (41%) | | >90% | |
| 99/193 (51%) | | <90% | |
| 17/59 (30%) | 654/984 (66%) | IBT >10% | 120 |
| 98/355 (28%) | | MAR test >10% | 131 |
| 28/170 (17%) | | >90% | |
| 40/113 (35%) | | >40% and <90% | |
| 30/72 (42%) | | <40% | |
| 53/105 (50%) | 93/128 (73%) | MAR test >20% | 121 |
| (42%) | (73%) | MAR test >10% | 122 |
| 124/165 (75%) | 978/1412 (69%) | IBT >20% | 126 |
| 209/544 (38%) | 380/558 (68%) | IBT >20% | 123 |
| 46/104 (44%) | 65/77 (84%) | MAR test >20% | 124 |
| 153/283 (54%) | | MAR test >50% | 188 |
| (66%) | (63%) | IBT >15% | 127 |
| (71%) | (73%) | MAR test >20% | 59 |

[1] Fertilized/total ova (Fertilization Rate)

well as in the presence of asthenozoospermia (63) or teratozoospermia (53). Secondly, the criteria employed to define the occurrence of immunological infertility were different from one series to another, and often inadequate. Also patients with low or moderate sperm autoimmunization were included in most series. Nevertheless, when the extent of sperm autoimmunization was taken into account, it was always inversely correlated to the overall fertilization rate (62-64). But, notably, in some individual patients, a high fertilization rate was achieved even in the presence of a high extent of sperm autoimmunization (52,54,55,60).

In conclusion, the analysis of human IVF results seems to indicate that ASA exert a *relative* impairment of fertilization, which, to some extent, is related to the degree of sperm autoimmunization. However, the degree of autoimmunization does not completely explain the variability of the antibody impairment. Seemingly, at the level of gamete interaction, more than at other levels (i.e., cervical mucus penetration) the interference of ASA exhibits *qualitative,* apart from quantitative, differences among patients, suggesting that this interference also depends on the relevance of the specific antigens, targeted by natural ASA, to the fertilization process.

### 3.4.2. Experimental laboratory-based studies

The IVF-model of study cannot give information about the level of the ASA-interference with the gametes interaction. Over the last two decades, several experimental laboratory-based studies have attempted to determine the level of this interference.

Some considerations could be helpful in analyzing the often-conflictual results reported. The effects of natural ASA on the fertilizing ability of human spermatozoa have been studied either by matching donor sperm suspensions exposed to sera from patients with circulating ASA against the same sperm suspensions exposed to control sera, or using spermatozoa coated "*in vivo*" with ASA. In both cases the results must be interpreted with caution. In fact, circulating ASA may differ from sperm-associated antibodies "*in vivo*" in their biological activity and affinity to sperm antigens, as locally produced secretory immunoglobulins occur in the genital tract in addition to serum-derived Ig. On the other hand, using spermatozoa coated "*in vivo*" with ASA, the concomitant presence of non-immunological sperm abnormalities raises doubts about the responsibility of ASA in affecting sperm functions. Using antibodies eluted from autoimmune ejaculates instead of circulating ASA is another and potentially more demonstrative approach. Although used in some studies, its feasibility is hindered by the difficulty in eluting sufficient amounts of antibodies.

#### 3.4.2.1. Effects on zona pellucida (ZP) interaction

There is general agreement that ASA can interfere with sperm capability to interact with the ZP. Circulating ASA have been shown to reduce the sperm binding to (65-68) and penetration through (69) the ZP. Using spermatozoa coated "*in vivo"* with ASA, Liu *et al*. (70) found a reduced binding to salt-stored human ZP as compared to donors' spermatozoa, in a small series, where, however, concomitant non-immunologic semen abnormalities could represent a confounding factor. Zouari and De Almeida (71) reported that antibodies eluted from 5 autoimmune ejaculates uniformly reduced the ZP-binding when transferred onto donor spermatozoa. A more substantial demonstration of the actual occurrence of ASA-interference with ZP-binding, was provided by a study by our group (72), who tested 22 patients exhibiting all ejaculated spermatozoa coated with antibodies against the sperm head. Excluding patients with abnormal semen from the analysis, an impairment of the ZP-binding was demonstrable in 50% of cases. As normal ZP-binding was observed even when all ejaculated spermatozoa were coated with both IgG- and IgA-antibodies, neither Ig class, even combined, appeared to unavoidably affect this sperm function. Notably, all normozoospermic patients with low ZP-binding showed circulating IgG-ASA with inhibitory effect, when transferred onto donor spermatozoa, while no patient with normal ZP-binding showed circulating ASA with inhibitory effect. Zouari and De Almeida (71) reported that the removal of either IgG or IgA antibodies eluted from autoimmune ejaculates did not change the inhibitory effect on ZP-binding. Altogether, these observations suggest that both humoral and local sperm auto-immunization exhibit the same behaviour in impairing or not ZP-binding. The fact that ZP-binding is not unavoidably inhibited by ASA, could explain the high fertilization rate reported in some cases treated with IVF-ET even in the presence of a high degree of sperm-autoimmunization (52,54,55,60). However, ZP-binding is only the first step in the more complex interaction between spermatozoa and ZP. Zona pellucida also triggers the acrosome reaction (AR) of bound sperm, which is required for ZP penetration and fertilization. We later demonstrated

that ASA can interfere with sperm-ZP interaction not only by inhibiting ZP-binding but also by inhibiting the induction of AR by ZP (73). While the inhibition of ZP-binding was always associated with the inhibition of ZP-induced AR, this latter interference could also occur in the absence of the inhibitory effect on ZP-binding. However, neither of these inhibitory effects might occur, even in the presence of a high ASA-titre. This may be explained by the polyclonal nature of the naturally occurring ASA in men. Sperm-antibodies inhibiting ZP-binding could mask or prevent the expression of specific receptors on the plasma membrane over the sperm heads for the ZP3-O-linked oligosaccharides. Since ZP3 serves both as a ligand for sperm binding and a trigger for acrosome reaction (74), ASA, which inhibit ZP-binding, also inhibit ZP-induced AR. When an inhibition of ZP-induced AR occurs in the absence of interference with ZP-binding, ASA could interfere with cross-linking of several antigenic sites recognized by ZP3 resulting in a blockage of their aggregation, which triggers the acrosome exocitosis (75). Another possibility is that ASA affect the fusogenic response to the biological signalling that triggers the AR. (see section 3.4.2.2)

