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Short-term FSH treatment and sperm maturation: a prospective study in idiopathic infertile men

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SUMMARY

The standard FSH treatment is based on a 3 months period, after which both quantitative/qualitative improvement of sperm parameters and increased pregnancy rate were reported. In this prospective clinical trial, for the first time, we studied (i) Sperm hyaluronic acid binding capacity after highly purified FSH (hpFSH) treatment; (ii) the effect after short-term and standard treatment on this functional parameter. As secondary objective, we analyzed three SNPs on *FSH*β and *FSHR* genes to define their potential predictive value for responsiveness. From a total of 210 consecutive patients, 40 oligo- and/or astheno- and/or teratozoospermic patients fulfilled the inclusion criteria. Treatment consisted in hpFSH 75 IU/L every other day for 3 months. To avoid potential biases derived from the lack of placebo, we analyzed each patient after 4–6 months of 'wash-out' period. After FSH treatment, we observed a statistically significant ($p < 0.001$) improvement of the percentage of hyaluronic acid bound spermatozoa from basal to T1 (after 1 month) and to T3 (after 3 months). Importantly, these values returned to near-baseline value after the wash-out. The same results were detected for total motile sperm count after 3 months with return to baseline after wash-out. Forty-two percent of patients responded to the therapy with increasing hyaluronic acid binding capacity above the double of the Intraindividual Variation (IV) while 24% of patients reached above the normal Sperm-Hyaluronan Binding Assay (HBA) value. Further increase in 'responders' was observed at T3. The responsiveness to treatment resulted independent from *FSHR*/*FSH*β polymorphisms. The significant positive effect on sperm maturity after 1 month opens novel therapeutic perspectives. In view of both the high cost and the relative invasiveness of treatment, the short protocol (1 month) could represent a viable FSH treatment option prior Assisted Reproductive Techniques since FSH, by acting on sperm maturation, increases the proportion of functionally competent cells.

INTRODUCTION

Infertility affects approximately 15% of couples and male factor is present in approximately 50% of cases. The etiology of impaired sperm production and function can be related to endocrine, genetic/epigenetic and environmental factors (Krausz, 2011; Tournaye *et al.*, 2016a). Despite progresses in diagnosing the causes of infertility, in about 30–50% cases, the etiology remains unknown and it is termed 'idiopathic infertility'. In this category of patients a number of empirical treatments have been proposed from antioxidant to hormonal therapies with controversial results (for review see Tournaye *et al.*, 2016b). A recent meta-analysis of 15 controlled clinical trials in which FSH was administered to idiopathic infertile men (compared with placebo or no treatment) showed that FSH administration to the male partner significantly improves sperm concentration and pregnancy rate both spontaneously or after Assisted Reproductive Techniques (ART; Santi *et al.*, 2015). However, because of heterogeneity of the studies, data quality is not considered optimal by the authors of the meta-analysis, leaving FSH treatment in idiopathic infertility still an open question. In addition, studies dealing with FSH therapy and sperm parameters have clearly shown that only a portion (approximately 50%) of treated subjects respond in terms of quantitative and qualitative improvement of spermatogenesis. The impossibility to predict responsiveness to treatment remains therefore a great limitation to this relatively expensive therapeutic approach and has prompted some authors to search for predictive parameters (Glander and Kratzsch, 1997; Foresta *et al.*, 2000). Pharmacogenetics seems to be a promising tool as two SNPs (*FSH*β c.-211G>T, rs10835638; *FSHR* c.2039A>G, rs6166) have been

proposed as predictive factors (Ferlin *et al.*, 2011; Selice *et al.*, 2011).

Data in the literature refer to the standard FSH treatment's length which is 3–4 months, covering the entire period of spermatogenesis. So far, no studies have been conducted to assess the effect of FSH treatment on the last phase of spermatogenesis. To define whether FSH treatment can positively affect sperm maturation, i.e. spermiogenesis, we selected a functional test, the Sperm-Hyaluronan Binding Assay (HBA; Huszar *et al.*, 2003). The hyaluronic acid (HA) binding capacity of spermatozoa appears to be correlated with cellular and chromatin maturity, DNA integrity, chromosomal aneuploidy frequency and sperm function (Huszar *et al.*, 2000, 2003, 2007; Cayli *et al.*, 2003; Roudebush *et al.*, 2004; Jakab *et al.*, 2005; Prinosilova *et al.*, 2009; Yagci *et al.*, 2010). Hyaluronic acid or hyaluronan is a molecule involved in the natural process of human fertilization as it seems to play a pivotal role in physiological sperm selection. The exposure of receptors for hyaluronic acid on sperm cells surface indicates that these cells have completed the remodeling of plasma membrane. Only mature spermatozoa which have correctly completed spermiogenesis expose on their surface a high density of HA receptors (Huszar *et al.*, 2003). Accordingly, the HA binding capacity can be considered a biomarker of fully completed spermiogenesis. By evaluating both HA binding capacity and sperm creatine kinase activity in the same sample it has been proposed that an HA binding over 60% is compatible with the use of a less invasive-assisted reproduction technique such as IUI or even with natural conception (Huszar *et al.*, 2002; Huszar *et al.*, 2007).

