



# Fibronectin as a new biomarker for human sperm selection in assisted reproductive technology

Mahnaz Heidari<sup>1\*</sup> , Sara Darbandi<sup>1\*</sup> , Mahsa Darbandi<sup>1</sup> , Mohammad Mehdi Akhondi<sup>2</sup> , Mohammad Reza Sadeghi<sup>2</sup>

**Cite this article as:** Heidari M, Darbandi S, Darbandi M, Akhondi MM, Sadeghi MR. Fibronectin as a new biomarker for human sperm selection in assisted reproductive technology. Turk J Urol 2019; 45(2): 83-90.

## ABSTRACT

**Objective:** Fibronectin (FN) is a multifunctional diametric glycoprotein on the surface of the sperm that plays an important role in the sperm-oocyte interaction and fertilization process. The aim of the present study was to assess the FN levels as a sperm surface biomarker for sperm selection in assisted reproductive technology.

**Material and methods:** Polyclonal antibody against human FN was produced in rabbit. Its quality, purity, and immune reactivity were assessed by SDS-PAGE and western blot. In addition, the presence of FN on the sperm surface was assessed through immunocytochemistry and flow cytometry. The amount of FN and the sperm quality were assessed in normozoospermia (N) (42 men) and asthenoteratozoospermia (AT) (72 men) groups through sperm chromatin dispersion (SCD), sperm chromatin structure assay (SCSA), and chromatin maturation index (CMI).

**Results:** The results showed the distribution of FN protein on the equatorial region of the human sperm surface. In addition, the FN levels were found to have a significant difference between the two groups with  $24.64 \pm 9.08\%$  in N and  $16.90 \pm 7.27\%$  in AT ( $p \leq 0.0001$ ). In addition, FN level negatively correlated with SCD ( $p \leq 0.0001$ ), SCSA ( $p \leq 0.0001$ ), and CMI ( $p \leq 0.001$ ). Threshold values of FN level and DNA fragmentation index (DFI) percentage were 16 and 30 and were identified as cut-off values to determine the N group with a specificity of 83.3% and 81.0% and a sensitivity of 16.8% and 19.0%, respectively. The specificity and sensitivity of FN-DFI were 91.2% and 8.8%, respectively.

**Conclusion:** It appears that FN can be used for the selection of sperm with suitable quality, although future studies are recommended.

**Keywords:** Asthenoteratozoospermia; fibronectin; normozoospermia; sperm chromatin; sperm selection.

### ORCID IDs of the authors:

M.H. 0000-0001-6075-8639;  
S.D. 0000-0002-1868-6712;  
M.D. 0000-0002-2243-9319;  
M.M.A. 0000-0002-8695-2138;  
M.R.S. 0000-0003-0401-3493

<sup>1</sup>Department of Embryology and Andrology, Reproductive Biotechnology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran

<sup>2</sup>Monoclonal Antibody Research Center, Avicenna Research Institute, ACECR, Tehran, Iran

\*Equal contribution: Mahnaz Heidari, Sara Darbandi

### Submitted:

10.10.2018

### Accepted:

14.11.2018

### Available Online Date:

22.01.2019

### Corresponding Author:

Mohammad Reza Sadeghi  
E-mail:  
mahsasaraa@hotmail.com

©Copyright 2019 by Turkish Association of Urology

Available online at  
www.turkishjournalofurology.com

## Introduction

It is estimated that approximately 15% of couples in the reproductive age are infertile; approximately half of them have male factor infertility. Assisted reproductive technologies (ARTs), such as intracytoplasmic sperm insemination (ICSI) and *in vitro* fertilization, are suitable and essential for most male infertility treatment.<sup>[1]</sup> In this method, sperm selection is usually based on microscopic criteria, such as motility and morphology with no sperm DNA integrity and chromatin maturity assessment.<sup>[2]</sup> It has been demonstrated that a sperm with normal motility and morphology may have DNA damage.<sup>[3]</sup> Additionally, some sperm abnormalities, especially at the molecular levels, cannot be detected by routine meth-

ods of sperm selection in ARTs.<sup>[4]</sup> Therefore, it is necessary to introduce new methods that enable to select sperm without DNA fragmentation and impaired chromatin integrity.<sup>[3]</sup> To overcome these constraints, many sperm selection methods based on sperm surface biomarkers including electrophoresis, zeta charge, hyaluronic acid (HA), annexin V sperm sorting by magnetic-activated cell sorting, and flow cytometry (FCM) are available and used at a clinical level for the diagnosis and treatment of severe male factor infertility, especially in cases of repeated implantation failure.<sup>[5-8]</sup> Some of the important potential biomarkers involved in zona pellucida penetration, sperm binding, and fertilization of the oocyte are heat shock protein family A (Hsp70) member 2, serum amyloid P compound, ubiquitin, fibronectin