**3.4.2.2. Effects on sperm capacitation, acrosome reaction and oocyte-fusion**

The possible effect of ASA on the sperm's ability to undergo capacitation and to exhibit a functional acrosome exocytosis leading to oocyte-fusion is more controversial.

It has been widely studied using the hamster egg penetration test (HEPT), which monitors the ability of capacitated human spermatozoa to fuse with zona-free hamster oocytes, thereby representing a very relevant biological measure of the sperm fertilizing ability.

Conflicting results have been reported when the effect of circulating ASA was tested with the conventional version of HEPT (i.e., without AR stimulation). In fact either inhibition (76-78), or enhancement (79), both inhibition and enhancement (35,80), or no effect at all were found (81,82). Some discrepancies may be explained by procedural differences. Generally, when inhibitory effects were reported, donor spermatozoa had been exposed to ASA after capacitation or directly in the insemination medium. Using this procedure, an interaction of ASA with internal sperm antigens that are revealed after the acrosomal loss has been supposed to explain the inhibitory effect (83). However, although this possible interference may occur in the presence of ASA in the female, it cannot occur when ASA are detected in the male. When donor sperm were washed following exposure to ASA and then capacitated (a procedure which is more suitable for studying the effects of naturally occurring ASA in the male), generally no effect (81,82) or even an enhancement of penetrations (79) was observed.

Zouari and De Almeida (71) reported that antibodies eluted from eight autoimmune ejaculates and transferred onto donor sperm, reduced sperm penetration in only three cases and a modest increasing effect was exhibited in one case. All 3 samples with inhibitory effect contained both IgG and IgA. The elimination of one of the two isotypes restored the ability of the sperm to penetrate into hamster oocytes.

Using spermatozoa coated *in vivo* with ASA, Haas *et al.* (84) reported a variable degree of impairment in penetrating hamster oocytes, but the concomitant presence of astheno- and/or-teratozoospermia may bias the results.

According to most data reported with conventional HEPT, when the effect of ASA on spontaneous AR rate under capacitation conditions was evaluated, no effect was found in most studies (65,80,81,85). In an extensive study by our group (81), the exposure either to serum or seminal ASA of all Ig-isotypes, even in association, did not modify the spontaneous AR rate of donor sperm used for the hamster egg penetration test, whose outcome was similarly not affected. However, in another report (86), spermatozoa coated "*in vivo"* with ASA exhibited a massive acrosome loss not only in capacitating conditions but also in native preparations. The omission of vitality assessment and the inexplicably high rate of AR, which was also found in the control group, arouse some concern in interpreting these results. High levels of spontaneous AR were also reported by Lansford et al. (87) in most patients with spermatozoa coated "*in vivo*" with IgG plus IgA antibodies, but vitality assessment was not carried out.

Actually, there is general agreement that, in human spermatozoa, spontaneous AR occurring during "*in vitro*" capacitation, represents a sporadic event with little biological efficacy (88). Likewise, the biological significance of conventional HEPT has been debated (89), as it only relies on spontaneous AR.

Sperm ability to undergo a complete AR in response to ionophore challenge has been reported as significantly related to fertility status (90) and to the human IVF outcome (91,92). Since the ionophore challenge bypasses the biological signalling that initiates the AR, and uncapacitated spermatozoa respond poorly (93), this test is accepted as a measure of the capacitation status and the integrity of the chain of events between entry on calcium and exocytosis. Likewise, enhanced versions of the HEPT, including procedures which stimulate acrosome exocytosis (ionophore challenge or sperm preincubation at 4°C in TEST-yolk buffer) would have a higher biological and clinical efficacy (89).

In the light of these considerations, some studies addressed the effect of ASA on AR induced by ionophore challenge. Zouari et al. (85) reported an inhibitory effect by sperm-eluted antibodies in a very small series. On the contrary, in a report by our group (82), circulating and seminal ASA caused a slight but constant and significant AR increase in response to ionophore challenge compared with that of the same donor sperm suspensions exposed to control sera. However, this effect was not reflected in the results of the HEPT also performed after ionophore challenge (82). Nevertheless, a stimulating effect by seminal ASA on AR induction after ionophore challenge was also reported in a more recent study by Bohring *et al.*

(94), where AR was assessed by flow-cytometry. In this study also spontaneous AR rate was higher than in controls; in no case ASA inhibited basal or stimulated AR .

Using the TEST-yolk buffer enhanced HEPT, we (72) found that 12/28 patients with all ejaculated spermatozoa coated with IgG- or IgG/IgA-antibodies exhibited a penetration index (PI = penetrations per oocyte) less than 2 (the lowest value exhibited by fertile controls). But excluding from the analysis 9 patients exhibiting abnormal semen in terms of oligo/astheno- and/or-teratozoospermia, only in 16% of cases was the PI slightly less than 2. Moreover, no serum from the same patients, containing high titre of sperm-agglutinating activity, produced an inhibitory effect great enough to cause a poor HEPT outcome, when transferred onto donor spermatozoa, and washed before capacitation.