Moreover, in subjects showing ≤65% of HA binding, the selection of Hyaluronan-Bound (HB) spermatozoa for Intra Cytoplasmic Sperm Injection (ICSI) led to a statistically significant reduction in pregnancy loss rate in respect to direct ICSI performed with a sperm selection based upon the visual examination of morphology and motility alone (Worrilow *et al.*, 2013).

Sperm HA binding capacity could therefore represent not only a good predictor for sperm maturity but also a better predictor of the probability to conceive in respect to 'classic' semen parameters. Our study is the first aiming to evaluate whether FSH therapy is effective in improving HA binding capacity in idiopathic infertile men and whether this effect is present already after 1 month of therapy (during spermiogenesis). As secondary endpoints, we analyzed two selected sperm parameters: total sperm number (TSN) and total motile sperm count (TMSC) in relationship with the 3 months treatment. Finally, we aimed at defining the predictive capacity of hormonal, seminal and genetic parameters to HA binding-response following FSH administration.

MATERIALS AND METHODS

Study population

Two hundred and ten consecutive infertile men attending the Sexual Medicine and Andrology Clinics of the University Hospital, Florence, Careggi underwent a comprehensive clinical examination: medical history, physical examination, hormonal dosage, semen analysis (according to WHO guidelines), karyotype and Y chromosome microdeletion analysis. In 107 patients all known causes of infertility have been excluded and 70 of them presented either impaired sperm number (oligozoospermia) or impaired sperm motility (asthenozoospermia) or reduced normal morphology (teratozoospermia) or the combination of the above anomalies (oligoasthenoteratozoospermia) with FSH value <8 IU/L. At baseline or during the treatment period 30 patients were excluded/dropped out for various reasons reported in Figure S1. A total of 40 patients completed the study. None of these men experienced a previous fatherhood, i.e. all cases were of primary infertility.

All patients received 75 IU/L of highly purified FSH (hpFSH Fostimon; IBSA, Lodi, Italy), every other day for 3 months and they were evaluated in four different time intervals: (i) at baseline (T0); (ii) after 1 month of FSH therapy (T1); (iii) after 3 months of FSH therapy (T3); (iv) 4–6 months after the end of therapy (wash-out or second baseline value).

Seminal samples were collected at T0, T1, T3 and wash-out to evaluate seminal parameters and to perform HBA. For each patient (except one), a blood sample was collected at T0 for molecular genetic analysis. During the study, one drop-out was registered, because of the patient's inability to participate at each visit planned for the study.

Semen analysis

All semen parameters were obtained from semen analysis in accordance with World Health Organization (WHO) criteria (WHO, 2010). The seminal fluid was obtained by masturbation. We selectively considered TSN and TMSC in the seminal fluid. The TSN was obtained considering the volume of ejaculate and the sperm concentration (calculated with Neubauer-improved chambers). The TMSC represents the total number of progressive motile spermatozoa in the seminal fluid; it can be considered a more reliable indicator of the severity of spermatogenic disturbance as it reflects both numerical and functional competence (progressive motility) of a given ejaculate. TMSC was calculated considering the TSN and the progressive sperm motility [(total sperm number × progressive sperm motility)/100].

Sperm-Hyaluronan Binding Assay

The HBA test (HBA Sperm-Hyaluronan Binding Assay; Biocoat, Horsham, PA, USA) distinguishes the mature from immature spermatozoa. The mature spermatozoa bind hyaluronan of the *cumulus oophorus* matrix. Therefore, these spermatozoa have also the capacity to bind hyaluronan chemically attached to a solid support like the HBA slide. The HBA slide has two identical hyaluronan-coated assay chambers. The HBA test was performed in accordance with the instructions for use. The seminal fluid, obtained by masturbation, was kept at 20–28 °C for 30 min to liquefy. Before use, the sample was mixed and a drop of 10 μL in the center of an assay chamber of the slide was loaded. Immediately the cover slip was installed with a viewing circle upward and over the chamber. Then the slide was incubated at room temperature and was read after 10–15 min allowing mature spermatozoa to bind the immobilized hyaluronan layer. Scoring was performed on at least 100 motile bound and unbound spermatozoa with the final calculation of the percentage of hyaluronan-bounded spermatozoa as follows: [Bound Motile Sperm/(Bound Motile Sperm + Unbound Motile Sperm)] × 100.

Definition of responders for TSN, TMSC and HBA values

To define 'true' responders, we first aimed to evaluate the Intraindividual Variation (IV) for each selected parameter in our

cohort. Semen analysis and HBA assay were performed twice (two semen collections at a distance from 1 to 6 months) in 50 untreated subjects (40 enrolled and 10 not-enrolled subjects). The Intraindividual Variation for each parameter was calculated as follows: [(parameter's mean analyzed 'basal 1'−parameter's mean analyzed at 'basal 2')/parameter's mean analyzed at 'basal 2'] × 100. The IV for TSN and TMSC was 84%, whereas for HBA it was 23%.

