Apoptotic sperm biomarkers and the correlation between conventional sperm parameters and clinical characteristics

R. Hichri1 | H. Amor1 | M. Khammari1 | M. Harzallah1 | S. El Fekih1 | A. Saad1 | M. Ajina2 | H. Ben Ali1

1Department of Cytogenetics, Genetics and Molecular Biology of Human Reproduction, CHU Farhat Hached, Sousse, Tunisia
2Reproductive Medicine Unit, CHU Farhat Hached, Sousse, Tunisia

Correspondence
Raja Hichri, Department of Cytogenetics, Genetics and Molecular Biology of Human Reproduction, CHU Farhat Hached, Sousse, Tunisia.
Email: hichriraja@yahoo.fr

Summary
The principal aim of this retrospective study was to examine the relationship between sperm apoptotic biomarkers and the patient’s biclincal characteristics, the conventional sperm parameters and the results of assisted reproductive technology. Sperm analysis, activated caspases, annexin V staining for phosphatidylserine (PS) externalisation and labelling assay for DNA fragmentation were assessed in 122 males of infertile couples. Fifty-seven couples were allocated to the natural conception group, and 65 couples underwent IVF or ICSI. Semen of IVF/ICSI patients showed a higher proportion of apoptotic spermatozoa in their spermatozoa when compared with a natural conception group (p < .05). Sperm apoptotic biomarkers correlated with age, FSH, and conventional sperm parameters. DNA fragmentation correlated positively with the percentage of semen having externalised PS (r = .78, p = 0) and activated caspases (r = .71, p = 0). Patients without clinical pregnancy had higher frequency of DNA fragmentation, externalised PS and activated caspases compared to patients with clinical pregnancy (p < .001). The best specificity and greater sensitivity were obtained with the test of the DNA fragmentation compared to the other biomarkers. Among the apoptotic biomarkers, only DNA fragmentation was found to predict natural or assisted pregnancy better than conventional sperm parameters.

KEYWORDS
apoptotic sperm biomarkers, conventional sperm parameters, IUI, patient clinical parameters, spontaneous pregnancy

1 | INTRODUCTION
Apoptosis is a complex process in which cell death signalling and regulatory pathways have been described exhaustively in somatic cells (Horvitz, 1999). Ultrastructural changes and expressions of typical markers of apoptosis have been described in human ejaculated spermatozoa (Baccetti, Collodel, & Piomboni, 1996). Although the germ cell apoptosis is necessary for the establishment and maintenance of normal spermatogenesis in the testes, the existence of apoptosis, which is an "active" cell death in spermatozoa, remains controversial (Lachaud, Tesarik, Canadas, & Mendoza, 2004). In effect, spermatozoa are highly differentiated cells which exhibit little transcriptional activity. However, many studies have shown a higher proportion of spermatozoa expressing apoptosis markers in the ejaculation of infertile patients, compared to fertile men (Said, Paasch, Glander, & Agarwal, 2004; Weng et al., 2002). This result is a testicular apoptosis initiated and aborted (Sakkas, Mariethoz, & St John, 1999), an apoptosis initiated and/or continued in the male genital tract (Grunewald et al., 2005). The meaning of the expression of markers of...
apoptosis in these cells is not clearly defined today. In addition, methods for measuring markers of apoptosis in somatic cells, particularly of activated caspases, were transposed human spermatozoa without prior approval. However, for sperm samples, the realisation of these measures shows some challenges such as the low amount of spermatozoa available, particularly in cases of oligozoospermia, and heterogeneous cell populations. To evaluate the functional quality of the sperm, we sought the relationship between the expression of these markers, sperm parameters and the results in assisted reproductive technology. The processes of apoptosis and stigma were assessed by measuring the spermatozoa of several biochemical markers of apoptosis (activated caspases, externalisation of PS and DNA fragmentation) in order to define the best marker for apoptosis and to optimise the management of infertile patients especially in assisted reproductive technology.

2 | MATERIALS AND METHODS

2.1 | Patients

We studied 122 male subjects who underwent seminal fluid evaluation at the Laboratory of cytogentic, molecular genetic and human reproductive biology CHU FarhatHached Sousse Tunisia. All subjects were the partners of women who had failed to conceive after 1 year of unprotected regular sexual intercourse. Patients’ information remained confidential and within the institution, and a written consent for the treatment was obtained from all patients. This study was conducted according to guidelines established for research on human subjects (Ethical committee, CHU FarhatHached Sousse).

2.2 | Study selection and inclusion criteria

The subjects were all nonsmokers, not using any medication and abstained from alcohol. The patients were ascertained to be in good health by means of their medical histories and a clinical examination including routine laboratory test and screening. Men with infection, varicoceles upon physical examination or azoospermia were excluded.

Women with an indication for IVF, such as tubal infertility, severe male factor infertility, severe endometriosis, unexplained infertility, immune infertility and anovulatory infertility, were also excluded. The inclusion criteria for the female partner were as follows: female age <40 years and female body mass index (BMI) <30 kg/m².

Individuals were divided into two groups:

- The normal semen group in which females were able to conceive naturally or after IUI constituted the “Natural conception group” (n = 57).
- The abnormal semen group (IVF/ICSI group) consisted of 65 couples: the cause of infertility was oligoasthenoteratozoospermia.

2.3 | Collection of semen samples

The samples from 122 subjects were collected by masturbation into sterile plastic jars, after 3 days of sexual abstinence. The basic semen parameters were evaluated according to the World Health Organization guidelines, a sperm concentration of $>15 \times 10^6$/ml, $>4\%$ morphologically normal cells, $>40\%$ motile spermatozoa (categories “a”, “b”, and “c”), and $>58\%$ alive spermatozoa (World Health Organisation, 2010).