On the whole these data seem to indicate that, in most cases, ASA do not exhert an inhibitory effect on sperm functions involved in oocyte-fusion, when they occur in the male; whereas, it is suggested that ASA tend to exert, at least in some cases, a promoting rather than an inhibitory effect on acrosome exocytosis, as an expression of accelerated capacitation. However, an interference with the capacitation-associated expression of sperm surface mannose receptors was reported by Benoff *et al.* (95) using circulating ASA. This effect was related to an inhibition of the reduction of membrane cholesterol content associated with sperm capacitation, which prevented the membrane fluidity changes needed for mannose receptors expression. An inhibition of the capacitation-related increase in the fluidity of the sperm plasma membrane was also recently reported by Nakagawa *et al.* (96): the incubation of donor motile sperm suspensions with IgG purified from sera containing sperm-immobilizing antibodies suppressed the increase in the internalization of an alkyl ester lysophospholipid probe across the plasma membrane, capacitation-related event (97). However, the reversibility of this effect, produced by a subsequent incubation of ASA-exposed spermatozoa in an antibody-free medium, is difficult to explain, as it is not possible to remove antigen/antibody complexes from the sperm surface in this way (see section 4.3.2).

A possible effect on sperm membrane functional integrity by means of the hypo-osmotic swelling (HOS) test has also been studied. Although Jairaj *et al.* (98) reported no significant reduction of HOS scores produced by circulating ASA, in a recent report by Rossato *et al.* (99), sperm samples from infertile men with ASA showed HOS test scores significantly lower than those of normozoospermic subjects despite similar sperm viability and motility. Furthermore, antibody-coated spermatozoa showed a reduced rise in intracellular calcium concentration and AR after hypo-osmotic challenge, suggesting that ASA can alter sperm membrane functionality, impairing transduction signalling pathways relevant to the sperm fertilizing ability.

**3.5. Post-fertilization effects**

Although in some IVF series (56,100), a lower cleavage rate was observed in the presence of female-ASA, most clinical data from IVF seem to indicate that when ASA occur in men, they do not interfere with post-fertilization events. No reduction in cleavage and pregnancy rates has generally been reported in IVF programs where the presence of sperm-bound antibodies was associated with a reduced fertilization rate (51,52,63,64). However, in disagreement with previous data, Vazquez-Levin *et al.* (55) reported a significant reduction both in the cleavage rate and pregnancy rate.

Data from ICSI, available so far, seem to confirm that ASA in men are not associated with a reduction in the cleavage and pregnancy rate (101-103), whereas, a poorer embryo quality (101) and a higher rate of pregnancy loss (102) have been reported or denied (103,104 ).

On the whole, data from ICSI on a possible interference of naturally-occurring ASA in the male with post-fertilization events seem to be more consistent than those from conventional IVF. This might suggest that a possible adverse effect of ASA on post-fertilization events concurs in most cases with that on the fertilization process. Bypassing fertilization failure with ICSI, post-fertilization effects might become more apparent. Further studies are needed to confirm that this is true.

Given the evidence produced in animals that embryos share epitopes with sperm antigens (105), antibodies occurring in the female against sperm antigens could interact with embryonic antigens, providing an attractive reason to hypothesize that ASA could adversely affect embryonic development, when they occur in the female. Antibodies against the cleavage signal (CS-1), sperm derived protein which should function as an extra-nuclear cleavage signal for early division of fertilized zygotes, could represent an attractive explanation for a possible post-fertilization effect of ASA when they occur in the male (106). However the actual role and occurrence of these antibodies have yet to be determined.

**3.6. Cognate antigens of ASA involved in their interference with fertility**

As has emerged from the analysis of human IVF results and laboratory-based studies, the effect of ASA on sperm functions involved in gamete interaction is not univocal, probably depending on their antigenic specificity. Therefore, there is general agreement on the need to identify sperm-antigens targeted by natural ASA, as well as the recognition of their relevance to the fertilization process (4,10,29,107). According to Bronson (10), "their availability through recombinant DNA technology should lead to the development of ELISA-based tests that may allow one to identify the specific locus of the fertilization blockade for individual couples".

Several methods have been used to characterize sperm proteins relevant to immunological infertility.

**3.6.1. Direct identification of sperm surface proteins**

Immunoblot and immunoprecipitation techniques have been employed in several studies to determine the relative molecular masses of sperm antigens recognized by ASA from infertile patients on one-dimensional electrophoresis of sperm extracts (108,109; see also 110 as review of previous studies). However, little information has been obtained concerning the clinical relevance of the host of identified proteins, as characterization did not proceed further in most studies, nor were sperm surface antigens identified. Therefore, the relationship between revealed immuno-dominant antigenic bands and immunological infertility was not defined by these studies.

Data obtained with two-dimensional (2-D) electrophoresis, which is currently the most powerful toll available for the analysis of complex mixture of polypeptides, are more promising (111). Following immunoblotting with ASA, the recognized proteins may be sequenced and characterized by MALDI-MS and peptide matching.

Nanby-Hansen *et al.* (112) created a 2-D protein database of human sperm proteins as a means of identifying sperm-surface proteins. Vectorial labelling of the cell surface by biotinylation or iodination identified a composite of 98 dual-labelled sperm-surface proteins. Using this 2-D system, Shetty *et al*.(113) identified 6 immuno-dominant surface antigens recognized by ASA-positive sera from infertile male and female patients, possibly relevant to immunological infertility.