IVs were used as threshold values to define 'responders' and 'non-responders' in an arbitrary way. For TSN and TMSC, we defined 'responders' those patients presenting improvement above the IV.

For HBA two types of 'responders' were defined: (i) 'responders A' with an improvement above the double of the IV value; (ii) 'responders B' with a % HA binding greater than an absolute HBA value equal or above 60% (compatible with lower limit value for normal semen according to Huszar *et al.*, 2002; Kovacs *et al.*, 2011; Molnar *et al.*, 2014).

Genotyping

Genomic DNA was extracted from EDTA-preserved blood using the Salting-out method. Concentration and purity of DNA were measured with Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Minor Allele Frequency of the three SNPs referring to the general population was obtained from 1000 Genomes, ExAC and GO-ESP. For one patient who has completed the study, it was not possible determining the genotypes.

Molecular analysis for polymorphism rs10835638 of FSHβ

The polymorphism rs10835638 in position −211 of *FSH*β gene was analyzed by PCR followed by RFLP analysis (Restriction Fragment Length Polymorphism analysis) using *Rsa*I restriction enzyme (for more details, see 'Supporting information'). All homozygous TT patients were confirmed by Sanger sequencing.

Molecular analysis for polymorphisms rs6166 and rs1394205 of FSHR

PCR and HRMA (High-Resolution Melting Analysis) using RotoreGene (QIAGEN, Hilden, Germany) were performed to analyze polymorphism rs6166 in position 2039 on exon 10 of *FSHR* gene and polymorphism rs1394205 in position −29 of the same gene (for more details, see 'Supporting information'). Sanger sequencing of samples with three different HRMA profiles was performed to validate the HRMA method. In addition all homozygous mutated samples and samples with ambiguous profile for the two SNPs were confirmed by Sanger sequencing.

Statistical analysis

All analyses were performed with statistical package SPSS (version 23.0; SPSS Inc, Chicago, IL, USA). Comparisons for parameters with normal distribution such as % HBA was performed using the 'Paired samples *T*-test': (i) mean HA binding values before and after 1 and 3 months of FSH treatment; (ii) mean HA binding values between subgroups after the stratification on the basis of: baseline sperm parameters (TSN and TMSC), HA binding, baseline FSH, testis volume and *FSH*β/*FSHR* polymorphisms. 'Non-parametric Mann–Whitney *U* test' for paired samples was used for comparisons of the medians of semen parameters before and after 3 months of FSH treatment. p values are reported and $p < 0.05$ was considered to indicate statistical significance.

Ethical approval

The local Ethics Committees of the AOUC of Florence approved the study (n. 89/12). All participants gave their informed written consent.

RESULTS

From 210 patients 70 were enrolled according to the criteria described in 'Materials and Methods'. During the study, 30 patients were excluded or dropped out for various reasons reported in Figure S1. Forty patients were finally evaluated for semen parameters and HBA at baseline (T0), after 1 (T1) and 3 (T3) months of FSH therapy and after 4–6 months from the FSH therapy discontinuation (*wash-out* or second baseline value). As HBA necessitates a minimum number of motile spermatozoa, this test was not possible to perform in 2/40 patients at T1. The mean values of each analyzed parameter in our study population are shown in Table S1. No drug-related adverse effect was observed.

FSH treatment vs. HBA

After 1 month of FSH therapy HBA test showed a significant improvement of spermatozoa % HA binding capacity from T0 (30.04 ± 2.23%) to T1 (41.22 ± 3.26%; $p < 0.001$) showing an average increase of 37% (Fig. 1A).

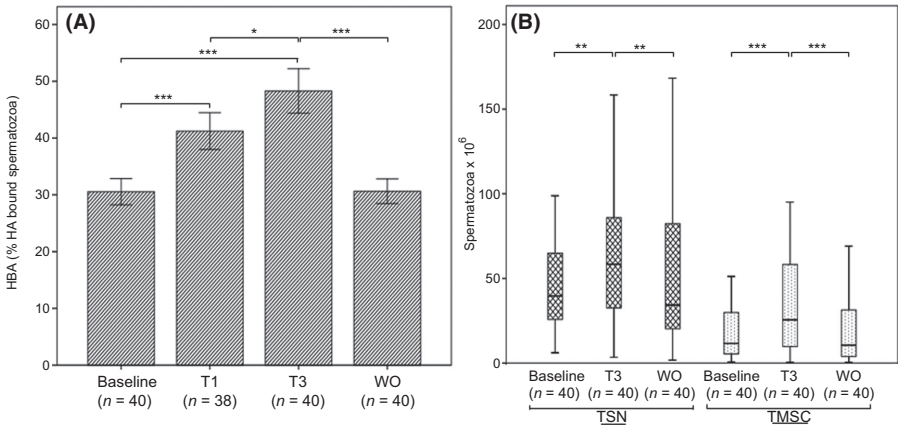
After 3 months of therapy, HA binding capacity further increased, and resulted significantly higher in respect to baseline (47.25 ± 3.79%; $p < 0.001$) with an average 57% increase. The T3 value was significantly higher in respect to T1 value ($p = 0.042$) and importantly, we observed a return to baseline value after the wash-out period of 4–6 months (Fig. 1A). For % HA binding we used two distinct criteria to define responsiveness as indicated in 'Materials and Methods': (i) 'responders A' with an increase equal or above the double of the Intraindividual Variation (>46%); (ii) 'responders B' with % HA binding equal or above an absolute value of 60%. Concerning 'responders A' we observed 42% of patients (16/38) at T1, with a further increase to 63% (25/40) at T3. 'Responders B' (who achieved normalization of % HA binding), were 24% (9/38) after 1 month with a further increase to 35% (14/40) after 3 months of FSH therapy.