2.4 | Detection of activated caspases in spermatozoa

FAM-FLICA® Caspase Assay Kit is commercially available as a component of the Fluorescein Caspase Activity Kit. Polycaspase FLICA® probe, FAM-VAD-FMK, is used as a general reagent to detect apoptosis as it is recognised by all types of activated caspases. Noncytotoxic fluorescent caspase inhibitor (FLICA) binds covalently and irreversibly to many activated caspases (caspase 1, 3, 4, 5, 6, 7, 8 and 9; Ekert, Silke, & Vaux, 1999). The fluorogenic substrate becomes fluorescent upon cleavage by the caspases (Vaux & Korsmeyer, 1999).

As specified in the kit, the FAM-VAD-FMK inhibitor was dissolved in dimethyl sulphoxide (DMSO) to obtain a 150× stock solution. Aliquots of this solution were stored at $-20\degree$C in the dark for 6 months protected from light. Before use, a 30× FLICA working solution was prepared by diluting the stock solution 1:5 in 200 μl PBS. For labelling $3 \times 10^5$ spermatozoa (dilute it to a total volume of 300 μl of PBS), it was incubated with 10 μl of 30× working solution for 1 hr at 37°C under 5% CO2 with tubes protected from light. Cells were washed twice with 1× wash buffer (PBS containing 0.5% bovine serum albumin and 0.05% sodium azide). Cell pellet was resuspended in 400 μl 1× wash buffer and incubated in 2 μl of propidium iodide (PI) for 15 min, protected from light at RT in order to assess viability. Finally, the nuclei of spermatozoa were stained with 2 μl of Hoechst 33,342 (Sigma) for 5 min, followed by washing and resuspension of cells in wash buffer.

Cells were observed under a fluorescence microscope using a band-pass filter (excitation 490 nm, emission $>610$ nm) to view green fluorescence.

A minimum of four hundred spermatozoa were randomly assessed per slide in at least five fields. Four patterns of fluorescence were measured:

1. Viable spermatozoa stained only with Hoechst, without staining of activated polycaspases: Casp−/PI− (blue).
2. Dead spermatozoa without staining of activated polycaspases: Casp−/PI+ (red and blue).
3. Viable spermatozoa with staining of activated polycaspases (early apoptotic spermatozoa): Casp+/PI− (blue and green).
4. Dead spermatozoa with staining of activated polycaspases (late apoptotic spermatozoa) Casp+/PI+ (blue, red and green).

2.5 | Annexin V binding assay

Translocation of PS to the outer leaflet of the plasma membrane was detected by annexin V–fluorescein isothiocyanate (FITC; Sigma, France). PS is normally found on the cytoplasmic face of the plasma membrane, and translocation to the extracellular leaflet involves a plasma membrane alteration. As the nuclear and plasma membranes of dead cells are also damaged, the assay includes staining with the DNA stain propidium
iodide (PI; Sigma) to assess viability. As cryopreservation is deleteri-
ous to the plasma membrane, this technique was only applied on fresh
sperm. By fluorescence microscopy, annexin V–FITC was observed as
green fluorescence and PI as red. For this assay, 1 μl of annexin V–FITC
(Sigma, France) was freshly mixed with 0.5 μl PI in 100 μl buffer solution
(10 mmol/L HEPES, 150 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl₂,
1.8 mmol/L CaCl₂) according to the instructions provided by the manu-
facturer (Sigma). An equal volume of this preparation was gently added
to the sperm aliquot, and the mixture was incubated in the dark, at room
temperature, for 15 min. Analysis of the sample was performed by con-
ventional epifluorescent microscopy (emission: 525–617 nm). At least
200 spermatozoa were randomly assessed per slide in a minimum of
five fields. As mentioned in the literature, the intra-observer and inter-
observer variabilities were <6% for this technique (Barroso, Morshedi,
& Oehninger, 2000). Four patterns of fluorescence were observed: (i) vi-
able spermatozoa with externalised PS (green) that stained only with an-
nexin V–FITC (AN+PI−) = apoptosis-live; (ii) dead spermatozoa without
externalised PS (red) that stained only with PI (AN−PI+) = intact-dead;
(iii) dead spermatozoa with externalised PS (red and green) that stained
both with annexin V–FITC and PI (AN+PI+) = apoptosis-dead; (iv) intact
spermatozoa without any staining (AN−PI−) = intact-live.

2.6 | TUNEL reaction

DNA fragmentation in spermatozoa was detected by the “in-situ cell
death detection kit” (Roche, Belgium) according to the instructions
of the manufacturer, as described by Stouffs et al. (2004). Ejaculated
sperm cells were washed twice in 5 ml of phosphate-buffered saline
(PBS) and once in 1 ml PBS, followed by at least one night and a maxi-
mum of 48-hr incubation at −20°C in acetic acid:methanol (1:3). The
samples were spread on polylysine slides, and the cells were permea-
bilised in 0.1 mol/L sodium citrate for 30 min at 70°C, incubated in the
TUNEL reaction mixture for 1 hr at 37°C after which the detection with
converter-alkaline phosphatase and nitroblue tetrazolium-5-bromo-4-
chloro-3-idoly phosphate (NBT-BCIP) (Roche) was performed. Slides
were counterstained by methyl green and analysed by light micros-
copy (data analysis performed on at least 400 spermatozoa). For each
test, positive (DNase: 2 UI/ml) and negative controls were included.

The degree of DNA fragmentation is measured with DNA frag-
mentation index (DFI).

2.7 | Ovarian stimulation and IUI or ICSI procedure

Women in IUI group received r-FSH and monitoring was performed
with urine LH surge along with TVS until hCG administration.
Ovulatory trigger with urinary hCG 5,000 IU was administered intra-
muscularly when there was at least one follicle of 18 mm or more.
Semen was prepared for insemination by double-density gradient
 technique. A single IUI was performed between 44 and 48 hr after
hCG injection.

A maximum of three cycles of IUI were performed.