Using 2-D electrophoresis, Auer *et al.* (114) demonstrated that 2 immuno-dominant protein zones of 37/36 kDa (P36) and 19/18 kDa (P18) recognized by sperm surface-eluted antibodies from infertile patients consisted of several peptides. Polyclonal antibodies, produced in rabbits against both P36 and P18 inhibited human sperm penetration into zona-free hamster oocytes. However, the apparent internal localization of these antigens (anti-P36 and P18 rabbit antibodies detected the corresponding proteins on most sperm heads in methanol-fixed but only in acrosome-reacted living spermatozoa with immunofluorescent –IF- studies), questions their involvement in the male immunological infertility.

Bohring and Krause (115), using 2-D electrophoresis of highly enriched sperm membranes, identified 18 antigens reactive with seminal plasma from infertile patients with ASA. Six of them, recognized by most samples, showed amino acid sequences that matched those of heat shock proteins HPS70 and HPS70-2, the disulphide isomerase ER60 (inactive form of caspase-3) and 2 subunits of the proteasome complex. However, neither their role in sperm function nor their localization on the sperm surface have been determined.

More recently Chiu *et al.* (116), using 2-D electrophoresis, identified a novel sperm protein recognized by serum ASA (SPRASA) from 2 patients who had remained infertile following vasectomy reversal, but not from fertile controls. Its amino acid sequences matched those of a protein derived from the C-type lysozyme/alfha-lactalbumin gene family. However, also in this case, the apparent localization in the inner acrosome membrane, as indicated by IF studies with a polyclonal antibody produced in rabbits, questions its involvement in the male immunological infertility.

**3.6.2. Identification of antigens in DNA expression libraries**

ASA from infertile patients have also been used to identify antigens expressed by recombinant bacteriophages in cDNA libraries. Using testis cDNA libraries (117-119), the testis/sperm-specificity of the antigens is assessed, but sperm membrane antigens acquired or changed during sperm passage through epididymis are not recognized. Isolation of relevant cDNAs provides the primary sequences of the identified antigens. Synthetic peptide epitopes and recombinant proteins can be employed to generate monoclonal or polyclonal antibodies, whose interfering effect on sperm fertilizing ability, when demonstrated, would provide evidence of the involvement of the native antigen in immunological infertility.

The BS-17 antigen, identified by the serum from an infertile woman with high titre of sperm agglutinating antibodies, is localized on the surface of the acrosomal region of human and other mammalian spermatozoa (120). Its peptide sequence, obtained by screening a human testis cDNA expression library, shares a high omology with calpastatin (121), whose RNA transcription occurs only in spermatids (122). Polyclonal antibodies against BS-17 inhibited human sperm ability to penetrate into zona-free hamster oocytes (120). Furthermore, mouse spermatozoa incubated with anti-DS-17 antiserum and instilled into the oviduct of ovulated female mice produced a number of embryos significantly lower than non-treated controls (120). As calpastatin is bound to calpain, a Ca++ - dependent endopeptidase by forming an inactive complex, it was hypothesized that anti-BS-17 antibodies could destabilize the calpastatine/calpain complex leading to a premature AR (123). However, it has not been reported whether this antigen is recognized by antibodies from male patients with immunological infertility.

Recently, Naz (124) identified seven unique and novel dodecamer aminoacid sequences, which reacted with ASA-containing sera from infertile patients, by screening the FliTrx random phage display library that has the expression of 1.77x108 different dodecamer peptide sequences. Three of the synthesized peptides demonstrated a stronger reaction with a high proportion of ASA-containing sera compared to control sera. Although the localization on sperm-surface as well as the involvement of these antigens in fertilization and in immunological infertility has not yet been demonstrated, according to the author the phage display technology

represents an exciting approach to delineate sperm epitopes involved in immunoinfertility.

### 3.6.3. ASA recognition of known antigens involved in fertilization

In an alternative strategy, sperm antigens, which over the last few years have been identified as playing a role in the fertilization process, can be tested for a possible involvement in human immunological infertility, by assessing their reactivity with ASA from infertile patients.

Both serum and seminal ASA from several infertile patients, but not ASA-negative samples, recognized FA-1 antigen (125,126), a sperm-specific surface glycoprotein.of testicular origin, localized on the post-acrosomal and tail of spermatozoa of various mammalian species including humans (127). The cDNA encoding for FA-1 was firstly cloned and sequenced in the mouse (128). Immunization of female mice with recombinant FA-1 (rFA-1) caused a long-term reversible reduction of fertility (129). Anti rFA-1 antibodies from immunized mice significantly blocked sperm binding to ZP and IVF in murine oocytes (129). Later, a human homologue of the murine FA-1 cDNA was also obtained and characterized (130). Antibodies raised against human rFA-1 caused a significant dose-response inhibition of human sperm capacitation and AR by blocking tyrosine phosphorilation of the FA-1 antigen. FA-1 antigen has also been applied to remove auto-antibodies from the surface of spermatozoa from immuno-infertile patients (131). Interestingly, absorption with FA-1 antigen increased immunobead-free swimming spermatozoa both for IgG and IgA ASA. This is surprising, as no "*in vitro*" sperm processing technique, unless inconsistent with sperm vitality (heating at 56°C or lowering the pH to less than 3), has been successfully utilized to disrupt antigen/antibody complexes on sperm surface (132,133).

Most sera and seminal plasma from ASA-positive infertile men, but not ASA-negative samples, also recognized the $YLP_{12}$ peptide sequence, obtained by screening a phage display library with solubilized human ZP preparations, in order to identify peptide sequences involved in ZP-binding (134). This peptide inhibited human sperm/ZP binding in a dose/response manner, when added to hemizone prior to spermatozoa, and its monovalent Fab' antibodies also inhibited human sperm/ZP binding (134). An inhibiting effect of $YLP_{12}$ Fab' antibodies on capacitation/AR was also claimed, as they inhibited AR following ionophore challenge (135). Immunization of female mice with $rYLP_{12}$ caused a long-term reversible reduction of fertility (136). As antibodies resulting from immunization recognized a protein band of about 72 kDa in testis extracts and a band of about 50 kDa in sperm extract, a modification or cleavage of the protein during the epididymal transit was suggested. The 50 kDa antigen is localized on the surface of the sperm acrosome and tail. (136).