(FN), cysteine-rich secretory proteins, fertilin beta, PH-20, DJ-1 (PARK7), and epididymis P34H protein.<sup>[3,9]</sup> Detection of these molecules on the sperm surface and separation of sperm according to them are dependent on the availability of a specific ligand for designing a commercial test. Today, HA as the PICSI dish is the only specific ligand available for sperm selection.<sup>[10]</sup> However, specific antibodies as powerful ligands for the detection and separation of sperm were used in different studies.<sup>[11–13]</sup> FN was a diametric multifunctional glycoprotein present on the sperm surface and base membrane of the seminiferous tubules.<sup>[11]</sup> Binding of FN to the sperm surface was mediated by integrin present in the extracellular matrix through recognizing the tripeptide amino acid sequence Arg-Gly-Asp (RGD) in FN. FN was incorporated into the sperm membrane development during the late stages of spermatogenesis in the testis and sperm maturation in the epididymis in which studies have demonstrated that FN appears on the surface of human sperm after capacitation presenting widespread expression over the surface sperm of living sperm.<sup>[14]</sup> The presence of FN receptor integrin on human oocyte played an important role in the sperm-oocyte interaction and fertilization process.<sup>[15]</sup> The aim of the present study was to assess the level of FN on the sperm surface and to evaluate its relationship with sperm quality and its potential for sperm selection in ART.

## Material and methods

### Sperm collection and preparation

Semen specimens were obtained from 42 normozoospermic (N) men (sperm concentration  $\geq 15$  million/mL, total sperm motility (progressive+nonprogressive)  $\geq 40\%$ , vitality  $\geq 58\%$ , and normal sperm morphology  $\geq 4\%$ ) and 72 asthenoteratozoospermic (AT) infertile men who were referred to the Avicenna Infertility Clinic affiliated with the Avicenna Research Institute (ARI), Tehran, Iran. The study was approved by the bioethics committees of the ARI. Informed consent was obtained from each healthy donor. Semen samples were collected after 48–72 h of sexual abstinence, and semen analysis was performed according to the World Health Organization guideline<sup>[1]</sup> manual to determine semen volume, pH, morphology, and sperm concentration. For the analysis of sperm motility, the computer-assisted semen analysis system was used.<sup>[16]</sup> Briefly, individual semen samples were allowed to liquefy at room temperature of 25°C, and the sperm was separated from seminal plasma, immature germ cells, and non-sperm cells through density gradient centrifugation (300g for 20 min) using PureSperm® solution (Nidacon, Gothenburg, Sweden). The sperm pellet containing normal sperm was washed twice using phosphate buffered saline (PBS), then capacitated sperm was prepared by incubation of the previously washed sperm in Ham's F10 medium (Sigma, Germany) supplemented with 3.5% human serum albumin for 3 h at 37°C, and the aliquot was used freshly in subsequent techniques.

### Production and characterization of polyclonal antibody against FN

Polyclonal antibodies of the FN against human sperm surface protein were generated in ARI, Tehran, Iran. Briefly, following immunization of rabbits, anti-FN antibody was purified by protein G-affinity chromatography column (Amersham Pharmacia Biotech). In order to assess the reactivity of anti-FN antibody against FN, immunochemical assays (ELISA, SDS-PAGE, western blot (WB), and immunocytochemistry) and FCM were performed and compared with the antibody FN commercial. Human liver cells (HepG2 cell line) were used as positive control of the FN expression in immunochemical assay. Samples without any primary and secondary antibodies (evaluation of autofluorescence) or only without primary antibodies were used as negative control.

### Detection of the FN level on the sperm surface

The presence of FN on the sperm surface was compared in the N and AT groups. First, semen samples were washed twice at 300 g for 10 min at 4°C with FCM buffer (ice-cold PBS pH 7.2, containing 1% goat serum and 2% fetal calf serum) and diluted to reach  $1 \times 10^6$  sperm/mL concentration. Then, a 100  $\mu$ L of affinity-purified rabbit anti-FN antibody (10  $\mu$ g/mL) was added to each fraction and incubated for 60 min. Sperm samples, which were washed as described previously, were incubated with a 100  $\mu$ L fluorescein isothiocyanate-conjugated goat anti-rabbit (Gibco Inc., USA) for 30 min at 4°C. In order to assess sperm viability, all sperm fractions were labeled with a 100  $\mu$ L propidium iodide (PI) (Sigma-Aldrich, Germany) at a final concentration of 15  $\mu$ g/mL for 5 min at room temperature. As a control, we used samples without any primary and secondary antibodies (evaluation of autofluorescence) or only without primary antibodies (negative control). Ten thousand sperms were analyzed per sample with a flow rate of FCM (Partec PAS, Germany).

Flow cytometry analysis was performed (Partec PAS) by excitation lasers at 488 nm (Coherent Sapphire 488-20 DPSS, filter 525/50, DM 505LP) and 561 nm (Melles Griot 85-YCA-25, filter 585/15, DM 565LP) to measure the fluorescent intensity in the Alexa Fluor 488 and Alexa Fluor 555 channels, respectively. Ten thousand sperms were analyzed per sample with a flow rate. Analysis was performed using FlowJo v7.5.4. software (Tree Star Inc., Ashland, OR, USA). The differences among individual samples in the percentage of sperm above the set threshold level of fluorescence intensity were assessed and statistically compared.<sup>[17]</sup> Briefly, samples were analyzed with FCM using a flow cytometer (Partec PAS). The sperm population of each sample was identified using the side scatter (SSC) and FL1 fluorescence intensities. Debris was gated out by establishing a region around the population of interest based on FL1-SSC scale histogram. Ten thousand

sperms per sample were analyzed. The sperms were labeled with anti-FN antibody (FN positive sperm). The positive and negative sperm populations were determined by comparing the population of control staining using rabbit IgG isotype control, instead of the antibody. The ratio of FN positive sperm (FN+) was then calculated by dividing the number of labeled sperm by the number of sperm analyzed, and the ratio of FN negative sperm (FN-) was determined by reducing the percentage of FN+ from 100. Total FN- count was calculated using the proportion of FN- and total sperm count.

