# Sperm DNA integrity and male infertility: current perspectives

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## Abstract

The sperm cell is designed to transmit the male DNA to the oocyte. The integrity of this DNA is an obvious requisite for normal embryo development and a successful pregnancy. Nevertheless, DNA quality, mainly the presence of DNA fragmentation, is not habitually assessed as part of sperm analysis due to the technical complexity of such an assessment and misunderstanding about its significance. Controversy still surrounds the relationship between DNA fragmentation rates in the sperm and fecundation, embryo quality and pregnancy. DNA damage may vary in type and degree. After sperm penetration, DNA damage tends to be repaired by the oocyte. The complex relationship between DNA damage and the extent and reliability of the different DNA repair pathways in the oocyte could explain the disparity of correlations with the different fertility parameters, reported in the different studies. The influence of DNA fragmentation on one or several fertility parameters seems clear. Moreover, assessment of DNA fragmentation should be considered not only in the context of infertility but integrated within the seminal study as a complementary parameter of sperm quality. This has been demonstrated in several andrological pathologies such as varicocele, infections and cancer. In addition, studies in various farm animal species show a clear influence of sperm DNA fragmentation on sperm quality and pregnancy outcome. The frequency of sperm cells with fragmented DNA tends to increase with time after sample collection or thawing of frozen straw and vary among species and among individuals within the same species. The evaluation in time may provide relevant information on the topic of sperm DNA fragmentation.

**Key words:** sperm, DNA damage, DNA fragmentation, DNA breaks, fertility.

## Introduction

The mature sperm is an extremely specialized cell, exclusively designed to transmit a haploid genome to the offspring. This is the result of a complex process that comprises the mitotic proliferation of spermatogonia, followed by two meiotic divisions of spermatocytes and the final differentiation from haploid spermatids during spermiogenesis.

This latter phase supposes a dramatic sequence of nuclear events, involving the replacement of histones by transition proteins TP1 and TP2 and then by protamines P1 and P2, in human and mouse at least. As a consequence, the chromatin looses its nucleosomal organization and acquires an extremely packed crystal-like structure [1, 2].

Sperm DNA and chromatin integrity obviously are essential for the accurate transmission of genetic information to subsequent generations, and accumulating evidence indicates that sperm chromatin abnormalities or DNA damage may influence human male fertility. Some sperm cells with extensive DNA breakage normally are present in human ejaculates, but infertile males are reported to have a higher fraction of sperm cells with DNA fragmentation than do fertile controls.

Sperm DNA fragmentation is the focus of very active research in andrology and fertility, and several excellent reviews exist on different aspects of the topic [3-5]. This report presents our personal view about the scientific and technical aspects of the determination of sperm DNA fragmentation, as well as the possible basic and clinical value of this research topic. As with all scientific pursuits, our interpretations may be revised as future research data become available.

## What are the mechanisms of sperm DNA fragmentation?

The basis of DNA fragmentation in mature sperm cells is not well understood, and three main hypotheses have been proposed. The first one is related to the exchange of histones by protamines in mid-spermiogenesis that would generate torsional stress in unconstrained supercoils. To eliminate this and facilitate protamine deposition, induction and subsequent repair of breaks arise in the DNA of the elongating spermatids of mice and possibly in the round spermatids of humans, probably mediated by topoisomerase II [6]. Abnormalities during spermiogenesis could result in an incomplete chromatin maturation process, whereby DNA breaks would remain unrepaired and persist in mature sperm cells. In fact, mice with targeted deletions of *Tnp1* or *Tnp2* genes or with disruption of one copy of the gene for either P1 (Prm1) or P2 (Prm2) show an increase in the frequency of sperm cells with damaged DNA [7, 8].

The second hypothesis proposes that DNA fragmentation is the consequence of oxidative stress in the male reproductive tract. High levels of reactive oxygen species (ROS) may be released by activated leukocytes and/or macrophages, for example, in an inflammatory-infectious process or by the immature sperm cells themselves with excessive cytoplasmic retention [9, 10]. Oxidative stress may develop when ROS generation overcomes the ROS antioxidant-scavenging activities of the seminal plasma exerted by enzymes like superoxide dismutase, catalase and gluthation peroxidase, as well as by chain-breaking antioxidants [10].