A monoclonal antibody (mAb), H6-3C4, with sperm agglutinating and complement-dependent sperm immobilizing activities, was immortalized from the lymphocytes of an infertile woman who exhibited high titre of sperm-immobilizing antibodies (137). Subsequently, the sperm-agglutinating S19 mAb, generated via the immunization of mice with human sperm extracts (138) was shown to react with the H6-3C4 cognate antigen. The H6-3C4/ S19 cognate antigen, designated Sperm Agglutination Antigen-1 (SAGA-1), was characterized as a GPI-anchored glycoprotein of epididymal origin, localized on the surface of human spermatozoa. Agglutinating S19 mAb exhibited complement-dependent sperm immobilizing activity, inhibited the ability of human sperm to penetrate cervical mucus, to bind zona pellucida and to fuse with zona free hamster oocytes. Purification with the S19 mAb followed by microsequencing demonstrated that the SAGA-1 core peptide is identical to that of CD52 lymphocyte antigen, but the N-linked glycosylation is tissue specific. All these studies are reviewed in ref.110. More recently, a recombinant single-chain variable fragment antibody (RASA) was engineered against SAGA-1, which exhibits a sperm agglutinating activity in a tangled pattern (head to head, head to tail, tail to tail) (139). Although SAGA-1 represents a very likely target for antibodies involved in the aetiology of clinical immunological infertility, the occurrence of its recognition by ASA from infertile patients has not yet been screened.

Other than sperm antigen of testicular origin, some of which may undergo modification during epididymal sperm transit, and sperm coating antigens of epididymal origin, ASA could also recognize prostasomes, organelles secreted by prostatic cells, that adhere to sperm surface (140). Chicken antiprostasome antibodies caused sperm-agglutination and all sera from 20 infertile patients with agglutinating ASA contained IgG-antibodies against prostasomes (140). More recently, these antigens where identified by means of 2D-electrophoresis and characterized (141). Prolactin-inducible protein and clusterin were the immunodominant prostasome antigens.

## 4. CLINICAL IMPLICATIONS

### 4.1. Are current ASA tests effective in screening and in quantifying sperm autoimmunization relevant to infertility ?

The lack of a standardized and universally accepted assay for the detection of ASA directed against known antigens has been repeatedly claimed as the main reason of the confusion over the actual role of ASA as well as their treatment in male infertility (4,10,142). In this section we will examine whether a standardized strategy in the use of current ASA tests could be effective in screening and quantifying sperm autoimmunization relevant to infertility, in spite of their claimed inadequacy.

The first assays to be utilized were indirect tests detecting the biological activities of circulating ASA, i.e., sperm agglutination techniques and complement dependent cytotoxicity techniques (see ref. 11 for review). Multicentric comparative studies (143,144) indicated that they determine largely the same antibody specificities but with different sensitivity, which was higher for sperm agglutination techniques, especially the Tray agglutination test-TAT (145).

Circulating ASA detected with these indirect tests ranged from 8.1% to 30.3% in unselected men with infertile marriages (14,33,146-148). At low titres they were also reported in 2.4% to 10% of fertile men (146,148). Low titres of sperm-agglutinating activity may be due to non-immunological factors (149), representing false positive results. When stricter criteria were used (i.e., the occurrence of sperm-immobilizing activity in addition to sperm-agglutinating activity (14,147) and/or occurrence of sperm-agglutinating activity in seminal plasma) (14), the prevalence of ASA in men with infertile marriages varied from 4.7% to 7.5%.

Over the last 2 decades widespread acceptance has been gained for direct tests developed for the detection of sperm-bound antibodies, including the IgG-mixed antiglobulin reaction (IgG-MAR) test (12), the immunobead test (IBT) (13) and the SpermMar test (150). Although the IgG-MAR test detects only antibodies belonging to IgG class while IBT detects IgG,A,M antibodies, comparative studies have generally demonstrated that all these tests are suitable as effective direct screening tests (12,14,15,151-155). In fact, sperm surface IgA are almost always found in association with IgG (151,153,156). As the IgG-MAR test and the commercially available SpermMAR are cheaper and quicker, according with the WHO (157) they are more suitable for routine screening of all semen analyses. The IBT should be performed on samples with a positive IgG MAR test, to determine whether and at what extent IgA-ASA are also bound to sperm surface.

Immunological screening by means of direct tests gave positive results in 7.8% to 20.1% (14,15,19,34,153,158-161), with the occurrence of strong positive results in about 6-7% of patients (14,15).

Although the higher frequency of ASA in males with infertile marriages than in fertile controls could imply a negative effect of ASA on fertility, the proof of a link between ASA and fertility impairment can only be produced by prospective studies comparing the occurrence of natural pregnancies in patients with ASA and those without. Some major peculiar reasons hinder the feasibility of these studies: 1) the low incidence of sperm autoimmunization in unselected infertile couples requires multi-centric studies including a suitable number of patients and a suitable number of observed cycles; 2) the inter-individual high variability of semen parameters, not related to the presence of ASA, makes it very difficult to obtain a study- and a control-population, homogeneous for semen quality.