### Sperm chromatin dispersion (SCD)

This test was performed using an SDFA kit (Dain Bioassay, Iran) according to the manufacturer's instruction. Briefly, a 50  $\mu$ L semen was diluted in Ham's F10 medium, and semen aliquot was mixed with 50  $\mu$ L agarose (6.5%). Then, a 20  $\mu$ L of the mixture was loaded onto a pretreated glass slide and placed on a cold surface at 4°C for 5 min. Then, the slides were treated with denaturing solution for 7 min and then treated with lysing solution for 15 min. Following this step, the slides were washed with distilled water for 5 min, dehydration was performed using increasing concentrations of ethanol (70%, 90%, and 100%), and finally, the air dried slide was stained. At least 200 sperms were assessed on a microscope with 100 $\times$  magnification. Sperms with a large or medium halo were classified as intact chromatin, and those with no halo or a small halo were classified as sperm with fragmented DNA. The results were presented as sperm DNA fragmentation index (DFI).<sup>[18]</sup>

### Sperm chromatin maturation index (CMI)

The FCM-based CMI staining assay was adapted from the slide-based method for the assessment of sperm CMI. Semen samples were washed with PBS and diluted to reach 1 $\times$ 10<sup>6</sup> sperm/mL. Each sperm sample was evaluated by fluorescent microscopy and FCM-based CMI staining assay. Briefly, a 1 $\times$ 10<sup>6</sup>/mL sperm was washed with PBS (300 g for 5 min) and fixed with Carnoy's solution (methanol and glacial acetic acid) for 5 min at 4°C. The fixed sperm was used for preparation of thin smears. Each slide was treated for 20 min with 100  $\mu$ L of 0.25 mg/mL chromomycin A3 (CMA3) (Abcam, USA) solution in McIlvaine buffer (0.1 M citric acid, 0.2 M Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, and 10 mM MgCl<sub>2</sub>) at room temperature. The stained slides were then washed and mounted for microscopic assessment with an appropriated filter at 460-470 nm (Olympus, Japan). In the next step, 200 sperms were assessed on each slide. CMI reacted sperms (CMI+) as immature sperm with protamine deficiency were identified with bright yellow stain; however, mature protaminated sperms (CMI-) were observed with yellowish green stain. For FCM, the sperm concentration was adjusted to 10<sup>6</sup>/mL and subsequently centrifuged and fixed with Carnoy's solution. The pellet was resuspended in 200  $\mu$ L

of CMA3 solution (0.25 mg/mL) for 1 h. Then, samples were washed twice with PBS and used for FCM. Fluorescence from CMA3 stained sperm was collected in fluorescence detector FL-3 with a 585/42 nm band-pass filter. At least 10,000 sperms were analyzed for each sample. Positive control was prepared by pre-incubating sperm with 200 mM dithiothreitol (Sigma-Aldrich) at 37°C for 10 min.<sup>[19]</sup>

### Sperm viability assay

Sperm viability was analyzed by FCM in rhodamine (Rh 123)-stained sperm. Semen aliquot was washed, and 10<sup>6</sup> sperms were incubated with rhodamine 123 (R123, Sigma) (0.01 mg/mL in water) at 25°C for 10 min without light exposure. The stained sperm was washed and centrifuged (300 g for 10 min), and subsequently, PI was added as previously described. Flow cytometric analysis was performed using an argon laser at 488 nm for excitation. Filter set-up included a 515 nm long-pass filter with a 457-505 nm laser blocker, a 550 nm dichroic beam splitter, and a combination of a 525 nm band pass with a 560 nm short-pass filter for R123. Sperm samples were treated with double distilled water to validate the assay. The mitochondrial activity changes in association with R123 fluorescence intensity changes.

### Sperm chromatin structure assay (SCSA)

Semen samples were prepared for SCSA, and all succeeding steps were performed at 4 °C. Samples were diluted with TNE buffer (0.15 mol/L NaCl, 0.01 mol/L Tris, 0.001 mol/L EDTA, and pH 7.4) to obtain a concentration of approximately 2 $\times$ 10<sup>6</sup> sperm/mL. A 200  $\mu$ L aliquot was removed and mixed with 400  $\mu$ L of a low pH detergent solution (0.15 mol/L NaCl, 0.08 N HCl, 0.01% Triton X-100, and pH 1.4). After 30 s, a 1.2 ml staining solution (6  $\mu$ g/mL AO, 0.2 M Na<sub>2</sub>HPO<sub>4</sub>, 1 mM disodium EDTA, 0.15 M NaCl, 0.1 M citric acid monohydrate, and pH 6.0) was added, and the stained sperm was placed into the FCM sample chamber. Abnormal chromatin structure was quantitated by the metachromatic shift from green to red fluorescence in FCM measurement.<sup>[20]</sup> The percentage of abnormal DNAs was reported as DFI.

$$DFI\% = \frac{\text{red fluorescence}}{\text{total (red+green) fluorescence}}$$

### Statistical analysis

All statistical analyses were made using the IBM Statistical Package for the Social Sciences version 19 (IBM SPSS Statistics Corp.; Armonk, NY, USA). Data were presented as mean $\pm$ SD and median. The two-paired t-test and independent paired t-test were used to analyze data, and computed correlation coefficients (r) between the individual parameters and methods were tested. A p-value of  $\leq$ 0.05 was considered as significant.