The third hypothesis is consistent with apoptosis-related DNA strand breaks, as in somatic cells. Thus, the presence of activated caspases 8, 1 and 3 in the postacrosomal region and of caspase 9 in the midpiece have been reported [11]. Moreover, recent evidence of the possible presence of an endogenous nuclease has been reported in sperm cells from human, hamster and mouse [12]. The presence of mature sperm cells with apoptotic markers, such as Fas, Bcl-x, p53, or annexin-V positivity in the cell membrane, especially in some infertile men, suggests an abortive apoptotic-like process by which some cells earmarked for elimination escape the action of the removal mechanism [13]. Nevertheless, no correlation exists between the presence of these typical apoptotic markers and the degree of DNA fragmentation [14].

This may suggest that the process of DNA fragmentation, if apoptotic, could be driven by a different mechanism than that operating in other cell types. In fact, caspase-independent pathways of DNA fragmentation and cell death seem to exist. We must bear in mind that the three main hypotheses are not exclusive or isolated phenomena. Apoptotic pathways and ROS may be associated, and the same could happen with abnormal spermiogenesis.

## Which DNA lesions should be expected?

According to the theories of the origin of DNA fragmentation in spermatozoa, different lesion types should be expected in DNA. Nucleases, either endogenous or exogenous, should produce DNA single-strand breaks (SSB) and/or DNA double-strand breaks (DSB). The DNA breaks produced by chromatin remodelling during spermiogenesis seem to correspond to DSB produced by topoisomerase II [15]. ROS and other radical molecules like those derived from nitric oxide should generate mainly SSB and many different DNA base damages [16]. 8-oxoguanine (8-oxoG) is one of the most common modified bases induced by ROS, utilized as a marker of oxidative damage.

DNA double-strand breaks are perhaps the most dangerous lesions. They are the main lesions responsible for creating chromosomal aberrations and possible apoptotic triggering in somatic cells. DNA double-strand breaks generally are not produced directly by ROS, unless an extreme and localized production occurs inside or very close to the nucleus. Nevertheless, ROS could elicit DSB through nuclease activation [17]. Some reports have speculated that DNA fragmentation (DSB)

corresponds to "primary" damage, whereas base lesions produced by oxidative stress are defined as "secondary" damage [18]. This is a distinction that does not have any support from the mutagenesis field.

## What are the expected consequences of different types of sperm DNA damage in fertilization?

The outcome of sperm DNA damage in fertilization and embryo development depends on the balance between the DNA damage from the sperm and the oocyte's repair capacity. Normal sperm DNA seems to contain a relatively higher density of background damage than do certain somatic nuclei like leukocytes [19]. This could be due to the presumed lack of repair activity in a compacted genome. Nevertheless, the oocyte should appropriately repair this background damage. Otherwise, variability in the extent of damage could be expected in those sperm nuclei with fragmented DNA, always within a high range.

Additionally, the type and/or complexity of DNA lesions in spermatozoa with DNA fragmentation can vary, and individual differences in type and complexity also occur. After penetration into the oocyte, sperm with extensively broken DNA, i.e., thousands of DSB as expected after an apoptotic-like-process, would exceed the oocyte's repair capacity by a wide margin. Consequently, sperm chromatin possibly may not be able to exchange protamines by histones and DNA replication due to cell-cycle block, preventing the normal formation or development of a male pronucleus, resulting in fertilization failure. This is supported by classical studies on cells exposed to very high doses of ionizing radiation that induce hundreds of DNA DSB. All these cells lose their proliferation capacity.

Conversely, when sperm DNA damage is composed mainly of a low level of DBS, SBS, abasic sites, and/or base damages, the oocyte's various specific DNA repair pathways may be effective, and the male pronucleus develops. Nevertheless, some misrepaired or unrepaired DNA lesions could lead to mutations or chromosome aberrations. Unrepaired SSB or other lesions also may lead to DSB when DNA is replicating, leading to structural chromosomal abnormalities [20]. If these aberrations are unstable, they affect the correct mitotic segregation of chromosomes, resulting in genomic instability and cell death, affecting embryo development. When DNA repair is complete, the morula and blastocyst stages can be achieved. The paternal genome may be expressed normally at this stage, so a pregnancy would be more likely. Otherwise, if the repair processes are not totally efficient, blastocyst arrest or spontaneous abortion may result [21].