Women in IVF/ICSI group underwent superovulation using a
gonadotropin-releasing hormone analogue suppression protocol and
recombinant gonadotropins (FSH per stimulated cycle varied between
1,800 and 3,500 UI depending on the individual response). As soon as
at least three follicles of R18 mm were detected, ovulation was induced
by 10,000 IU of human chorionic gonadotropin (hCG). Oocyte–cumu-
lus complexes were recovered 36 hr after administration of hCG. After
cumulus cell removal by treatment with 25 IU hyaluronidase (Origio,
Berlin, Germany), followed by pipetting to remove the innermost layer
of cells, the oocytes were then rinsed three times in flushing medium
(Origio, 10,845,060) and the morphology of the metaphase II oocytes
was evaluated at the time of ICSI (from 2 to 4 hr after retrieval) under
inverted microscope at ×400 magnification. Only metaphase II oocytes
with normal and clear zona pellucida, small size of perivitelline space,
without vacuoles or cytoplasmic granularity and normal polar body
were injected with motile spermatozoa into the ooplasm according to
previously described procedures (Bungum et al., 2004).

Around 18–20 hr post-ICSI, fertilisation was assessed by the pres-
ence of pro-nuclei. The fertilisation rate was calculated from the ratio
of fertilised oocytes to the total number of viable injected metaphase
II oocytes. Segmentation rate was calculated from the ratio of cleaved
embryos to the number of fertilised oocytes. Embryo quality was as-
essed at day 3 post-oocyte retrieval. The nonfragmented embryos
with eight regular blastomeres were considered the best quality and
named type I embryos. On day 3, type I embryos were transferred
after a nonselective quarter laser-assisted hatching technique, using
a solid-state diode laser (ZILOS-tK; Hamilton Thorne Biosciences,
Beverly, MA, USA) in G-2. Pregnancy rate was defined from positive
blood HCG and ultrasonography findings showing at least one embryo
with a foetal heart beat 5 weeks after transfer.

2.8 | Statistical analysis

Statistical analysis was performed using SPSS 19.0 software. All vari-
ables were initially tested to determine variance homogeneity and data
normality. The values are expressed as mean ± mean ± SD (range) or
percentage (%). The Student’s t test was used for the comparison of
percentages between IVF/ICSI group and natural conception group.
Pearson’s correlation was performed to examine the relationship be-
tween sperm apoptotic markers and, respectively, male age, BMI (body
mass index), FSH (follicle-stimulating hormone), conventional sperm
parameters and the achievement of the natural outcome of the first
IVF/ICSI attempt (fertilisation rate, segmentation rate, of type I em-
bryos and pregnancy). A receiver-operating characteristic (ROC) curve
was used to calculate the area under the ROC curve (AUC) to deter-
mine the optimal threshold for the prediction of pregnancy with the
best sensitivity and specificity. Statistical significance was set at p < .05.

3 | RESULTS

3.1 | Clinical characteristics and conventional sperm
parameters

Bioclinical characteristics and conventional sperm parameters of the
natural conception group (n = 57) and IVF/ICSI group (n = 65) are
presented in Table 1. We found statistically significant differences between the natural conception group and the IVF/ICSI group with regard to both conventional sperm parameters (concentration, motility \([a + b]\) and atypical forms) and FSH. However, clinical characteristics values did not appear to be significantly different between the two treatment groups.

### 3.2 Comparison of apoptotic sperm biomarkers between natural conception group and IVF/ICSI group

Results of apoptotic marker analysis in semen of IVF/ICSI patients and natural conception group (control) are presented in Table 2. An average of 500 spermatozoa per subject were counted. As mentioned, semen of IVF/ICSI patients showed a higher proportion of both viable and dead spermatozoa with activated caspases (respectively, 39.21% versus 19.01% and 28.65 versus 21.85) viable and dead spermatozoa with externalised PS (respectively, 21.26% versus 14.68 and 25.39 versus 9.82%) and DNA fragmentation (18.46% versus 6.22%) in their spermatozoa when compared with natural conception group \((p < .05)\). Moreover, intact viable spermatozoa (viable spermatozoa without activated caspases or externalised PS) were more frequent in males with altered spermatozoa (IVF/ICSI patients) than in those with normal spermatozoa \((p < .05)\). However, the rate of intact-dead spermatozoa (dead spermatozoa without externalised PS or activated caspases) did not differ significantly from IVF/ICSI patients and control (respectively, \(p = .98\) and \(p = .45\)). The detailed results of the correlation analyses between apoptotic markers and both clinical and bioclinical parameters in ejaculated sperm samples of IVF/ICSI patients and potential fertile patients are given in Table 3. Viable spermatozoa with activated caspases correlated positively with age \((r = .23; p = .013)\) and abnormal forms \((r = .56; p = .00)\) and negatively with FSH \((r = -.15; p = .04)\), sperm count \((r = -.41; p = .00)\) and motility \([a + b]\) \((r = -.49; p = .00)\). Viable spermatozoa having externalised PS \((AN+, PI−)\) correlated positively with the percentage of spermatozoa with abnormal forms \((r = .25; p = .005)\) and negatively with FSH \((r = -.23; p = .02)\) and sperm motility \([a + b]\) \((r = -.42; p = .00)\). DNA fragmentation correlated positively with age \((r = .24; p = .008)\) and percentage of abnormal forms \((r = .56; p = .00)\) and negatively with FSH \((p = .04, r = -.33; p = .04)\), sperm concentration \((r = -.27; p = .002)\) and sperm motility

| TABLE 1 Bioclinical characteristics and semen analysis results for natural conception group and IVF/ICSI group |
|-------------------------------|-------------------------------|-----------------|
| Natural conception group \(n = 57\) | IVF/ICSI group \(n = 65\) | p-value       |
| Male age (years)               | 34.58 ± 5.7                   | 36.97 ± 6.35    | .06             |
| Duration of infertility (years)| 2.6 ± 2.4                     | 5.8 ± 3.5       | .65             |
| BMI (kg/m²)                    | 25.36 ± 4.6                   | 24.84 ± 5.27    | .57             |
| FSH (IU/L)                     | 7.2 ± 3.98                    | 4.7 ± 1.24      | .00*            |
| Volume (ml)                    | 3.29 ± 0.96                   | 2.92 ± 1.26     | .1              |
| Concentration \((×10⁶/ml)\)    | 185 ± 92.84                   | 48.07 ± 39.67   | .00*            |
| Motility a + b (%)             | 64.58 ± 17.19                 | 24 ± 14.23      | .00*            |
| Atypical forms (%)             | 42.6 ± 26.32                  | 92.31 ± 6.4     | .00*            |