## Results

### Characterization of the anti-FN antibody

The production of antibody against FN protein in rabbit and its reactivity against pure FN protein were examined by ELISA before and during immunization. The reactivity of affinity-purified antibody was observed as a single band at 220 kDa by WB (Figure 1a). Indirect immunofluorescence

assays were performed using affinity-purified anti-FN antibodies. The green fluorescence on the surface of the sperm and HepG2 cell line was an indicator for the presence of FN (Figure 1b).

### Semen analysis and FN detection

A total of 114 samples (42 in the N group and 72 in the AT group) are assessed and summarized in Table 1. Data

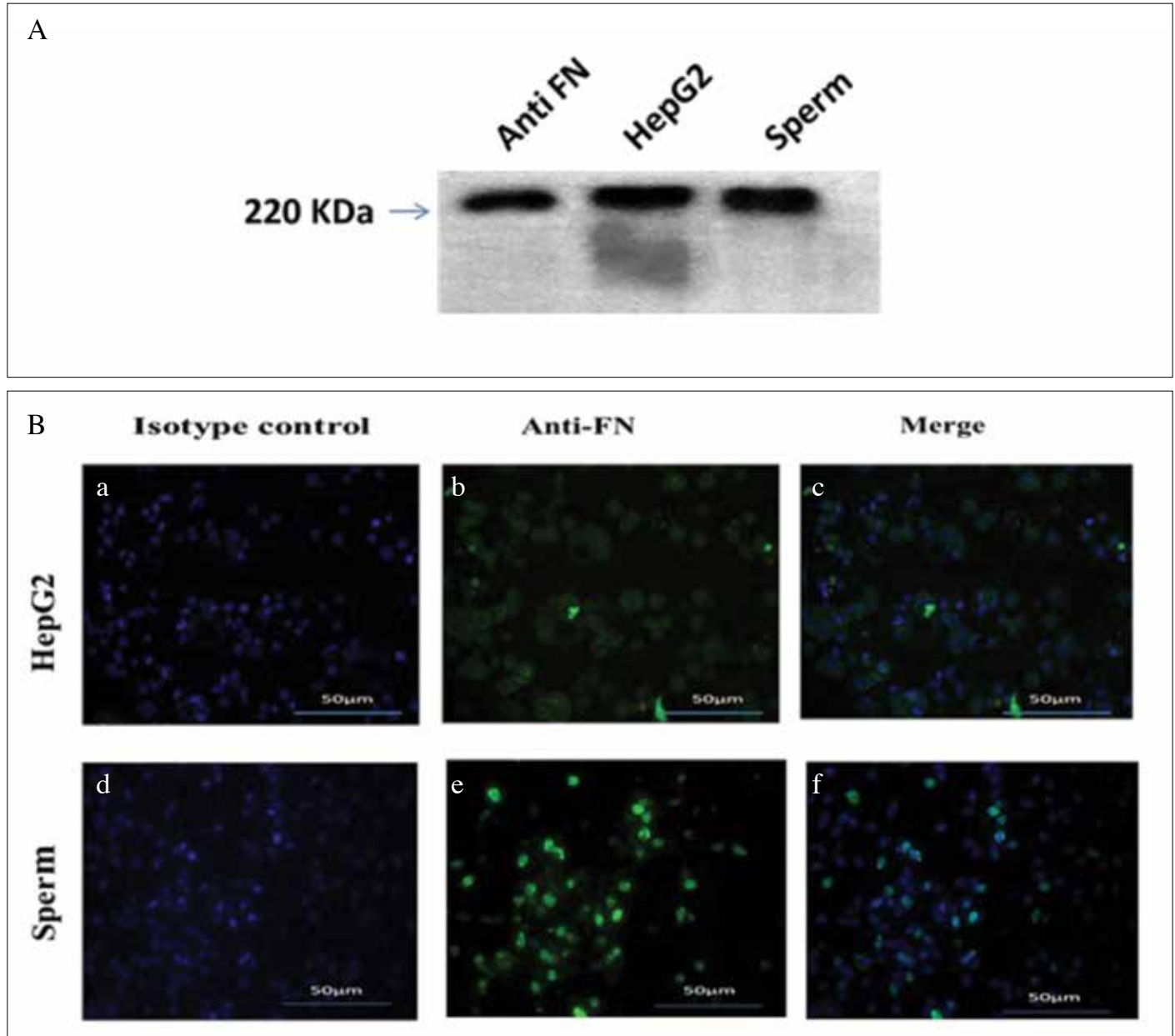


Figure 1. A, B. Affinity purification of FN in the sperm samples and positive control (HepG2 and FN protein). (A) Position of the FN protein as recognized by western blot. (B) Immunocytochemistry assay using anti-FN antibody. Anti-FN antibody was used to detect the presence of relevant proteins on the surface of HepG2 (a, b, c) and sperm (d, e, f). Fluorescein isothiocyanate (green) and DAPI (blue), a and d (negative controls)  
FN: fibronectin