The extreme complexity resulting from qualitative and/or quantitative DNA damage from each sperm joined with the variable DNA repair capacity of each oocyte may explain the different correlations obtained by different studies. Recently, this interaction was experimentally evidenced by mating male mice with sperm cells matured from irradiated spermatids with females with disrupted DSB DNA repair pathways [22].

Animal models in which *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are performed with strict DNA lesions on oocytes of similar quality should provide a reliable correlation with the possible effects in human fertilization, embryo development and pregnancy outcome. Accordingly, a recent report in mice about ICSI with a high percentage of spermatozoa with fragmented DNA (induced by freezing-thawing without cryoprotection) showed decreased oocyte survival and pregnancy rates [23]. The freezing-thawing mechanism increased DNA fragmentation from between 4 and 7% to between 16 and 24%. Nevertheless, the type of DNA breakage constituting the extra fragmentation is not clear; the majority of sperm have no detectable DNA fragmentation. Intriguingly, a significant proportion of adult offspring evidenced abnormal behavioral tests, malformations, tumors and premature aging.

## Should direct and indirect methodologies for sperm DNA fragmentation analysis be distinguished?

Actually, several techniques exist to detect sperm DNA fragmentation, i.e, DSB and SSB. One group relies on the enzymatic addition of labelled nucleotides in an end of a break, such as the terminal deoxynucleotidyl transferase-mediated nick-end labelling (TUNEL), or the *in situ* nick translation (ISNT) with *Escherichia coli* DNA polymerase I. The single-cell gel electrophoreis (SCGE), or comet assay, consists of the electrophoresic mobilization of deproteinized nuclei, resulting in an image of a "comet", with a head and a tail of chromatin in the direction of the anode. DNA breaks can be assessed from the mobilized tail, since they increase its DNA length and/or mass.

In the sperm chromatin structure assay (SCSA) [3], the older reported procedure, sperm cells are incubated in suspension with an acid solution. This process denatures only the fragmented DNA; after staining with acridine orange, it emits red fluorescence by flow cytometry. Spermatozoa without fragmented DNA, i.e, with undenatured, double-stranded DNA, emit green fluorescence. The acid treatment sometimes is reported to induce DNA breakage in susceptible sites [18, 24]. This is not correct since DNA breaks are natively present before acid treatment, as evidenced through their

behaviour as origins of denaturation, "unwinding", i.e., production of single-stranded DNA by lowering the pH [25]. Low pH is a relatively soft DNA denaturant in comparison with alkaline (≥12) pH. Only abundant DNA breaks promote a significant denaturation by acid treatment under the conditions employed. The short acid incubation of spermatozoa barely produces detectable DNA denaturation in intact sperm. Even DNA from blood leukocytes with histone-nucleosome organized chromatin, much less compacted that that from spermatozoa, is very slightly denatured by the short acid treatment. In fact, 0.24 N HCl for 30 min at 37°C (much stronger conditions than employed in the SCSA and SCD test, 0.08 N HCl) denatures only an estimated 17% of the DNA in a slide [26]. Thus, the concept that enzymatic labelling and comet assay directly detect DNA breaks, while SCSA is an indirect method does not follow, from our point of view. Susceptibility of sperm DNA to acid denaturation is given by DNA breaks, as well as susceptibility to electrophoresic migration or susceptibility to attach nucleotides. All seem indirect methods to evidence DNA breaks, and attaching labelled nucleotides in ISNT or TUNEL also is not direct but mediated by an enzyme.

The sperm chromatin dispersion (SCD) test recently has been reported to be a simple, rapid procedure to determine sperm DNA fragmentation [27]. The spermatozoa embedded in an inert agarose microgel on a slide are incubated in HCl for acid denaturation of only those sperm nuclei with fragmented DNA, followed by treatment in a lysing solution for removal of nuclear proteins to produce a halo of the spread of the nuclear DNA loops. After staining, spermatozoa without fragmented DNA produce nucleoids with large halos, whereas those with fragmented DNA are easily distinguished by a small halo or absence of a halo. The SCD test showed an excellent correlation with SCSA and TUNEL assays [28].