Among patients who showed an increase in HBA value during treatment, we observed three distinct patterns (Figure S2): (i) 'rapid responders' (patients with a response to FSH therapy after 1 month and a subsequent *plateau* of HB values at T3); (ii) 'slow responders' (patients with a pronounced improvement of HB values only after 3 months); (iii) 'progressive responders' (patients in whom the HBA values improve progressively throughout the duration of FSH therapy).

FSH treatment vs. selected semen parameters

We evaluated two semen parameters reflecting the entire spermatogenic cycle: TSN and TMSC. We found a statistically significant improvement of TSN and TMSC after 3 months of treatment and a return to near-baseline values after FSH therapy wash-out (Fig. 1B). As far as TSN is concerned, a statistically significant improvement was observed from the basal (median:

Figure 1 Dynamic of responsiveness to FSH treatment and comparison of selected semen parameters at different time points of observation: (A) effect of treatment on Hyaluronan Binding capacity of spermatozoa reported as %HBA: the bars show the HBA mean values, the whiskers cover the SEM (Standard Error of Mean) of the raw data; (B) effect of treatment on Total Sperm Number (TSN) and Total Motile Sperm Count (TMSC): the boxes represent the 25th and 75th percentiles, the whiskers cover the minimum and maximum of the raw data, the median value is denoted as the line that bisects the boxes. HBA, Hyaluronan Binding Assay; HA, hyaluronic acid; Baseline = before the therapy; T1 = after 1 month of therapy; T3 = after 3 months of therapy; WO = second baseline after 4–6 months from hpFSH withdrawal. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$. p values referring to the comparison between WO vs. T1/T3 are the same as for T0; no significant differences between T0 vs. WO.



39.72×10^6, min–max: 6.13–221×10^6) vs. T3 value (median: 58.38×10^6, min–max: 3.49–300.60×10^6) ($p = 0.002$). Similarly, we found a significant increase in TMSC when we compared the T0 value (median: 11.59×10^6, min–max: 0.64–85.69×10^6) with T3 value (median: 25.55×10^6, min–max: 0.37–95.04×10^6) ($p = 0.000$) with an average increase of 121%. In addition, we found a significant increase in sperm morphology from baseline (median: 1%, min–max: 1–5%) to T3 (median: 2%, min–max: 1–7%) ($p = 0.008$) after FSH therapy.

On the basis of the previously defined Intraindividual Variation (IV), we divided the patient cohort into 'responders' (>IV) and 'no responders' (<IV) for the two selected parameters. Patients responding in terms of TSN and TMSC were 30% (12/40) and 53% (21/40), respectively.

Search for predictive parameters for HBA responsiveness

Baseline sperm parameters

Total sperm number. In this study, we included infertile males, regardless the TSN at baseline. Consequently, the study population is made up by subjects with TSN below and above the normal range (39 million, as defined according to WHO, 2010 guidelines): 19 men belonged to the >39 million category (48%), with impaired motility/morphology (asthenoteratozoospermic, AT), and 21 (52%) were oligoasthenoteratozoospermic (OAT) patients. We sought to analyze whether HBA responsiveness to FSH therapy was related to basal sperm number.

Sperm-Hyaluronan Binding Assay baseline values were not significantly different between AT and OAT patients. In AT patients, we assessed a significant improvement of HBA value from T0 to T1 ($p = 0.01$) and T3 ($p = 0.001$) showing an average 44 and 77% increase, respectively (Table 1). Similarly, in the OAT group HBA values significantly increased from T0 to T1 ($p = 0.001$) and T3 ($p = 0.004$) with an extent of the improvement of HBA values of 33 and 57%, respectively (Table 1). Finally, the frequency of 'responders' (both type 'A' and 'B') was similar in the two subgroups (AT vs. OAT) after 1 and 3 months of FSH treatment (Table 2). The distribution of the two semen categories in the 'responder' groups is reported in Table S2.