**Table 1. The results of parameters evaluated between the N and AT groups**

| Parameter                                  | N (n=42)    | AT (n=74)     | p           |        |
|--|-------------|---------------|-------------|--------|
| Semen volume (mL)                          | 4±1.04      | 4.54±1.76     | NS          |        |
| Age (year)                                 | 32.54±4.21  | 31.98±4.66    | NS          |        |
| Sperm concentration (×10 <sup>6</sup> /mL) | 45.57±11.42 | 32.40±9.41    | 0.001       |        |
| Normal morphology (%)                      | 5.60±1.73   | 2.54±1.09     | 0.001       |        |
| Progressive motility (%)                   | 50.57±10.00 | 28.57±11.30   | 0.001       |        |
| FN (%)                                     | 23.88±6.93  | 16.76±7.35    | <0.001      |        |
| DFI (%)                                    | SCD (%)     | 21.10±7.75    | 30.42±15.13 | <0.001 |
|  | SCSA (%)    | 21.98±10.46   | 31.32±14.16 | <0.001 |
| CMI- (%)                                   | 23.79±6.34  | 32.33±11.1567 | 0.001       |        |
| Rh 123 (%)                                 | 77.45±10.99 | 66.76±9.08    | <0.001      |        |
| Viability (%)                              | 81.43±8.019 | 77.45±10.99   | NS          |        |

Values are expressed as mean±SD. NS: not significant; N: normozoospermia; AT: asthenoteratozoospermia; SCD: sperm chromatin dispersion; SCSA: sperm chromatin structure assay; CMI: chromatin maturation index; Rh 123: rhodamine; DFI: DNA fragmentation index; FN: fibronectin

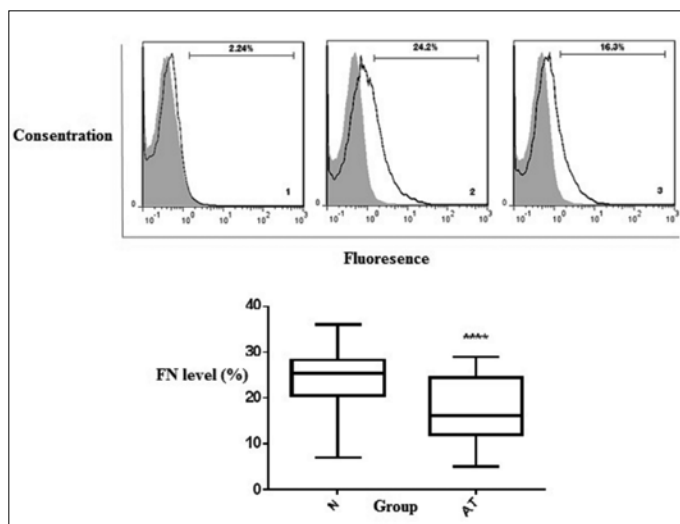


Figure 2. The level of FN on sperm surface 1, Isotype control sperm; 2, sperm sample of the N group; 3, sperm sample of the AT group  
FN: fibronectin; N: normozoospermia; AT: asthenoteratozoospermia

showed that sperm concentration ( $p \leq 0.0001$ ), progressive motility ( $p \leq 0.001$ ), and normal morphology ( $p \leq 0.001$ ) in the AT group had significant differences when compared with the N group. The percentage of the FN level in the N group ( $24.64 \pm 7.43\%$ ) was significantly higher than that in the AT group ( $16.90 \pm 7.27\%$ ) as was shown in Table 1 and Figure 2 ( $p \leq 0.0001$ ). As shown in Table 2, the cut-off value of the FN expression was estimated as 16 with 83.3% specificity and 16.7% sensitivity in the N group and 47.2% specificity and 52.8% sensitivity in the AT group.

### Sperm chromatin integrity

In the N and AT groups, the percentage of CMI was  $23.79 \pm 6.34$  versus  $32.33 \pm 11.15$ , DFI using SCD was  $21.10 \pm 7.75$  versus  $30.42 \pm 15.13$ , and DFI by using SCSA was  $21.98 \pm 10.46$  versus  $31.32 \pm 14.16$ , respectively. Data showed that sperm CMI ( $p \leq 0.0001$ ), SCD ( $p \leq 0.0001$ ), and SCSA ( $p \leq 0.001$ ) percentages in the AT group had significant differences when compared with the N group (Table 1).

### Vitality

Sperm vitality was assessed by eosin-nigrosin and rhodamine 123. The percentage of sperm vitality in the N and AT groups was  $81.43 \pm 8.019$  versus  $77.45 \pm 10.99$  and  $79.44 \pm 10.72$  versus  $66.76 \pm 9.08$ , respectively. As was shown in Table 1, the sperm vitality percentage did not significantly change between the two groups ( $p > 0.05$ ). To determine the possible effect of the FN level on sperm quality, the correlations between FN expression level and chromatin integrity index were analyzed (Table 3). Significant negative correlations were found between FN expression level and SCD ( $r = -0.722$ ,  $p < 0.0001$ ), CMI ( $r = -0.742$ ,  $p < 0.0001$ ), and SCSA ( $r = -0.744$ ,  $p < 0.0001$ ).