Based on theoretical considerations, TUNEL and ISNT assays require the use of enzymes with potential irregular activity and accessibility to DNA breaks, unlike physicochemical based methods. Only those DNA breaks that have a hydroxyl free group as their 3' end are susceptible to being labelled. Although free 3'-OH are constitutive of DNA breaks originated by nucleases, this is not typical in those breaks produced by ROS, since they usually contain modified ends. The comet assay and SCSA do not have this presumed limitation because they act independently of the chemical nature of the DNA break end, i.e. all DNA break types are detected.

Nevertheless, despite the theory, all seem good procedures to analyze sperm DNA fragmentation in practice, with excellent correlations in comparative studies [28, 29] when adequately and confidently performed. For example, the percentage of TUNEL-positive spermatozoa assessed by flow cytometry has been reported 2.6 times higher than that detected in microscopy [30]. Nevertheless, a correlation between both detections systems is certain.

## Is there a distinction between "real" versus "potential" DNA breaks or a coding versus non-coding location of DNA breakages?

It has been proposed occasionally that the enzymatic tests that incorporate modified nucleotides to DNA break-ends measure "real" DNA breaks, whereas those that require DNA denaturation to detect DNA breaks would measure "potential" DNA breaks [18, 31]. Techniques that evaluate "real" breaks would be more predictive of pregnancy outcome than those that detect "potential" breaks. This idea was based on the misinterpretation that DNA damage in one strand would be of little or no consequence for pronuclei formation after fertilization of the oocyte because DNA is not denatured inside the oocyte due to its neutral pH.

The distinction between "real" and "potential" damage is not logical from biochemical-mutagenesis studies, and, as reported in the previous section, DNA breaks are not produced in susceptible sites secondary to the acid incubation performed in the SCSA or SCD test. Enzymatic and denaturing systems both detect "real" DNA breaks, either through their susceptibility to being a target for the enzymatic polymerisation of nucleotides or by their enhancement of DNA denaturation. From early research in the 70s on alkaline unwinding assays for determination of ionizing radiation-induced DNA breaks, it is suggested that DNA breaks behave as starting points for DNA denaturation [25]. As indicated previously, acid is much less a denaturant than alkali, so only extensive DNA breaks result in significant DNA denaturation after a short incubation, producing short pieces of single-stranded DNA beginning from the ends of the break. Strong and prolonged acid incubation may induce some loss of purines, which does not correspond to any previous "potential" lesion. If it happens, this is irrelevant to the test result since apurinic sites are not transformed into new DNA breaks.

Moreover, DNA is never denatured by pH changes inside any cell, with or without DNA breaks or other lesions, in one or both strands. And obviously, the impairment for pronucleus development has no relationship to DNA denaturation. This impairment should be a consequence of cell-cycle arrest or cell death after recognition of DNA damage.

The only concept in the mutagenesis field that could resemble "potential DNA breaks" is that

of alkali-labile sites. These are "real"-mutagenic DNA lesions (abasic sites and some deoxyribose damages) that alkaline treatment *in vitro* may transform into SSB [32]. This does not happen for the short acid unwinding treatment. Possibly there is confusion about the presumed greater severity, in terms of lethality, of DSB in comparison with SSB or base damages. Nonetheless, enzymatic labelling tests do not distinguish between SSB and DSB, as is the case for procedures that rely on DNA denaturation.

Conceptually, the comet assay performed under non-denaturing conditions is preferentially detecting DSB, although SSB also may contribute in part to migration, due to more relaxation of the chromatin. The alkaline comet assay may be problematic since native intact DNA from deproteinized human sperm nucleus is strongly sensitive to alkaline unwinding, possibly as a result of a specific chromatin-DNA conformation [19]. A variant of the SCD test using only the lysing solution without previous acid unwinding treatment should also preferentially detect DSB. This latter corresponds to a non-denaturing, diffusion-like assay, where massive DSB allow a wide dissemination of the DNA spots or fragments from the residual central core of the sperm head [33]. In any case, a good correlation has always been obtained between electrophoresic, enzymatic and denaturing detection systems, at least with ejaculated sperm [28, 29]. Another proposition is that the majority of DNA breaks would not be dangerous since most of DNA is non-protein coding, meaning that the probability of impact on the exonic regions would be low [18]. This could explain the occurrence of viable pregnancies with high levels of sperm DNA fragmentation. Nevertheless, this is not correct since DSB or other DNA lesions, especially when these are abundant, may trigger cell arrest or an apoptotic pathway after fertilization. Moreover, DNA lesions may be unrepaired or misrepaired, resulting in structural chromosomal aberrations that can lead to cell death in the first or subsequent cell cycles. Apoptosis triggering, misrepair, chromosome aberrations and cell death occur independently of the coding or non-coding nature of the DNA region where the initial DNA lesions were located. As described previously, the relationship between sperm DNA lesions and fertility is a very complex scenario that needs extensive research.