Total motile sperm count. Given that for this parameter there is no normal reference value given by the WHO, we have used a commonly accepted value of TMSC 10×10^6/mL (Ombelet *et al.*, 2014 and references herewith). On this basis, we stratified our cohort in two groups patients with a low baseline TMSC ($<10 \times 10^6$/mL, 19/40, 48%) and patients with 'normal' TMSC ($\geq 10 \times 10^6$/mL, 21/40, 52%). No differences in HA binding baseline values were observed between the two groups. In both groups, a significant increase in HA binding was observed after 1 and 3 months of therapy in respect to baseline: (i) in patients with 'normal' TMSC from T0 to T1 ($p = 0.005$) and T3

Table 1 Comparison of the mean% hyaluronic acid binding before and after FSH therapy in different subgroups based on baseline semen characteristics

	Baseline (mean% ± SD)	T1 (mean% ± SD)	T3 (mean% ± SD)
AT ($n = 19$)	29.46 ± 17.74	41.75 ± 23.24**	50.05 ± 26.38***
OAT ($n = 21$)	30.11 ± 13.08	40.75 ± 17.32***	44.73 ± 21.93**
TMSC < 10×10^6 ($n = 19$)	30.40 ± 13.35	41.04 ± 17.20**	45.88 ± 21.34**
TMSC ≥ 10×10^6 ($n = 21$)	29.72 ± 15.08	41.39 ± 22.79**	48.50 ± 26.61***
Baseline HBA < 30% ($n = 21$)	18.45 ± 6.66	28.79 ± 16.11**	34.78 ± 21.48***
Baseline HBA ≥ 30% ($n = 19$)	42.85 ± 7.21	53.66 ± 15.54*	61.04 ± 18.73**

Baseline = mean value obtained from two observations: prior therapy and wash-out (after therapy); T1 = after 1 month of therapy; T3 = after 3 months of therapy; SD, standard deviation; AT, asthenoteratozoospermic; OAT, oligoasthenoteratozoospermic; TMSC, total motile sperm count; HBA, Hyaluronan Binding Assay. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

Table 2 Comparison of the frequency (number) of 'responders A' and 'B' in different subgroups based on baseline semen characteristics

	AT vs. OAT	TMSC < 10×10^6 vs. TMSC ≥ 10×10^6	HBA < 30% vs. HBA ≥ 30%
Responders			
'A'			
Baseline-T1	44% (8/18) vs. 40% (8/20)	39% (7/18) vs. 45% (9/20)	47% (9/19) vs. 37% (7/19)
Baseline-T3	68% (13/19) vs. 57% (12/21)	58% (11/19) vs. 67% (14/21)	67% (14/21) vs. 58% (11/19)
'B'			
Baseline-T1	28% (5/18) vs. 20% (4/20)	22% (4/18) vs. 25% (5/20)	5% (1/19) vs. 42% (8/19)*
Baseline-T3	42% (8/19) vs. 29% (6/21)	26% (5/19) vs. 43% (9/21)	14% (3/21) vs. 58% (11/19)**

'Responders A' = hyaluronic acid (HA) binding more than double Intraindividual Variation (46%); 'Responders B' = HA binding ≥60%; Baseline = mean value obtained from two observations: prior therapy and wash-out (after therapy); T1 = after 1 month of therapy; T3 = after 3 months of therapy; AT, asthenoteratozoospermic; OAT, oligoasthenoteratozoospermic; TMSC, Total Motile Sperm Count; HBA, Hyaluronan Binding Assay.
*$p \leq 0.05$; **$p \leq 0.01$.

($p < 0.001$) showing an average 39 and 63% increase, respectively (Table 1); (ii) in patients with a low TMSC from T0 to T1 ($p = 0.002$) and T3 ($p = 0.007$) showing an average 35 and 51% increase, respectively (Table 1). The extent of the observed improvements was not different between the two subgroups. Also for this parameter the frequency of 'responders' (both type 'A' and 'B') was similar in the two subgroups after FSH treatment (Table 2). The distribution of the two semen categories in the 'responder' groups are reported in Table S3.

Sperm-Hyaluronan Binding Assay. On the basis of the basal median % HA binding at T0, we divided our cohort into two groups: patients with a baseline % HA binding <30% (21/40, 53%) and patients with % HA binding ≥30% (19/40, 47%). This cut-off value was chosen based on a previous article by Mokánszki *et al.* (2012), reporting the HBA value of 30% as the mean value in oligoasthenozoospermic patients. An additional reason for using this value is that this cut-off divided our study population into two groups with equal number of patients, making statistical analysis more reliable.

Both groups displayed a significant improvement for HBA values: (i) patients with HBA baseline values <30% from T0 to T1 ($p = 0.002$) and T3 ($p = 0.001$) showing an average 56 and 89% increase, respectively; (ii) patients with HBA baseline values ≥30% from T0 to T1 ($p = 0.01$) and T3 ($p = 0.002$) showing an average 25 and 42% increase, respectively (Table 1). Despite a more pronounced improvement in the group of patients with baseline HBA < 30%, the average increase for any time point was not statistically different between the two subgroups.

The frequency of 'responders A' between the two subgroups was similar, whereas as expected, there was a significant difference concerning 'responders B' (Table 2). In fact, when we considered the threshold of % HA binding ≥60% ('normalization' of HBA), a significantly higher percentage of patients with baseline % HA binding ≥30% showed a normalization of the % HA binding both after 1 (42% vs. 5%, $p = 0.019$, OR = 13.09 with 95% CI = 1.44–119.34) and 3 months (58% vs. 14%, $p = 0.007$, OR = 8.25 with 95% CI = 1.80–37.88). Of note, 5/8 (63%) of 'responders B' showed a baseline % HA binding <50%, with a mean of 36 ± 4.7%.