BMI, Body Mass Index.
*Show significantly different for \(p < .01\).

| TABLE 2 Measurements of proportion of spermatozoa with activated caspases, externalisation PS and DNA fragmentation in ejaculated spermatozoa from IVF/ICSI patients and control group (IUI group) |
|-------------------------------|-------------------------------|-----------------|
| Caspase assays                | IUI group \(n = 57\)         | IVF/ICSI group \(n = 65\) | p-value       |
| Casp+/PI− (%)                 | 19.01 ± 13.01                 | 39.21 ± 21.3     | .00**          |
| Casp+/PI+ (%)                 | 21.85 ± 16.1                  | 28.65 ± 15.76    | .025*          |
| Casp−/PI+ (%)                 | 7.02 ± 4.5                    | 7.7 ± 5.49       | .45            |
| Casp−/PI− (%)                 | 50.92 ± 24.47                 | 22.10 ± 19.45    | .000*          |
| Annexin V binding assay       | AN+/PI− (%)                   | 14.68 ± 12.40    | .023*          |
| AN+/PI+ (%)                   | 9.82 ± 9.19                   | 25.39 ± 14.57    | .000**         |
| AN−/PI+ (%)                   | 12.04 ± 6.71                  | 12.03 ± 6.7      | .98            |
| AN−/PI− (%)                   | 64.02 ± 17.95                 | 41.05 ± 21.6     | .001**         |
| TUNEL                         | DFI (%)                       | 6.22 ± 4.67      | 18.46 ± 12.9   | .000**         |

AN+/PI− = viable spermatozoa with externalised PS, apoptosis-live; AN+/PI+ = dead spermatozoa with externalised PS, apoptosis-dead; AN−/PI+ = dead spermatozoa without externalised PS, intact-dead; AN−/PI− = intact sperm, intact-live. Casp+/PI− = viable spermatozoa with activated caspases, apoptosis-live; Casp−/PI− = dead spermatozoa with activated caspases, apoptosis-dead. Casp−/PI+ = dead spermatozoa without activated caspases, intact-dead; Casp−/PI− = intact sperm, intact-live; PI = propidium iodide; DFI: DNA fragmentation index.

*Show significantly different for \(p < .05\). **For \(p < .01\).

\(r = -.61; p = .00\). Correspondingly, a highly significant \((p < .001)\) correlation was observed between apoptotic dead spermatozoa (Casp+/PI− or AN−/PI+) and, respectively, sperm motility and percentage of atypical forms. The percentage of normal viable spermatozoa (Casp−/PI− or AN−/PI−) showed a significant correlation with age, FSH, sperm concentration, motility and abnormal forms \((p < .05)\). In contrast, no correlation was found between, respectively, the percentage of apoptotic sperm, normal viable sperm, dead sperm, DNA fragmentation, and both BMI and semen volume \((p > .05)\). The percentage of spermatozoa showing signs of apoptosis showed strong and positive correlations
Correlations between apoptotic markers and clinical, hormonal and conventional sperm parameters of all patients (n = 122)

| Caspase assay | Age (%) | BMI | FSH | Volume | Concentration (×10^6/ml) | Sperm motility (% | Atypical forms |
|---------------|---------|-----|-----|--------|---------------------------|------------------|---------------|
| Casp+/PI− (%) | p = .013 | NS  | p = .04 | NS  | p = .00 | p = .00 | p = .00 |
|              | r = .23*|     | r = −.15*| NS | r = −.41** | r = −.49** | r = .56** |
| Casp+/PI+ (%) | NS      | NS  | NS  | NS  | p = .00 | p = .00 | p = .046 |
|              |         |     |     |     | r = −.3** | r = −.18* |             |
| Casp−/PI+ (%) | NS      | NS  | NS  | NS  | NS  | NS  | NS  |
| Casp−/PI− (%) | p = .016 | NS  | p = .003 | NS  | p = .47** | r = .54** | r = −.62** |

Annexin V binding assay

| AN+/PI− (%) | NS | NS | NS | NS | p = .018 | p = .00 | p = .005 |
|            |    |    |    |    | r = −.23* | r = −.11* | r = .25** |
| AN+/PI+ (%) | p = .019 | NS | p = .000 | NS | p = .00 | p = .00 | NS  |
|              | r = .23* |    | r = −.45** |    | r = −.63** |     | r = .53** |
| AN−/PI+ (%) | NS  | NS | NS  | NS  | NS  | NS  | NS  |
| AN−/PI− (%) | p = .014 | NS | p = .000 | NS  | p = .46** | r = .68** | NS  |
|              | r = −.26* |    | r = −.25** |    | r = −.49** |     |     |

TUNEL

| DFI (%) | NS | NS | NS | NS | p = .002 | p = .00 | p = .00 |
|---------|----|----|----|----|----------|--------|--------|
|         | p = .008 |     | p = .04 |     | p = .22** | r = −.33* | r = .56* |

AN+/PI− = viable spermatozoa with externalised PS, apoptosis-live; AN+/PI− = dead spermatozoa with externalised PS, apoptosis-dead; AN−/PI+ = dead spermatozoa without externalised PS, intact-dead; AN−/PI− = intact sperm, intact-live. Casp+/PI− = viable spermatozoa with activated caspases, apoptosis-live; Casp+/PI− = dead spermatozoa with activated caspases, apoptosis-dead. Casp−/PI+ = dead spermatozoa without activated caspases, intact-dead; Casp−/PI− = intact sperm, intact-live; PI = propidium iodide; DFI, DNA fragmentation index.