### Discussion

In the present study, sperm chromatin maturity and DNA integrity were assessed by SCSA, SCD, and CMI and compared with the FN level on the sperm surface. These findings showed that the AT group had a lower percentage of sperm FN level, higher percentage of sperm DNA damage, and abnormal chromatin packaging than the N group. It appears that the expression level of this protein was dependent on sperm DNA integrity and chromatin maturity. Regarding that the sperm FN level

**Table 2. The cut-off values in the N and AT groups**

| Group     |                 | DFI  |      |      |                   |
|-----------|-----------------|------|------|------|-------------------|
|           |                 | FN   | SCD  | SCSA | FN-DFI            |
| N (n=42)  | Cut-off value   | >16  | <30  | <30  | FN>16 and DFI <30 |
|           | Specificity (%) | 83.3 | 81.0 | 81.0 | 91.2              |
|           | Sensitivity (%) | 16.7 | 19.0 | 19.0 | 8.8               |
| AT (n=72) | Cut-off value   | <16  | >30  | >30  | >30               |
|           | Specificity (%) | 47.2 | 51.4 | 51.4 | 81.1              |
|           | Sensitivity (%) | 52.8 | 48.6 | 48.6 | 18.9              |

N: normozoospermia; AT: asthenoteratozoospermia; SCD: sperm chromatin dispersion; SCSA: sperm chromatin structure assay; DFI: DNA fragmentation index; FN: fibronectin

**Table 3. The correlation between sperm FN level and sperm parameters**

| Parameters                                      | r       | P       |
|---|---------|---------|
| Sperm concentration ( $\times 10^6/\text{mL}$ ) | 0.053   | 0.656   |
| Progressive                                     | 0.315*  | 0.007   |
| Morphology                                      | 0.192   | 0.106   |
| CMI   | -0.626* | <0.001  |
| DFI   | SCSA    | -0.660* |
|   | SCD     | -0.738* |
| Rh 123  | 0.280   | 0.017   |
| Mortality                                       | -0.398* | 0.001   |

\* $p \leq 0.05$  was considered to be statistically significant. r: indicates the Pearson correlation coefficient; SCD: sperm chromatin dispersion; SCSA: sperm chromatin structure assay; CMI: chromatin maturation index; Rh 123: rhodamine; DFI: DNA fragmentation index; FN: fibronectin

had very wide range in men with normal and abnormal sperm parameters, in the present study, we demonstrated a cut-off value for categorizing sperm based on FN levels, with value estimated as 16 with a specificity of 83.3% and 47.2% and a sensitivity of 16.7% and 52.8%, respectively, in N and AT men. According to this data, it appears that FN can be used for the selection of sperm with suitable quality and chromatin compaction. To our knowledge, these results had not been reported by other investigators. Today, several studies presented new positive and negative biomarkers to separate mature sperm with the best DNA compaction and maturation. For negative biomarkers, only defective sperms were recognized, whereas positive biomarkers could select the best forms for ICSI. Healthy sperm could be selected from defective and abnormal forms; however, sperm motility and functions were damaged by most of these methods. Therefore, methods need to be improved and optimized before being applied routinely in daily practice of ART.<sup>[21]</sup> FN was expressed during the late stages of spermatogenesis in the testis and localized on the testicular and epididymal sperm.<sup>[22]</sup> Previous studies had shown that the presence of FN

on sperm played a significant role in the fertilization process.<sup>[23]</sup> FN, as a multifunctional adhesive glycoprotein, was widely distributed in human tissues and was known to assist in various cellular regulation processes, including cell growth and migration, differentiation, hemostasis, and male reproduction.<sup>[23]</sup> It could exist in both plasma and cell surface, with similarity in the physiological functions of cell adhesion to various biological molecules.<sup>[24]</sup> In addition, FN on the sperm head could bind to integrins and on specific RGD sequences on the oocyte surface.<sup>[15]</sup> In order to evaluate the efficacy of FN as a positive or negative biomarker, a gold standard for sperm function and male fertility was needed. However, as mentioned previously, there were not any efficient standard methods, and only parameters, such as morphology, concentration, and motility, were used as standard. However, these parameters did not have suitable reliability to accurately evaluate male fertility. Therefore, we evaluated FN levels as a usable biomarker to select sperm according to their fertilizability. As high levels of FN were found in healthy sperm, and significant negative correlation has been detected between FN level and sperm DFI and CMI, therefore, FN could be regarded as a positive biomarker to select more functional sperm in ARTs, especially in ICSI. The present study found that the level of FN was not only related to routine parameters of human sperm but also associated to functional parameters of sperm, such as mitochondrial status, chromatin maturation, and DNA integrity. This result was in accordance with studies showing that localized FN on the human sperm surface was an essential factor for sperm-egg adhesion that could be correlated with normal morphology and functional integrity of the sperm.<sup>[25,26]</sup> The positive correlation between FN level, chromatin maturation, and DNA integrity in N might be due to the role of FN in sperm protamination and suitable folding of chromatin during sperm maturation in the testis and epididymis.<sup>[27,28]</sup> While a significant lower level of FN in defective sperm in AT might be an indicator for spermatogenesis failure, leading to disorder in sperm function and male infertility. One of the essential steps in spermatogenesis was DNA packaging and condensation, which was synchronic with FN expression on sperm during