Baseline FSH and testis volume

We did not find any difference concerning baseline FSH values or testicular volumes between HBA 'responders' and 'non-responders' groups (both type 'A' and type 'B') (Table S1).

FSHβ and FSHR polymorphisms

We stratified our population according to *FSH*β −211G>T, to *FSHR* 2039A>G and to *FSHR* −29G>A genotypes. Baseline characteristics of patients with different genotypes are listed in Table S3. No significant difference was found between patients with 'wild-type' and at least one mutant allele when we compared baseline HA binding, TSN, seminal volume, sperm morphology, TMSC, FSH, LH, testosterone and testicular volume.

We compared the allelic distribution of the three SNPs in our cohort vs. that reported in the general population (data from 1000 Genomes, ExAC and GO-ESP): (i) the frequency of the mutated T-allele for *FSH*β −211G>T SNP was significantly increased in our patients vs. the general population (15.2% vs. 8.4%, respectively, $p = 0.028$); (ii) a slightly higher (but no statistically significant) frequency of the mutated G-allele in our cohort for *FSHR* 2039A>G SNP (53% vs. 41% in the general population); (iii) similar allelic distribution for *FSHR* −29G>A SNP in the two groups (21% vs. 34%).

Considering *FSH*β −211G>T SNP genotypes, 25 patients (64%) were GG homozygotes ('wild-type group') and 14 (36%) were GT heterozygotes or TT homozygotes ('mutant group'). In both groups there was a significant difference concerning the HBA values between baseline and after 1 ($p = 0.001$ and $p = 0.019$) and 3 months ($p < 0.001$ and $p = 0.016$) of FSH treatment (Table 3).

Considering *FSHR* 2039A>G SNP genotypes, eight patients (21%) were AA homozygotes ('wild-type group') and 31 (79%) were either AG heterozygotes or GG homozygotes ('mutant group'). Concerning the 'wild-type' genotype, there was a statistical difference in HBA value only between baseline and after 3 months ($p = 0.012$) of FSH treatment. In the 'mutant' (AG and GG) genotype subgroup, there were statistical differences in HBA values between baseline and after both 1 and 3 months therapy ($p < 0.001$ for both) (Table 3).

Considering *FSHR* −29G>A SNP genotypes, 21 patients (54%) were GG homozygotes ('wild-type group') and 18 (46%) were GA heterozygotes or AA homozygotes ('mutant group'). There was a significant difference in HBA value in both groups between baseline and after 1 ($p = 0.028$ and $p < 0.001$) and 3 months ($p = 0.003$ and $p = 0.002$) of FSH treatment (Table 3).

The extent of improvement of HBA values from T0 to T1 and to T3 were similar in the different genotypes, with the exception of *FSHR* 2039A>G at T1 (Table S4).

Table 3 Comparison between mean% hyaluronic acid binding at T0, T1 and T3 between patients stratified on the basis of *FSHβ* −211G>T, *FSHR* 2039A>G and *FSHR* −29G>A genotypes

	HAB (mean% ± SD)		
	Baseline	T1	T3
FSHβ −211G>T			
GG (n = 25)	31.55 ± 15.41	41.45 ± 20.05***	47.30 ± 20.52***
GT-TT (n = 14)	27.92 ± 12.02	40.78 ± 20.91*	48.77 ± 30.12*
FSHR 2039A>G			
AA (n = 8)	27.74 ± 12.07	37.64 ± 16.36	47.19 ± 16.14*
AG-GG (n = 31)	30.90 ± 14.84	42.03 ± 20.96***	47.99 ± 25.88***
FSHR −29G>A			
GG (n = 21)	31.44 ± 13.14	41.20 ± 20.86*	48.84 ± 21.6**
GA-AA (n = 18)	28.85 ± 15.67	41.25 ± 19.7***	46.64 ± 27.15**

Baseline = mean value obtained from two observations: prior therapy and wash-out (after therapy); T1 = after 1 month of therapy; T3 = after 3 months of therapy; SD, standard deviation. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

DISCUSSION

FSH and Testosterone are crucial hormones for a quantitatively and qualitatively normal spermatogenesis. Consequently, both FSH and human chorionic gonadotropin (able to stimulate the Testosterone synthesis in Leydig cells) are necessary in patients affected by hypogonadotrophic hypogonadism. In case of idiopathic infertility the treatment is limited to FSH because Testosterone level in these patients is normal. Two meta-analyses suggest that therapy with exogenous FSH (for at least 3 months) in idiopathic infertile men with normal FSH levels promotes a quantitative and qualitative improvement of semen parameters and pregnancy rate (Attia *et al.*, 2013; Santi *et al.*, 2015).