Due to these limitations, little information has been produced by follow-up studies comparing ASA-positive and negative patients, where conflicting results were reported (18,147,162-166). However, worth noting is that when the degree of sperm autoimmunization was considered, a significant inverse correlation was found between either the titre of circulating ASA (147,167) or the percentage of sperm bound antibodies (168) and the incidence of pregnancies. A poor prognostic value of low to moderate levels of sperm-bound antibodies was also confirmed by Barrat *et al.* (169).

Altogether, the analysis of epidemiological and prognostic studies indicate that ASA are a *relative*, rather than absolute, cause of infertility (11,29), that appears to be related to the degree of sperm-autoimmunization. It determinates the degree of sperm-agglutination and, mainly, the interfering effect on cervical mucus penetration, independently from the isotype and antigenic specificity of ASA. The interfering effect on cervical mucus penetration should be verified and quantified by a carefully performed *post coital test* and, possibly, by an *in vitro* cervical mucus penetration test.

In this light, if ASA-screening tests (IgG-MAR and/or IBT) are negative or weakly positive (less than 50% of the motile spermatozoa carrying ASA), an immunological infertility may be excluded and no further immunological tests are needed. On the other hand, if all, or the majority, of spermatozoa are coated by ASA, the effects on fertility are more difficult to evaluate, first of all because these tests cannot quantify the Ig density on sperm surface, which could play a role in the ASA interference with fertility. In these cases, titration of ASA in serum and seminal plasma may be useful. High titres in serum or an excess of free ASA in seminal plasma very likely indicate that the amount of ASA on sperm surface would also be high. Sperm agglutination techniques or indirect IBT should be preferred for this purpose. Enzyme-linked immunosorbent assay (ELISA) (170-173) must be discouraged, since its sensitivity and specificity for the detection of sperm surface-directed ASA is hindered by fixation of whole spermatozoa or membrane extracts.

Actually, flow cytometry (FCM) has been proposed as an objective method to quantify the amount of IgG and IgA on individual viable spermatozoa (174-179). Although some technical limitations have been claimed (180), its improvement for wide clinical use should be encouraged. The regional specificity of antibody link, not detected by FCM, could be assessed by immunofluorescent test (IFT) on the same sperm suspension used for FCM. The use of living sperm suspensions instead of fixed smears makes the immunofluorescent test highly specific for surface antigens-directed ASA. It is usually utilized in the direct form (156) in our lab for a better evaluation of the regional specificity of the antibody link in all samples positive at screening ASA tests.

Radiolabeled antiglobulin assay (181,182), although highly sensitive and specific in quantify the antibody load, does not detect the regional specificity of antibody link, neither does it determine the proportion of ASA-positive spermatozoa, thereby representing a less attractive diagnostic tool than FCM.

### 4.2. Clinical conditions associated with a higher prevalence of ASA

Using various diagnostic techniques, a high prevalence of ASA has been observed in some clinical conditions, mainly, acquired genital tract obstructions,

thereby recognized as identifiable causes of their development. Among them, vasectomy is the most common, with a prevalence of ASA ranging from 34% to 74% (148,159,183-185), and with their persistence in 38% to 60% following successful vasovasostomy (184,186,187). On the contrary, the association of ASA with obstructive azoospermia due to congenital causes is not yet well established, as conflicting data have been reported (188-192). Antisperm antibodies have also been associated with acute and chronic genitourinary infections (193-197). Some studies have focused on the relationship between ASA and asymptomatic Chlamydia trachomatis infections. Although a high incidence of ASA was reported in the presence of Chlamydia in genital secretion as detected by means of culture (198) or the polimerase chain reaction (199), circulating chlamydial antibodies were not associated (200) or only weakly associated (201) with the presence of sperm bound antibodies, whereas both a strong correlation (201) as well as no association (202) were reported between the presence of seminal chlamydial antibodies and ASA. More recently, a significant correlation was reported between chlamydial antibodies and ASA in patients with genital chlamydial infections, whereas no association was found in those with ocular chlamydial infections (203). On the whole, these data suggest that chlamydial infections may play a role in the induction of ASA, as a result of the inflammatory process rather than of a cross reactivity between sperm and Chlamydia antigens. Although in the past a higher prevalence of ASA was reported in men treated and not treated with orchidopexy for cryptorchidism (204,205), this was not confirmed in a more recent study (206). Repeatedly, a higher incidence of ASA has been reported in men with testicular carcinoma (207-209) and spinal cord injury (210-214). Reports also exist on a higher incidence in homosexual men (215,216). Finally, conflicting results have been reported on the association of ASA with varicocele (217-221) However, in a multivariate analysis of men from infertile couples with and without ASA, only prior vas reversal and a history of genital tract infection were significantly associated with the presence of sperm-bound antibodies (222).

### 4.3. Implications for treatment

The effectiveness of the treatments for male immunological infertility can be proved only by evaluating the cumulative pregnancy rate in prospective studies conducted according to *evidence-based medicine.* However, the feasibility of such studies is hindered by the same peculiar causes that make it hard to obtain the proof of a link between ASA and fertility impairment (see section 4.1).

Nevertheless, both clinical and experimental data so far produced can provide a rationale for a treatment strategy to be offered to infertile patients with ASA, *even in the absence of this proof.*

In the evaluation of possible therapeutic modalities for infertile patients with ASA, it is important to keep some considerations in mind: 1) some mechanisms of antibody-interference with fertility, namely sperm-agglutination and inhibition of cervical mucus penetration, are well established and are related to the degree of autoimmunization; 2) other mechanisms that may occur downstream mucus penetration are suggested by experimental data: they could or could not occur depending on Ig-isotype (e.g., complement-mediated sperm injury) or on the specific antigen(s) involved in immune response (e.g., interference with gametes interaction); 3) It is not possible to prevent or disrupt antigen/antibody complexes on sperm surfaces by means of "*in vitro*" sperm processing techniques unless methods inconsistent with sperm vitality (heating at 56°C or lowering the pH to less than 3) are utilized (132,133).