## Is there a threshold for pregnancy and an "iceberg" effect?

Using the SCSA, a threshold for pregnancy has been suggested. Thus, when the frequency of sperm cells with fragmented DNA is ≥30%, the probability of viable pregnancy would be extremely low. An "iceberg effect" has been postulated, suggesting that spermatozoa without fragmented DNA in the presence of spermatozoa with fragmentation also should contain some type of DNA damage not compatible with viable pregnancy [3]. It has been suggested that this could be oxidative base damage like 8-oxoG [18, 31]. Nevertheless, ROS attack results not only in base lesions but also is accompanied by DNA breaks. From this it would be expected that both DNA damage types would be coupled in the same sperm cell. The possibility exists of a low level of DNA damage, practically undetectable by the actual procedures, in sperm cells not positive for DNA fragmentation. However, this small damage would be relevant only in the case of fertilization of an oocyte with defective DNA repair pathways. Otherwise, the threshold for pregnancy has not been found in recent studies with SCSA, many successful pregnancies have been reported with high sperm DNA fragmentation values [34, 35]. It remains to be established whether there are specific groups of patients in whom the presumed "iceberg effect" is operative.

## Comments on clinical results in fertility

Some of the most extensive data to date from a prospective, multi-center study analysing 729 couples using the SCD test [36] support other previous studies [37] demonstrating a correlation between the frequency of sperm cells with fragmented DNA in the sample and the fertilization rate of the oocyte, the embryo quality, blastocyst rate and implantation rate. Although a relationship between frequency of sperm cells with fragmented DNA and pregnancy rate would be expected, it was not manifested. The reason for this is obvious as embryos of better morphological quality, i.e., with better prognosis, are the ones transferred in IVF or ICSI-assisted reproduction procedures. Due to the observed correlation between DNA fragmentation yield and embryo quality, embryos fertilized by sperm containing fragmented DNA were not selected for transfer. Possibly, the influence of sperm DNA fragmentation on pregnancy exists and would be unmasked if embryos were not selectively transferred.

The assessment of sperm DNA damage might be efficient for couples with previous cycle cancellation or ART failure, as well those female partners with poor prognostic criteria such as advanced age, poor response, implantation failure or poor embryo quality in previous cycles. It may also serve as a useful criterion for embryo selection for transfer. In any case, it seems possible that certain specific subgroups of patients could benefit significantly from a sperm DNA integrity assessment. This has been suggested in a recent

systematic review and meta-analysis in which a small but statistically significant association between sperm DNA integrity test results and pregnancy in IVF and ICSI cycles was confirmed [38]. In any case, many different studies have established correlations with different endpoints of fertility (i.e., oocyte fertilization rate, embryo development, embryo quality, blastocyst rate, implantation, pregnancy outcome, abortion rate). The surprising fact is that correlations are diverse in the different studies, even using the same tests, evidencing the complexity of the topic. In animals, probably due to the fact that a strong selection is performed on those species used for reproductive purposes, correlations between a high level of sperm DNA fragmentation and fertility are usually more clearly established. More thorough research needs to be done to address these important questions.

## Why limit sperm DNA fragmentation analysis to fertility assessment?

Perhaps the debate has been excessively focused on the relationship between sperm DNA fragmentation and pregnancy outcome, since most of this research has been performed by groups dedicated to assisted reproduction. Nevertheless, sperm DNA integrity also has an undeniable value in sperm quality assessment in most andrological pathologies, like varicocele, cancer and infections. It provides valuable information on disease severity and therapeutic efficacy, as well as genetic toxicology at the sperm DNA level in population group studies [39, 40]. For example, the determination of the profile of categories of halos obtained with the SCD test has been found to be relevant in samples from subjects with varicocele [41]. These patients exhibited a much higher proportion of sperm nucleoids of the degraded type, i.e., with the stronger nuclear damage level, in the total amount of spermatozoa with fragmented DNA. It would be of interest to expand this study to examine whether this peculiarity is exclusive to varicocele patients. If confirmed, the SCD test would be a valuable tool for diagnostics and follow-up in varicocele.