Our study is the first focusing on the effect of hpFSH treatment on a functional sperm parameter called HA binding capacity. HA binding capacity is a faithful biomarker of the entire cycle of spermatogenesis, in particular of the final phase of this process, called 'spermiogenesis'. In fact, this sperm parameter reflects the degree of maturation, the morpho-structural, functional and genomic integrity of spermatozoa. Accordingly, our aim was to evaluate the effect of FSH treatment not only after the entire cycle of spermatogenesis (3 months) but also to test whether spermiogenesis (the last 1 month of spermatogenesis) would be also affected positively by the drug. In fact, several experimental evidences support the concept that FSH in concert with Testosterone is essential also in the regulation of the latest phases of spermatogenesis. In vitro studies on human germ cells showed that FSH stimulates meiosis II and round spermatid flagellum extrusion, whereas Testosterone potentiates FSH action and stimulates late spermatid differentiation (Sousa *et al.*, 2002). Both hormones are considered as survival factor for spermatocytes and spermatids through the regulation of both the intrinsic and the extrinsic apoptotic pathways (Ruwanpura *et al.*, 2008). In FSH receptor knockout mice, there is a disturbance in the normal replacement of histones by protamines during spermiogenesis, leading to poor condensation of spermatid nuclei (Krishnamurthy *et al.*, 2000). Previous human studies demonstrated a positive effect of FSH therapy on ultrastructural sperm characteristics and DNA condensation (Bartoov *et al.*, 1994; Baccetti *et al.*, 1997, 2004; Kamischke *et al.*, 1998; Ben-Rafael *et al.*, 2000; Piomboni *et al.*, 2009). Moreover, recent studies showed that FSH therapy improves sperm DNA integrity in men with idiopathic oligoasthenozoospermia by reducing DNA fragmentation index (Palomba *et al.*, 2011; Colacurci *et al.*, 2012; Ruvolo *et al.*, 2013).

To date, no data are available on the effect of FSH therapy on HA binding capacity in idiopathic infertile males. HA is the main constituent of the extracellular matrix surrounding the cumulus, crucial in the natural fertilization process. Only mature spermatozoa have the ability to bind HA and thus the cumulus since only after the completion of the maturation process they exhibit HA receptors on their surface. The ability of spermatozoa to bind HA indicates adequate maturation and morphology, a lower level of chromosomal aneuploidies, a lower DNA fragmentation level, a greater chromatin integrity and, consequently, a greater fertilizing potential (Huszar *et al.*, 2003; Jakab *et al.*, 2005; Prinosilova *et al.*, 2009; Yagci *et al.*, 2010; Worrilow *et al.*, 2013).

In our study, we evaluated the percentage of spermatozoa able to bind HA after 1 (T1) and 3 months (T3) of purified FSH treatment (75 IU/L every other day) in patients affected by idiopathic infertility with oligo-, astheno- or teratozoospermia, normal FSH values (1.5–8 IU/L) and baseline HBA values <60%. We found a significant improvement of HA binding capacity at T1 and at T3 with a 37 and 57% increase, respectively. We observed a high proportion of responsive patients both after one (46% 'responders A') and 3 months of therapy (62% of 'responders A'), reaching in 35% of them a value higher than the HBA 'normality' threshold (>60%). It is important to note that although it is not a double-blind placebo controlled trial, the study design included two baseline evaluations: prior and after the end of the FSH therapy (wash-out). After the wash-out, the observed return of the values to the baseline (pre-therapy) values, supports a potential causative relationship between the drug administration and the observed improvement of the sperm parameters.

Besides the effect on a functional parameter such as HA capacity, we focused our attention on selected sperm parameters, i.e. TSN and the TMSC. After 3 months of therapy (corresponding to an entire spermatogenic cycle), we observed a statistically significant increase in both TSN ($p < 0.001$) and TMSC ($p < 0.001$), with an extent of improvement of 44 and 138% compared with baseline, respectively. Similarly, to HBA also for these parameters we observed a return to the baseline values after 4–6 months from the end of therapy. The percentage of 'responders' (response greater than the Intraindividual Variation in our cohort) for TSN and TMSC was equal to 28 and 53%, respectively. We therefore showed, in line with previous studies that FSH therapy is able to substantially improve semen parameters in about 50% of men affected by idiopathic infertility (Foresta *et al.*, 2002, 2005; Ferlin *et al.*, 2011; Selice *et al.*, 2011).

Our study demonstrates the efficacy of hpFSH administration in idiopathic infertile men, in improving not only TSN and TMSC (after 3 months of treatment) but also functional parameters, such as HA capacity (both after 1 and 3 months of treatment). However, as previously reported, this improvement was found in approximately 30–50% of subjects, depending on the parameter analyzed. The reason behind 'responsiveness' remains largely unclear. We have taken into consideration a series of baseline parameters to evaluate their predictive value for predicting HBA responsiveness. To this purposes we compared subgroups based on baseline values of sperm count

(oligozoospermic vs. astheno/teratozoospermic patients), TMSC (< or >10 millions spermatozoa), FSH value, bilateral testis volume and HBA value. A significant increase in the HBA value was found in all subgroups indicating a lack of predictive power of the above baseline parameters. Concerning 'responders B', i.e. patients achieving HBA absolute values ≥60%, which has been proposed as a 'cut-off' for a less invasive-assisted reproduction techniques such as IUI or even natural conception (Huszar *et al.*, 2002, 2007), we did observed differences between the two subgroups. As expected, a significantly higher percentage of 'responders B' belonged to the subgroup of baseline HBA value 30–60%. In particular, patients with higher HBA baseline values showed a probability almost 13 times higher (OR = 13.09; 95% CI = 1.44–119.34) to reach and/or exceed the clinical cut-off of 60% after 1 month of therapy. Interestingly, also 14% of subjects with HBA value <30% showed a 'normalization' at T3. This percentage reached to 58% in the subgroup with HBA 30–60% with a mean baseline HBA value of 36 ± 4.7%. Our finding shows that the 'normalization' of the HBA values is an achievable objective with FSH treatment both after 1 and 3 months.