*p Significantly correlation for p < .05.
**For p < .01.

with each other. In fact, DNA fragmentation was significantly positively associated with, respectively, the percentage of dead and viable spermatozoa with externalised PS (r = .78, p = 0) and dead and viable spermatozoa with activated caspases (r = .71, p = 0) (Table 4).

3.3 | Relationship between apoptotic biomarkers and the outcome of IUI and IVF/ICSI

Overall, the 57 couples underwent IUI; there were 12 pregnancies (21.1%) of which five were ongoing. There was no difference in male bioclinical characteristics and conventional spermatozoa (p > .05) when couples with positive and negative reproductive outcome were compared. However, the analysis of apoptotic markers in semen of patients with and without clinical pregnancy showed that patients without clinical pregnancy had significantly higher frequency of viable apoptotic spermatozoa with activated caspases (p = .008), externalised PS (p = .004) and DNA fragmentation (p = .038), in their spermatozoa when compared with patients with clinical pregnancy (p < .001).

Correlation between conventional sperm parameters, apoptotic markers according to fertilisation, segmentation, type I embryos rate and pregnancy in the IVF/ICSI group is given in Table 5. In the whole group of IVF/ICSI, the rate of fertilisation, segmentation and type I embryos decrease with DNA fragmentation (p < .001). Viable apoptotic spermatozoa with externalised PS (AN+/PI−) correlates negatively with both fertilisation (r = −.33; p = .01) and segmentation rate (r = −.37; p = .03) while viable spermatozoa with activated caspases (Casp+/PI−) correlates only with the fertilisation rate (r = .46; p = .00). A positive correlation was also found between the percentage of viable intact spermatozoa (Casp−/PI− or AN−/PI−) and, respectively, fertilisation, segmentation and type I embryos rate. However, no correlation was obtained between dead spermatozoa and fertilisation, segmentation and embryos development. In addition, patients without clinical pregnancy had significantly higher frequency of, respectively, percentage of a typical forms, DNA fragmentation and viable and dead spermatozoa with activated caspases and externalised PS.

ROC analysis (Figure 1) showed that DFI >15.5% (DFI; AUC = 0.79 with 100% sensitivity, 74.7% specificity) and AN+ >42% (AN+; AUC = 0.72 with 87% sensitivity, 81% specificity) and AN+ >42% (AN+; AUC = 0.7 with 70% sensitivity and 60% specificity) were reliable predictors of pregnancy (p < .001). In addition, DNA fragmentation had the best predictive value in pregnancy when compared with the other apoptotic markers.

4 | DISCUSSION

As far as we know, our study is the first research that combines and compares three different apoptotic tests to analyse fresh semen of
IFV/ICSI patients who did not have any identifiable systemic or anatomical causes of sperm parameter abnormalities. When compared with control (natural conception group), the first test used in this study evaluated the integrity of plasma membrane (annexin V binding assay), the second showed the cytoplasm quality (caspase assays) and the last one gave information about the DNA fragmentation (TUNEL).

Our results confirm that infertile patients had lower sperm density, motile spermatozoa and normal forms, compared with the control group. Additionally, semen of patients with lowered fertilising ability contained higher percentages of spermatozoa with apoptosis markers. These findings are in accordance with the results of others studies (Grunewald, Said, Paasch, Glander, & Agarwal, 2008; Marchetti, Gallego, Defossez, Formstecher, & Marchetti, 2004).

This study shows that sperm apoptotic markers are correlated positively with age, and that apoptosis at least in part plays an important role in the ageing process and age-related infertility in men. Previous reports demonstrated the association between advancing age and the expression of early apoptotic markers as evidenced by the significant increase in plasma membrane translocation of PS, as well as with a more subtle proportion of spermatozoa carrying DNA fragmentation (Moskovtsev et al., 2010; Wyrobek et al., 2006). Moreover, it has been shown that apoptosis increases with age, producing an accelerated germ cell loss (Kimura et al., 2003), related to the falling androgen levels and/or an increase in oxidative stress in the tissue (Samanta, Sahoo, & Chainy, 1999).

In the current study, we have not shown a correlation between BMI and the percentage of apoptotic cells. This is not in accordance with the results obtained by others who found that men with BMI ≥30 kg/m2 had more apoptotic, fewer normal viable spermatozoa and lower percentage of spermatozoa with normal mitochondrial