spermatogenesis.<sup>[29,30]</sup> The defect in chromatin packaging using protamine during spermatogenesis and increasing DNA fragmentation in AT sperm might be simultaneous with lower FN level, which could directly or indirectly affect the process of fertilization.<sup>[31]</sup> Moreover, our results were in accordance with studies estimating that infertile men with low sperm motility and morphology had increased DNA fragmentation, DNA damages, and protamine deficiency.<sup>[32]</sup> Another concern was that in contrast to the PH-20 receptor, which banded to HA ligand in PICSi dish, anti-FN antibody was used in our assays despite the presence of integrin and other FN receptors in the connective tissue. Since this antibody had an animal origin (mouse or rabbit), it was not possible to be used in routine preselection or processing of human sperm in ARTs. Future studies could be designed using the three RGD peptide sequences on a suitable support of stationary phase to select normal and healthy sperm with FN at its surface for sperm selection in ARTs.

Sperm chromatin maturation and DNA integrity were correlated with FN level in sperm. The present data strongly suggested that FN levels in sperm had a predictive value in determining the quality of sperm in infertile men. The present study implied that the FN levels in sperm could be potentially used as a biomarker in sperm selection and assessing the quality of sperm in ART. These results might help clinicians and scientists to further understand the clinical values of FN levels. However, further studies are needed to fully explain the feasibility and efficacy of this biomarker in improving ART outcomes. In addition, further studies on fertile men, unexplained infertility, pure asthenozoospermia, and teratozoospermia failures can determine the position of this biomarker in male infertility and sperm selection in the future.

**Ethics Committee Approval:** Ethics committee approval was received for this study from the Medical Ethics Committee of Avicenna Research Institute (IR.ACECR.Avicenna.REC.4886/93).

**Informed Consent:** Written informed consent was obtained from patients who participated in this study.

**Peer-review:** Externally peer-reviewed.

**Author Contributions:** Data interpretation – M.H., S.D., M.D.; Execution – M.H., S.D., M.D.; Analysis – M.H., S.D., M.D.; Manuscript Drafting – M.H., S.D., M.D. M.R.S., M.M.A.; Manuscript Revision – M.H., S.D., M.D., M.R.S., M.M.A.; Supervision – M.R.S., M.M.A.

**Conflict of Interest:** The authors have no conflicts of interest to declare.

**Financial Disclosure:** This project was financially supported by Avicenna Research Institute (Tehran, Iran).

## References

1. The World Health Organization. Examination and processing human semen. 2010;286. Available from: [http://apps.who.int/iris/bitstream/handle/10665/44261/9789241547789\\_eng.pdf](http://apps.who.int/iris/bitstream/handle/10665/44261/9789241547789_eng.pdf).
2. Simopoulou M, Gkoles L, Bakas P, Giannelou P, Kalampokas T, Pantos K, et al. Improving ICSI: A review from the spermatozoon perspective. *Syst Biol Reprod Med* 2016;62:359-71. [\[CrossRef\]](#)
3. Toriki-Boldaji B, Tavalae M, Bahadorani M, Nasr-Esfahani MH. Selection of physiological spermatozoa during intracytoplasmic sperm injection. *Andrologia* 2017;49:DOI: 10.1111/and.12579. [\[CrossRef\]](#)
4. Paasch U, Grunewald S, Glander H. Sperm selection in assisted reproductive techniques. *Soc Reprod Fertil Suppl* 2007;65:515-25.
5. Simon L, Ge SQ, Carrell DT. Sperm selection based on electrostatic charge. *Methods Mol Biol* 2013;927:269-78. [\[CrossRef\]](#)
6. Saylan A, Duman S. Efficacy of Hyaluronic Acid in The Selection of Human Spermatozoa with Intact DNA by The Swim-up Method. *Cell J* 2016;18:83-8.
7. Grunewald S, Paasch U. Sperm selection for ICSI using annexin V. *Methods Mol Biol* 2013;927:257-62. [\[CrossRef\]](#)
8. Barrier Battut I, Kempfer A, Becker J, Lebailly L, Camugli S, Chevrier L. Development of a new fertility prediction model for stallion semen, including flow cytometry. *Theriogenology* 2016;86:1111-31. [\[CrossRef\]](#)
9. Widlak W, Vydra N. The Role of Heat Shock Factors in Mammalian Spermatogenesis. *Adv Anat Embryol Cell Biol* 2017;222:45-65. [\[CrossRef\]](#)
10. Witt KD, Beresford L, Bhattacharya S, Brian K, Coomarasamy A, Cutting R, et al. Hyaluronic Acid Binding Sperm Selection for assisted reproduction treatment (HABSelect): study protocol for a multicentre randomised controlled trial. *BMJ Open* 2016;6:e012609. [\[CrossRef\]](#)
11. Capkova J, Kubatova A, Ded L, Tepla O, Peknicova J. Evaluation of the expression of sperm proteins in normozoospermic and asthenozoospermic men using monoclonal antibodies. *Asian J Androl* 2016;18:108-13. [\[CrossRef\]](#)
12. Kaya A, Memili E. Sperm macromolecules associated with bull fertility. *Anim Reprod Sci* 2016;169:88-94. [\[CrossRef\]](#)
13. Rahman MS, Kwon WS, Pang MG. Prediction of male fertility using capacitation-associated proteins in spermatozoa. *Mol Reprod Dev* 2017;84:749-59. [\[CrossRef\]](#)
14. Ekhlasi-Hundrieser M, Schäfer B, Philipp U, Kuiper H, Leeb T, Mehta M, et al. Sperm-binding fibronectin type II-module proteins are genetically linked and functionally related. *Gene* 2007;392:253-65. [\[CrossRef\]](#)
15. Bronson RA, Fusi FM. Integrins and human reproduction. *Mol Hum Reprod* 1996;2:153-68. [\[CrossRef\]](#)
16. Aksoy E, Aktan TM, Duman S, Cuce G. Assessment of spermatozoa morphology under light microscopy with different histologic stains and comparison of morphometric measurements. *Int J Morphol* 2012;30:1544-50. [\[CrossRef\]](#)
17. Evenson DP, Darzynkiewicz Z, Melamed MR. Simultaneous measurement by flow cytometry of sperm cell viability and mitochondrial membrane potential related to cell motility. *J Histochem Cytochem* 1982;30:279-80. [\[CrossRef\]](#)
18. Fernandez JL, Muriel L, Goyanes V, Segrelles E, Gosálvez J, Enciso M, et al. Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. *Fertil Steril* 2005;84:833-42. [\[CrossRef\]](#)