#### 4.3.1. Corticosteroid therapy

The rationale for this treatment is to reduce the production of ASA, thereby obtaining a proportion of antibody-free sperm sufficient for fertilization and/or reducing the density of ASA on sperm surface. Either long-term low-dose treatment (e.g., prednisolone, 5 mg three times daily for at least 6 months) (223), or intermittent high-doses of metilprednisolone (96 mg/day for 7 days) (224) were widely used in the past. Unfortunately, most of those early studies lack a placebo control group (225 for review). In a double-blind, placebo-controlled study, intermittent high-doses of metilprednisolone did not produce a favourable effect over placebo on the men's subsequent fertility (226). In the same study, a significant reduction in sperm-associated IgG was reported using radiolabelling antiglobulin assay, with no effect either on sperm-associated IgA or on circulating IgG-ASA levels. Because of the risk of serious adverse effects of high doses of corticosteroid treatment (227), an intermediate-dose cyclic regimen was evolved. In a double-blind crossover trial, prednisolone treatment, at 20 mg twice daily on days 1-10 of the female partner's menstrual cycle, followed by 5 mg on days 11 and 12, was associated with a cumulative pregnancy rate of 31% during 9 months, which was significantly higher than the rate of 9.5% for placebo (228). Unfortunately, with the same treatment and the same study design, no pregnancy was achieved during three months in a subsequent report (229). In both studies, the circulating ASA-titres were not significantly modified by steroid treatment, while a significant fall in antibody titres in seminal plasma was found in the former. Finally, in a placebo-controlled flow cytometric study, the antibody levels measured before and after treatment with prednisolone (20 mg/day) or with placebo were not statistically different, but in 2/10 patients a marked decrease in the proportion of spermatozoa positive both for IgG and IgA was observed (230).

On the whole, evidence of the suppressive effect of corticosteroid in more recently employed regimes is scarce. A meta-analysis by Kamischke & Nieschlag (231) on 4 randomized trials (226,229,232,233), including 190 patients, revealed no significant influence of corticosteroid treatment on pregnancy rates. However, according to the authors, conclusions from this meta-analysis have to be drawn with caution, as the number of patients in qualitative adequate studies and the power of meta-analysis were low. The doubtful efficacy judged against the potential adverse effects has strongly reduced the use of corticosteroid

treatment in favour of procedures of assisted procreation. Nevertheless, as argued by Check (45), the risks for women undergoing assisted procreation should also be considered as well as the fact that there are patients for whom assisted procreation techniques are not options, for financial, personal or religious reasons. In this light, a better understanding of the potential benefits together with the potential side effects of corticosteroid treatment would be of clinical value.

**4.3.2. Assisted Reproduction Techniques (ARTs)**

ICSI has been claimed as the primary choice of treatment in immunological infertility, as it overcomes any potential interference of ASA with sperm fertilizing ability (8,9). However, given its high cost and invasive nature, as well as the heterogeneity of infertile male patients with ASA, ISCI should rather be reserved for patients for whom achieving a pregnancy with less invasive techniques would be very unlikely. To establish an ART treatment strategy the main point to be addressed is the following:

When could intrauterine insemination (IUI) represent effective first line ART treatment?

The rationale for the use of IUI for the treatment of the male immunological infertility is that it overcomes the most established interference of ASA with fertility, represented by their impairment of cervical mucus penetration. Considering the reported results as a whole (232,234-241), the usefulness of IUI may appear controversial, as pregnancy rate/couple ranged from 0% (236,239) to 64% (240). Several reasons may be advocated to explain these controversial results, including different inclusion criteria with respect to the assessment and degree of sperm autoimmunization, confounding effects of other concomitant non-immunological semen abnormalities, different IUI protocols or procedures (e.g., stimulated or non-stimulated cycles, different sperm recovery and manipulation techniques). Nevertheless, a main datum emerges from the analysis of reported studies: good results can be obtained when moderate sperm autoimmunization is also included. Using more than 50% positive MAR test as the inclusion criterion, in a crossover, randomized trial, Lahteenmaki *et al.*(232) reported that IUI in non-stimulated cycles (maximum 3 cycles/couple) was significantly more effective than cyclic, low dose prednisone treatment in 40 couples (9 pregnancies vs. 1 pregnancy, respectively). Furthermore, using the same inclusion criterion, in another prospective non-randomized study, where the effectiveness of IUI with ovarian stimulation was compared with that of IVF in 29 couples, Ombelet *et al.* (240) reported that 64.3% of the patients conceived after a maximum of three IUI cycles, while 46.6% of patients conceived during the first IVF cycle. Cost benefit analysis favoured a course of four IUI cycles, indicating this treatment as a valuable first-choice method to be used before starting more invasive and expensive ART options. In a randomized, cross-over study comparing IUI in superovulated cycles with natural intercourse in men receiving cyclical intermediate-dose steroid therapy for immunological subfertility (immunobead binding levels more than 50% in either seminal plasma or serum), a cumulative pregnancy rate of 39.4% over four cycles of IUI was achieved, compared with only 4.8% over four cycles of timed intercourse with the same regimen of steroid therapy (241). The effectiveness of IUI in cases of moderate sperm autoimmunization may be due to the proportion of antibody-free spermatozoa in the semen (functional oligozoospermia), whose meeting with the egg is favoured by a well timed IUI. Furthermore, a low density of antibodies on the sperm surface, although impairing mucus penetration, might not dramatically affect sperm survival in the female tract and gamete interaction.