| Bioclinical characteristics | Natural conception group (n = 57) | 18/31.5% |  |  |  |
|-----------------------------|-----------------------------------|----------|---|---|---|
| Pregnancy                   | No Pregnancy                      | p-value* |
| Age                         | 34.5 ± 5.4                        | 34.9 ± 4.8 | .8 |
| BMI                         | 25.88 ± 4.7                       | 24.2 ± 4.42 | .23 |
| FSH                         | 4.7 ± 1.2                         | 4.8 ± 1.3 | .86 |
| Conventional sperm parameters|                                   |          |   |   |   |
| Volume                      | 3.3 ± 1.03                        | 3.1 ± 0.7 | .63 |
| Sperm concentration         | 211.75 ± 97.86                    | 148.8 ± 72.5 | .007** |
| Atypical forms              | 42.92 ± 26.82                     | 42.06 ± 26.75 | .91 |
| Motility a + b (%)          | 66.5 ± 17.08                      | 61.17 ± 17.46 | .28 |
| Caspase assay               |                                   |          |   |   |   |
| Casp+/PI− (%)               | 16.72 ± 12.61                     | 29.8 ± 17.94 | .008** |
| Casp+/PI+ (%)               | 16.47 ± 13.96                     | 22.95 ± 10.52 | .065 |
| Casp+/PI− plus Casp+/PI+ (%)| 32.58 ± 21.02                     | 49.96 ± 18.13 | .003** |
| Casp−/PI+ (%)               | 6.08 ± 4.5                        | 8.49 ± 4.22 | .06 |
| Casp−/PI− (%)               | 60.54 ± 25.15                     | 39.12 ± 18.3 | .003** |
| Annexin V binding assay     |                                   |          |   |   |   |
| AN+/PI− (%)                 | 10.5 ± 9.5                        | 20.04 ± 13.75 | .004* |
| AN+/PI+ (%)                 | 9.54 ± 9.66                       | 10.20 ± 8.7 | .78 |
| AN+/PI− plus AN+/PI+ (%)    | 20.04 ± 16.45                     | 29.83 ± 13.7 | .018* |
| AN−/PI+ (%)                 | 12.47 ± 7.52                      | 11.46 ± 5.5 | .56 |
| AN−/PI− (%)                 | 68.1 ± 18.6                       | 58.4 ± 15.57 | .038* |
| TUNEL                       |                                   |          |   |   |   |
| DFI (%)                     | 7.94 ± 3.9                        | 16.83 ± 4.8 | .038* |

AN+/PI− = viable spermatozoa with externalised PS, apoptosis-live; AN+/PI− = dead spermatozoa with externalised PS, apoptosis-dead; AN−/PI+ = dead spermatozoa without externalised PS, intact-dead; AN−/PI− = intact sperm, intact-live. Casp+PI− = viable spermatozoa with activated caspases, apoptosis-live; Casp+PI− = dead spermatozoa with activated caspases, apoptosis-dead. Casp−/PI+ = dead spermatozoa without activated caspases, intact-dead; Casp−/PI− = intact sperm, intact-live; PI = propidium iodide; DFI, DNA Fragmentation Index.

*Significantly different for p < .05.

**For p < .01.
membrane potential (MMP) than men with BMI <30 kg/m² (Chavarro, Toth, Wright, Meeker, & Hauser, 2010; Zorn, Golob, Ihan, Kopitar, & Kolbezen, 2012). Our study revealed a positive correlation between FSH and the percentage of spermatozoa with externalisation PS, activated caspases and nuclear DNA damage. Moreover, we found a negative correlation between FSH and viable spermatozoa without externalised PS or activated caspases. Other studies also found that DNA fragmentation and sperm plasma membrane alterations decrease with FSH (Ruwanpura, McLachlan, Matthieson, & Meachem, 2008; Zorn et al., 2012); however, the relationships between FSH and activated caspases in spermatozoa have never been studied before. The correlation between apoptosis and FSH could be explained by the role of FSH, the main hormonal regulator of spermatogenesis that plays an important role in germ cell survival. Indeed, it has been shown that both extrinsic and intrinsic apoptotic death pathways are operative in the germ cells following the decrease in FSH and testosterone levels. Therefore, FSH and testosterone maintain spermatogenic homoeostasis by inhibiting death signals for the germ cells (Pareek, Joshi, Sanyal, & Dighe, 2007; Shaha, 2008).

Using a triple test measuring semen apoptosis, our study confirms that apoptotic markers are suitably sensitive to analyse the functional capacity of human spermatozoa. In addition, we have observed a highly significant positive correlation between conventional sperm parameters, dead sperm, apoptotic spermatozoa with activated caspases or externalised PS, apoptotic-dead. Casp-/PI− = dead spermatozoa without activated caspases, intact-dead; Casp-PI− = viable spermatozoa with externalised PS, apoptosis-live; Casp-/PI− = dead spermatozoa with activated caspases, apoptosis-dead. Casp-/PI− = dead spermatozoa without activated caspases, intact-dead; Casp-PI− = intact sperm, intact-live; PI = propidium iodide; DFI, DNA Fragmentation Index.

| TABLE 5 Correlation between conventional sperm parameters, apoptotic markers according to fertilisation, segmentation, type I embryos rate and Pregnancy in the IVF/ICSI group |
|-----------------------------------------------|
| IVF/ICSI group (n = 65) | Fertilisation rate | Segmentation rate | Type I embryos % | Pregnancy |
|-----------------------------------------------|
| Pregnancies (n/%) | 9/13.84 |
| Bioclinical characteristics |  |  |  |  |
| Age | NS | NS | NS | NS |
| BMI | NS | NS | NS | NS |
| FSH | NS | NS | NS | NS |
| Conventional sperm parameters |  |  |  |  |
| Volume | NS | NS | NS | NS |
| Sperm concentration (%) | NS | NS | NS | NS |
| Atypical forms (%) | NS | NS | NS | NS |
| Motility a + b (%) | NS | NS | NS | NS |
| Caspase assay |  |  |  |  |
| Casp+/PI− (%) | p = .00, r = −.46* | NS | NS | p* = .048 |
| Casp+/PI+ (%) | NS | NS | NS | NS |
| Casp−PI+ (%) | NS | NS | NS | NS |
| Casp−/PI− (%) | p = .00, r = .49* | p = .016, r = .29* | p = .003, r = .27** | p* = .024 |
| Annexin V binding assay |  |  |  |  |
| AN+/PI− (%) | p = .01, r = −.33* | p = .03, r = −.37* | NS | p* = .005 |
| AN+/PI+ (%) | NS | NS | NS | p* = .008 |
| AN−/PI− (%) | NS | NS | NS | NS |
| AN−/PI− (%) | NS | NS | NS | NS |
| TUNEL |  |  |  |  |
| DFI (%) | p = .00, r = −.59** | p = .01, r = −.52** | p = .001, r = −.58** | p* = .001 |

AN+/PI− = viable spermatozoa with externalised PS, apoptosis-live; AN+/PI− = dead spermatozoa with externalised PS, apoptosis-dead; AN−/PI− = dead spermatozoa without externalised PS, intact-dead; AN−/PI+ = intact sperm, intact-live; Casp+PI− = viable spermatozoa with activated caspases, apoptosis-live; Casp+/PI− = dead spermatozoa with activated caspases, apoptosis-dead. Casp−/PI− = dead spermatozoa without activated caspases, intact-dead; Casp−/PI− = intact sperm, intact-live; PI = propidium iodide; DFI, DNA Fragmentation Index.