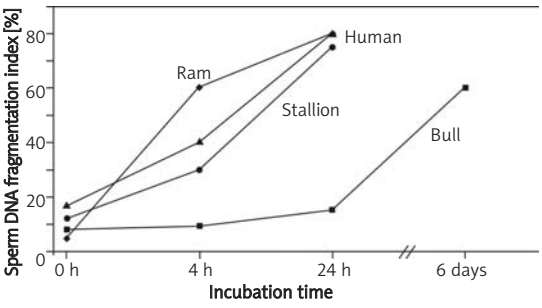


**Figure 1.** Comparative dynamics of sperm DNA fragmentation in frozen sperm samples incubated at 37°C for a variable period of time. Each line represents average of sperm DNA fragmentation from ten individuals for each species. Besides the evident differences for the diverse species, variations were also found for individuals within each species

In addition, oncological patients, mainly with lymphoma, seminoma and other non-seminomatous testicular cancer, exhibit increased frequency of sperm cells with fragmented DNA [42]. This suggests that the presence of cancer, *per se*, may affect sperm quality and DNA fragmentation. Finally, a recent study of samples from patients with genitourinary infection by *Chlamydia trachomatis* and *Mycoplasma* demonstrated a significant increase in the frequency of spermatozoa with fragmented DNA, whereas standard seminal parameters were scarcely affected. Furthermore, antibiotic treatment tended to decrease this condition [43]. Therefore, the determination of sperm DNA fragmentation may be useful to monitor therapeutic effectiveness in genitourinary infections. DNA fragmentation should be considered a parameter of sperm quality, complementary to the conventional evaluations. It should be of great interest to evaluate if the effect of sterilization agents like furacin [44] is partly mediated by induction of DNA fragmentation in sperm cells.

## Is sperm DNA fragmentation a stationary parameter?

Standard seminal parameters like motility and vitality usually are evaluated at different periods of time. Some reports indicate that when sperm DNA fragmentation is assessed over time, i.e., sperm DNA fragmentation dynamics, it is evident that DNA degrades progressively when incubated in semen extenders at 37°C [45]. From a biological viewpoint, this indicates that when the semen sample is used for intrauterine insemination (IUI) or IVF, the level of sperm DNA fragmentation in the sperm sample inoculated or co-incubated with the oocyte may be higher at the time of fertilization than that assessed before the clinical practice. In routine IVF, oocytes are frequently exposed to sperm overnight with a maximum exposure to 24 h. In some cases, this long period of co-incubation has been shown to produce problems in normal embryonic development.

## Should the differential velocity among individuals to produce DNA damage be taken into account at the time of reproductive practice?

The dynamics of sperm DNA fragmentation in terms of velocity of DNA degradation vary among species and among individuals within species [46, 47]. Figure 1 shows the differences in the tendency to increase sperm DNA damage in different mammalian species. In some cases such as human, stallion or ram, some individuals may double the basal level of sperm DNA determined before ejaculation in only 5 h, while in other species such as boar or bull this effect is

delayed even for some days. This specific aspect of DNA damage deserves more attention for two main reasons: 1) the actual level of sperm DNA fragmentation when the sperm has penetrated the cell membrane of the oocyte could be higher than that determined immediately after ejaculation or thawing; and 2) the comparison of the results from different laboratories or even those obtained within the same laboratory may be biased if clear references to the time that have passed from ejaculation or thawing to analysis are not given.

## Conclusions

Fertility is a multi-factorial phenomenon that usually involves both members of the couple, and assessment of sperm DNA integrity is only one piece of a complex puzzle. Tests that assess sperm quality should not only identify the ability of spermatozoa to reach the oocyte but also their ability to fertilize the oocyte and activate embryo growth, i.e., not only the carrier but also the content. Sperm DNA fragmentation should be considered a parameter of sperm quality. Moreover, its determination may provide beneficial information in andrological pathology, complementary to that from standard seminal parameters and must be evaluated concurrently and examined within the clinical context of each patient or couple.

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