19. Shamsi MB, Imam SN, Dada R. Sperm DNA integrity assays: diagnostic and prognostic challenges and implications in management of infertility. *J Assist Reprod Genet* 2011;28:1073-85. [\[CrossRef\]](#)
20. Evenson DP. The Sperm Chromatin Structure Assay (SCSA®) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility. *Anim Reprod Sci* 2016;169:56-75. [\[CrossRef\]](#)
21. Sutovsky P, Aarabi M, Miranda-Vizuete A, Oko R. Negative biomarker-based male fertility evaluation: sperm phenotypes associated with molecular-level anomalies. *Asian J Androl* 2015;17:554-60. [\[CrossRef\]](#)
22. Schaller J, Glander H, Dethloff J. Andrology: Evidence of  $\beta$ 1 integrins and fibronectin on spermatogenic cells in human testis. *Hum Reprod* 1993;8:1873-8. [\[CrossRef\]](#)
23. Martinez-Leon E, Osycka-Salut C, Signorelli J, Pozo P, Perez B, Kong M, et al. Fibronectin stimulates human sperm capacitation through the cyclic AMP/protein kinase A pathway. *Hum Reprod* 2015;30:2138-51. [\[CrossRef\]](#)
24. Attia A, Hassan A, Zalata A, Hagag M, Yousef K, Mostafa T. Seminal fibronectin in fertile and infertile males. *Andrologia* 2011;43:387-91. [\[CrossRef\]](#)
25. Glander HJ, Herrmann K, Hausteil UF. The equatorial fibronectin band (EFB) on human spermatozoa-a diagnostic help for male fertility? *Andrologia* 1987;19:456-9. [\[CrossRef\]](#)
26. Hoshi K, Sasaki H, Yanagida K, Sato A, Tsuiki A. Localization of fibronectin on the surface of human spermatozoa and relation to the sperm-egg interaction. *Fertil Steril* 1994;61:542-7. [\[CrossRef\]](#)
27. Huszar G, Sbracia M, Vigue L, Miller DJ, Shur BD. Sperm plasma membrane remodeling during spermiogenetic maturation in men: relationship among plasma membrane beta 1, 4-galactosyltransferase, cytoplasmic creatine phosphokinase, and creatine phosphokinase isoform ratios. *Biol Reprod* 1997;56:1020-4. [\[CrossRef\]](#)
28. Varghese A, Bragais F, Mukhopadhyay D, Kundu S, Pal M, Bhat-tacharyya A, et al. Human sperm DNA integrity in normal and abnormal semen samples and its correlation with sperm characteristics. *Andrologia* 2009;41:207-15. [\[CrossRef\]](#)
29. Miranda PV Tezon JG. Characterization of fibronectin as a marker for human epididymal sperm maturation. *Mol Reprod Dev* 1992;33:443-50. [\[CrossRef\]](#)
30. Gaucher J, Reynoird N, Montellier E, Boussouar F, Rousseaux S, Khochbin S. From meiosis to postmeiotic events: the secrets of histone disappearance. *Febs J* 2010;277:599-604. [\[CrossRef\]](#)
31. Ni K, Spiess AN, Schuppe HC, Steger K. The impact of sperm protamine deficiency and sperm DNA damage on human male fertility: a systematic review and meta-analysis. *Andrology* 2016;4:789-99. [\[CrossRef\]](#)
32. Lopes S, Jurisicova A, Casper RF. Gamete-specific DNA fragmentation in unfertilized human oocytes after intracytoplasmic sperm injection. *Hum Reprod* 1998;13:703-8. [\[CrossRef\]](#)