*Significantly correlation for p < .05.
**For p < .01.
*aSignificantly different for p < .05.
In our study, caspases activation and PS externalisation appear to have a negative association with fertilisation and embryo development. Previous studies have shown that successful fertilisation requires also sperm plasma membrane with normal integrity (Cincik et al., 2007; Flesch & Gadella, 2000) and the numerous functions of the membrane are related to cell metabolism, for maintaining sperm motility, capacitation, acrosome reaction and sperm–oocyte interaction (Cross & Hanks, 1991).

It is known that assisted reproductive technologies (ART) have provided a treatment choice for many couples with unexplained infertility; however, the current success rates of these procedures remain suboptimal. One potential reason for these low rates may be the inclusion of apoptotic spermatozoa during intracytoplasmic sperm injection (ICSI) (Seli et al., 2004).

Elimination of the apoptotic spermatozoa before ART may be a safe method resulting in higher fertilisation rate and embryo quality in patients diagnosed with infertility.

5 | CONCLUSION

The positive correlation between respective semen with activated caspases and externalised PS and DNA fragmentation suggested that cytoplasm and plasma membrane integrity should be considered as a direct indicator of DNA integrity. In the light of these findings, apoptotic markers may replace conventional sperm parameters and should be taken into account when assessing male fertility potential and decision on the type of assisted reproduction technology.

ACKNOWLEDGEMENTS

The authors give special thanks to the technical staff of the Reproductive Medicine Unit of the University Hospital of FarhatHached, Sousse, particularly to Mr Mounir Ajina for his valuable contribution to the study, and also thank the reviewers for their helpful comments and suggestions.

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

AUTHORS’ CONTRIBUTIONS

BH conceived the idea and supervised the study. HR conducted the literature review, writing of the manuscript, experimentation, data analysis and calculations, and prepared the article for submission. AH, KM, ES, HM and AM helped with the reviewing and editing of the manuscript. SA approved its release to be published. All authors declared that they participated, read and approved the final manuscript.

REFERENCES

Almeida, C., Cardoso, M. F., Sousa, M., Viana, P., Goncalves, A., Silva, J., & Barros, A. (2005). Quantitative study of caspase-3 activity in semen...
and after swim-up preparation in relation to sperm quality. Human Reproduction, 20(5), 1307–1313.

Baccetti, B., Collodel, G., & Piomboni, P. (1996). Apoptosis in human ejaculated sperm cells (notulae seminologicae 9). Journal of Submicroscopic Cytology and Pathology, 28(4), 587–596.

Barroso, G., Marshedhi, M., & Oehninger, S. (2000). Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. Human Reproduction, 15(6), 1338–1344.

Barroso, G., Taylor, S., Marshedhi, M., Manzur, F., Gavino, F., & Oehninger, S. (2006). Mitochondrial membrane potential integrity and plasma membrane translocation of phosphatidylserine as early apoptotic markers: A comparison of two different sperm subpopulations. Fertility and Sterility, 85(1), 149–154.

Banchalib, M., Lornage, J., Mazoyer, C., Lejeune, H., Salle, B., & Francois, G. (2007). Sperm deoxyribonucleic acid fragmentation as a prognostic indicator of assisted reproductive technology outcome. Fertility and Sterility, 87(1), 93–100.

Bungum, M., Humaidan, P., Spano, M., Jepson, K., Bungum, L., & Giwercman, A. (2004). The predictive value of sperm chromatin structure assay (SCSA) parameters for the outcome of intraterrine insemination, IVF and ICSI. Human Reproduction, 19(6), 1401–1408.

Chavarro, J. E., Toth, L. T., Wright, D. L., Meeke, J. D., & Hauser, R. (2010). Body mass index in relation to semen quality, sperm DNA integrity, and seminal reproductive hormone levels among men attending an infertility clinic. Fertility and Sterility, 93(7), 2222–2321.

Chen, Z., Hauser, R., Trbovich, A. M., Shifren, J. L., Dorer, D. J., Godfrey-Bailey, L., & Singh, N. P. (2006). The relationship between human semen characteristics and sperm apoptosis: A pilot study. Journal of Andrology, 27(1), 112–120.

Cincik, M., Ergur, A. R., Tutuncu, L., Muhcu, M., Kilic, M., Balaban, B., & Urman, B. (2007). Combination of hypoosmotic swelling/eosin Y test for sperm membrane integrity evaluation: Correlations with other sperm parameters to predict ICSI cycles. Archives of Andrology, 53(1), 25–28.

Cross, N. L., & Hanks, S. E. (1991). Effects of cryopreservation on human sperm acrosomes. Human Reproduction, 6(9), 1279–1283.

Ekert, P. G., Silke, J., & Vaux, D. L. (1999). Caspase inhibitors. Cell Death and Differentiation, 6(11), 1081–1086.

Evgeni, E., Charalabopoulos, K., & Asimakopoulos, B. (2014). Human sperm DNA fragmentation and its correlation with conventional semen parameters. Journal of Reproduction & Infertility, 15(1), 2–14.

Flesch, F. M., & Gadella, B. M. (2000). Dynamics of the mammalian sperm plasma membrane in the process of fertilization. Biochimica et Biophysica Acta, 1469(3), 197–235.

Grunewald, S., Paasch, U., Said, T. M., Sharma, R. K., Glander, H. J., & Agarwal, A. (2005). Caspase activation in human spermatozoa in response to physiological and pathological stimuli. Fertility and Sterility, 83(Suppl. 1), 1106–1112.