The effectiveness of IUI in the case of strong autoimmunization (the totality of ejaculated spermatozoa coated with ASA, associated with high titres of serum ASA and/or an excess of free ASA in seminal plasma), is less established, as such inclusion criterion has rarely been used. Using this inclusion criterion, our group (239) did not obtain any pregnancy with 110 IUIs in 19 couples, while a pregnancy rate/couple of 25.6% was obtained in the control group (n°=86) without ASA. Both groups were homogeneous for both clinical data and seminal parameters, having excluded teratozoospermic patients (with and without ASA) from the analysis, since teratozoospermia had been proved to strongly impair the IUI outcome (242). The opsonizing effect through the female genital tract and/or the effects on the sperm functions involved in the gamete interaction by a high density of antibodies on the sperm surface could account for this failure. In disagreement with these results, in the above cited report by Lahteenmaki *et al.*(232) 6 out of 9 patients with ASA, whose wives conceived following IUI, exhibited a strong positive (more than 90%) IgG-MAR test associated with a variable degree of serum sperm agglutinating activity.

Although it is impossible to prevent or disrupt antigen/antibody complexes on sperm surfaces, interestingly, in a prospective study by Bollendorf *et al.* (243), incubation of antibody-coated spermatozoa (direct IgG-MAR test more than 50%) with the protein digestive enzyme chymotrypsin before IUI was more effective in achieving pregnancies than without digestion before IUI (15% versus 3% pregnancy/cycle). In a more recent retrospective analysis from the same group (244), pregnancy rate/cycle of 10.7% was achieved with the same treatment in cases where all ejaculated spermatozoa were antibody-coated. The effectiveness of this proteolytic treatment in neutralizing the interfering effects of ASA should be further investigated against possible negative effects (e.g. on other sperm surface proteins relevant to fertilization). Interestingly, Lenzi *et al.* (245) observed a capacitation-related significant reduction of sperm-antibodies bound to the acrosomal region, as evaluated by IBT. A rearrangement of the plasma membrane, including a loss of membrane molecules, occurs during sperm capacitation (246). The authors hypothesis was that some of these molecules might be antigens against which antibodies are directed. Therefore, the authors proposed that *in vitro* capacitation could provide an *in vitro* therapy able to remove whole immunocomplexes from the sperm-surface, without damaging the sperm-membrane.

The resort to IFV procedures is mandatory when other less invasive approaches have failed or they may also be chosen as a first-choice method in cases of strong sperm autoimmunization. ICSI, where the reported fertilization rates are similar to those in other indications (102,103) or even significantly higher (101), should be preferred to conventional IVF-ET, especially after IUI failure, given the qualitative other than quantitative ASA effect on the sperm/egg interaction and the unavailability of current tests to determine the antigenic specificity of ASA.

Since the inability of antibody-coated spermatozoa to bind the zona pellucida is apparently the main but not unavoidably occurring obstacle to fertilization (see section 3.4.2), the outcome of the ZP-binding test could identify those patients with immunological infertility who profit by conventional IVF. However, the demonstration that ASA can inhibit the induction of AR by ZP in the absence of an inhibitory effect on ZP-binding (73) indicates that the ZP-binding test does not completely explore the interference of ASA with the sperm-ZP interaction. Therefore, testing AR-induction by ZP could be usefully included in the diagnostic screening before IVF. But simpler tests should be developed, and recombinant human ZP3 would be a convenient tool in such development.

## 5. CONCLUSIONS AND PERSPECTIVES

Although a vast body of literature has provided evidence that ASA can affect the sperm fertilizing ability at various levels, it is still hard to establish in each individual patient, whether, or to what extent, these interfering effects occur, apart from sperm-agglutination and the impairment of cervical mucus penetration. The main reasons are the difficulty in quantifying the antibody density on the sperm surface and the unavailability of diagnostic tests to determine the antigenic specificities of ASA. Due to the persistence of these limitations, the interest in clinical studies has strongly diminished in the last few years.

However, flow cytometry is a promising objective method to quantify the amount of ASA on individual viable spermatozoa, and its improvement for a wide clinical use should be encouraged. Alternatively, in a standardized diagnostic strategy, a high antibody density on the sperm surface could be inferred by the occurrence of the totality of ejaculated spermatozoa coated with ASA (as determined by direct MAR test and/or IBT), associated with high titres in serum and/or an excess of free ASA in seminal plasma (as determined by sperm agglutination techniques or indirect IBT).

Furthermore, as far as the antigenic specificities of ASA are concerned, it is expected that ever-growing knowledge will be produced by studies aimed at immunocontraception. Antisperm-antibodies develop in post-vasectomized men or they spontaneously occur in men without physiopathological and clinical complications, apart from infertility, despite their persistence for years. Thus, ASA induced by immunization of men or women with antigens involved in natural immunoinfertility might similarly be without side effects. With this assumption, the study of clinical infertility due to ASA, regarded as "experiments of nature" in fertility reduction, has been approached to identify candidate sperm antigens for immunocontraceptive development (247). Most of data on the identification of sperm-surface antigens recognized by natural ASA and their role in fertilization, revised in section 3.6., have been produced in studies aimed to develop an immunocontraceptive strategy. The availability of sperm antigens involved in fertilization through recombinant DNA technology should lead to the development of ELISA-based tests that may determine the antigenic specificities of ASA occurring in each patient and their relevance to the various steps of the fertilization process.

This would strongly renew interest in the study of clinical infertility mediated by ASA and finally settle the controversies on the significance of naturally-occurring ASA in men.

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**Send correspondence to:** Professor Felice Francavilla, Dipartimento di Medicina Interna, Università de L'Aquila, Blocco 11, Coppito, 67100 L'Aquila, Italy, Tel: 0862.368338, Fax: 0862.368342, E-mail: francavi@cc.univaq.it