In the last few years, several studies highlighted the influence of *FSH*β and *FSHR* polymorphisms on men's gonadic function and reproductive parameters (Grigorova *et al.*, 2008, 2010, 2011, 2013, 2014; Tüttelmann *et al.*, 2012). To date, only three pharmacogenetic studies have been conducted in relationship with two polymorphisms. Selice *et al.* (2011) and Ferlin *et al.* (2011) reported a strict correlation between the presence of *FSH*β (−211G>T) and *FSHR* (Asn680Ser and Thr307Ala) polymorphisms and response to treatment. In their studies, patients carrying the mutated allele of *FSH*β and *FSHR* showed a significantly higher improvement of the 'classical' seminal parameters (total sperm count, motility and morphology) than patients with 'wild type' genotype. On the other hand, a recent paper from Simoni *et al.* (2016) demonstrated that FSH administration is effective in reducing sperm DNA fragmentation in men carrying the combined *FSHR* p.N680S N homozygous and *FSH*β −211G>T G homozygous genotypes (i.e. 'wild type' genotypes).

We have analyzed three different SNPs: *FSH*β −211G>T, *FSHR* Asn680Ser and *FSHR* −29G>A. Ferlin *et al.* (2011) reported that 100% of patients with the mutated genotype responded to the therapy in terms of doubling of the sperm count after 3 months of treatment. Such an effect was not detectable in our two patients carrying the mutated 'T' allele in homozygosis and both resulted 'non-responders' for TSN after 3 months of treatment. Although our study is not powered to detect relatively milder effect of the genotype on FSH responsiveness, based on our data we can conclude that there is no clear-cut effect of the genotype in predicting response to treatment, neither with regard to classical semen parameters, nor to HA binding capacity. Also concerning the two other SNPs we were unable to detect a 'responsive' or 'non-responsive' genotype; hence, we did observe a significant improvement of HBA values regardless to which genotype subgroups belonged our patients. The combination of different alleles is likely to be relevant not only to testicular volume and circulating FSH level (Grigorova *et al.*, 2008, 2010, 2011, 2013, 2014; Tüttelmann *et al.*, 2012) but also in regard to FSH responsiveness. The analysis of an exceptionally large study population is needed to address this issue, i.e. to establish the importance of *FSH*β and *FSHR* genotyping prior FSH therapy (Busch *et al.*, 2015).

This study allows us to predict that about 50% of subjects treated with FSH will present a quantitative and qualitative improvement in selected and functional sperm parameters (TMSC and HA binding capacity). More importantly, approximately 30% of all patients treated with hpFSH will reach 'normal' HBA values (>60%) after 3 months. An additional novelty of our study, besides the biomarker analyzed in relation to FSH therapy, is represented by the length of treatment as previous studies have considered a minimum period of 3 months of therapy. We have shown that hpFSH is able to improve both spermatogenesis and spermiogenesis implying that both short and standard regimens are viable therapeutic options. The standard protocol of FSH therapy (3 months), through a quantitative/qualitative improvement of seminal parameters, could be a valuable tool to increase the probability of both natural and ART conception. On the other hand, in view of both the cost and the relative invasiveness of treatment, the short cycle (1 month) would represent an innovative FSH treatment option to increase the proportion of functionally competent cells in the ejaculate prior ART.

In conclusion, our manuscript supports the previously reported positive effect of FSH therapy on spermatogenesis and in addition opens novel exciting perspectives on the efficacy of the short-term FSH treatment in couples undergoing different types of Assisted Reproductive Techniques.

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CONFLICT OF INTEREST

The authors declare that no conflicts of interest are present.

AUTHORS' CONTRIBUTIONS

C.K. designed the study and coordinated the work. E.S., S.B., F.L., M.M., M.E.C., G.F. and C.K. selected and treated the patients. M.G.F. performed semen analysis. S.V. performed Sperm-Hyaluronan Binding Assay (HBA). E.C. performed genetic analysis and with E.S. collected/examined data. C.K., E.S. and E.C. wrote the manuscript. All authors contributed to the final version and approved the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Study population (flow chart).
Figure S2. Different patterns of responsiveness to FSH therapy in terms of HBA values variations.
Table S1. Baseline characteristics of study population.
Table S2. Distribution of the two semen categories in the responders groups.
Table S3. Baseline characteristics of patients with different genotypes.
Table S4. Extent of improvement of % HA binding from T0 to T1 and to T3.
Appendix S1. Materials and methods.