Grunewald, S., Said, T. M., Paasch, U., Glander, H. J., & Agarwal, A. (2008). Relationship between sperm apoptosis signalling and oocyte penetration capacity. International Journal of Andrology, 31(3), 325–330.

Horvitz, H. R. (1999). Genetic control of programmed cell death in the nematode Caenorhabditis elegans. Cancer Research, 59(7 Suppl.), 1701s–1706s.

Kimura, M., Itoh, N., Takagi, S., Sasa, T., Takahashi, A., Masumori, N., & Tsukamoto, T. (2003). Balance of apoptosis and proliferation of germ cells related to spermatogenesis in aged men. Journal of Andrology, 24(2), 185–191.

Lachaud, C., Tesarik, J., Canadas, M. L., & Mendoza, C. (2004). Apoptosis and necrosis in human ejaculated spermatozoa. Human Reproduction, 19(3), 607–610.

Marchetti, P., Ballot, C., Jouy, N., Thomas, P., & Marchetti, C. (2012). Influence of mitochondrial membrane potential of spermatozoa on in vitro fertilisation outcome. Andrologia, 44(2), 136–141.

Marchetti, C., Gallego, M. A., Defossez, A., Formstercher, P., & Marchetti, P. (2004). Staining of human sperm with fluorochrome-labeled inhibitor of caspases to detect activated caspases: Correlation with apoptosis and sperm parameters. Human Reproduction, 19(5), 1127–1134.

Moskovtsev, S. I., Mullen, J. B., Lecker, I., Jarvi, K., White, J., Roberts, M., & Lo, K. C. (2010). Frequency and severity of sperm DNA damage in patients with confirmed cases of male infertility of different aetiologies. Reproductive BioMedicine Online, 20(6), 759–763.

Pareek, T. K., Joshi, A. R., Sanyal, A., & Dighe, R. R. (2007). Insights into male germ cell apoptosis due to depletion of gonadotropins by GnRH antagonists. Apoptosis, 12(6), 1085–1100.

Ruwangura, S. M., McLachlan, R. I., Matthiessen, K. L., & Meachem, S. J. (2008). Gonadotrophins regulate germ cell survival, not proliferation, in normal adult men. Human Reproduction, 23(2), 403–411.

Said, T. M., Paasch, U., Glander, H. J., & Agarwal, A. (2004). Role of caspases in male infertility. Human Reproduction Update, 10(1), 39–51.

Sakkas, D., Mariethoz, E., & St John, J. C. (1999). Abnormal sperm parameters in humans are indicative of an abortive apoptotic mechanism linked to the Fas-mediated pathway. Experimental Cell Research, 251(2), 350–355.

Samanta, L., Sahoo, A., & Chainy, G. B. (1999). Age-related changes in rat testicular oxidative stress parameters by hexachlorocyclohexane. Archives of Toxicology, 73(2), 96–107.

Seli, E., Gardner, D.K., Schoolcraft, W.B., Moffatt, O., & Sakkas, D. (2004). Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after in vitro fertilization. Fertility and sterility. Fertil Steril, 82(2), 378–383.

Shaha, C. (2008). Estrogens and spermatogenesis. Advances in Experimental Medicine and Biology, 636, 42–64.

Shen, H. M., Dai, J., Chia, S. E., Lim, A., & Ong, C. N. (2002). Detection of apoptotic alterations in sperm in subfertile patients and their correlations with sperm quality. Human Reproduction, 17(5), 1266–1273.

Stouffs, K., Lissens, W., Verheyen, G., Van Landuyt, L., Goossens, A., Tournaye, H., ... Liebaers, I. (2004). Expression pattern of the Y-linked PRY gene suggests a function in apoptosis but not in spermatogenesis. Molecular Human Reproduction, 10(1), 15–21.

Tavaalee, M., Deemeh, M. R., Arbabian, M., Kiyani, A., & Nasr-Esfahani, H. M. (2014). Relationship between fertilization rate and early apoptosis in sperm population of infertile individuals. Andrologia, 46(1), 36–41.

Taylor, S. L., Weng, S. L., Fox, P., Duran, E. H., Morshedhi, M. S., Oehninger, S., & Beebe, S. J. (2004). Somatic cell apoptosis markers and pathways in human ejaculated sperm: Potential utility as indicators of sperm quality. Molecular Human Reproduction, 10(11), 825–834.

Vaux, D. L., & Korsmeyer, S. J. (1999). Cell death in development. Cell, 96(2), 245–254.

Weng, S. L., Taylor, S. L., Morshedhi, M., Schu nnfer, A., Duran, E. H., Beebe, S., & Oehninger, S. (2002). Caspase activity and apoptotic markers in ejaculated human sperm. Molecular Human Reproduction, 8(11), 984–991.

World Health Organisation. (2010). WHO laboratory manual for the examination and processing of human semen, 5th ed. York: Cambridge University Press.

Wyrobek, A. J., Eskenazi, B., Young, S., Arnhem, N., Tiemann-Boege, I., Jabs, E. W., ... Evenson, D. (2006). Advancing age has differential effects on DNA damage, chromatin integrity, gene mutations, and aneuploidies in sperm. Proceedings of the National Academy of Sciences of the United States of America, 103(25), 9601–9606.

Zorn, B., Golob, B., Ihan, A., Kopitar, A., & Kolbezen, M. (2012). Apoptotic sperm biomarkers and their correlation with conventional sperm parameters and male fertility potential. Journal of Assisted Reproduction and Genetics, 29(4), 357–364.

How to cite this article: Hichri R, Amor H, Khammari M et al. Apoptotic sperm biomarkers and the correlation between conventional sperm parameters and clinical characteristics. Andrologia. 2018;50:e12813. https://doi.org/10.1111/and.12813