TREATMENT WITH HUMAN, RECOMBINANT FSH IMPROVES SPERM DNA FRAGMENTATION IN IDIOPATHIC INFERTILE MEN DEPENDING ON THE FSH RECEPTOR POLYMORPHISM p.N680S: A PHARMACOGENETIC STUDY

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STUDY QUESTION: Does the sperm DNA fragmentation index (DFI) improve depending on the FSH receptor (FSHR) genotype as assessed by the nonsynonymous polymorphisms rs6166 (p.N680S) after 3 months of recombinant FSH treatment in men with idiopathic infertility?

SUMMARY ANSWER: FSH treatment significantly improves sperm DFI only in idiopathic infertile men with the p.N680S homozygous N FSHR.

WHAT IS KNOWN ALREADY: FSH, fundamental for spermatogenesis, is empirically used to treat male idiopathic infertility and several studies suggest that DFI could be a candidate predictor of response to FSH treatment, in terms of probability to conceive. Furthermore, it is known that the FSHR single nucleotide polymorphism (SNP) rs6166 (p.N680S) influences ovarian response in women and testicular volume in men.

STUDY DESIGN, SIZE AND DURATION: A multicenter, longitudinal, prospective, open-label, two-arm clinical trial was performed. Subjects enrolled were idiopathic infertile men who received 150 IU recombinant human FSH s.c. every other day for 12 weeks and were followed-up for a further 12 weeks after FSH withdrawal. Patients were evaluated at baseline, at the end of treatment and at the end of follow-up.
**PARTICIPANTS/MATERIALS, SETTING, METHODS:** Eighty-nine men with idiopathic infertility carrier of the FSHR p.N680S homozygous N or S genotype, FSH < 8 IU/l and DFI > 15%, were enrolled. A total of 66 patients had DFI analysis completed on at least two visits. DFI was evaluated in one laboratory by TUNEL/PI (propidium iodide) assay coupled to flow cytometry, resolving two different fractions of sperm, namely the ‘brighter’ and ‘dimmer’ sperm DFI fractions.

**MAIN RESULTS AND THE ROLE OF CHANCE:** Thirty-eight men (57.6%) were carriers of the p.N680S homozygous N and 28 (42.4%) of the homozygous S FSHR. Sperm concentration/number was highly heterogeneous and both groups included men ranging from severe oligozoospermia to normozoospermia. Total DFI was significantly lower at the end of the study in homozygous carriers of the p.N680S N versus p.N680S S allele (P = 0.008). Total DFI decreased significantly from baseline to the end of the study (P = 0.021) only in carriers of the p.N680S homozygous N polymorphism, and this decrease involved the sperm population containing vital sperm (i.e. brighter sperm) (P = 0.008). The dimmer sperm DFI fraction, including only nonvital sperm, was significantly larger in p.N680S S homozygous patients than in homozygous N men (P = 0.018). Total DFI was inversely related to total sperm number (P = 0.020) and progressive sperm motility (P = 0.014). When patients were further stratified according to sperm concentration (normozoospermic versus oligozoospermic) or -211G>T polymorphism in the FSHB gene (rs10835638) (homozygous G versus others), the significant improvement of sperm DFI in FSHR p.N680S homozygous N men was independent of sperm concentration and associated with the homozygous FSHB -211G>T homozygous G genotype.

**LIMITATIONS, REASONS FOR CAUTION:** The statistical power of the study is 86.9% with alpha error 0.05. This is the first pharmacogenetic study suggesting that FSH treatment induces a significant improvement of total DFI in men carriers of the p.N680S homozygous FSHR; however, the results need to be confirmed in larger studies using a personalized FSH dosage and treatment duration.

**WIDER IMPLICATIONS OF THE FINDINGS:** The evaluation of sperm DFI as a surrogate marker of sperm quality, and of the FSHR SNP rs6166 (p.N680S), might be useful to predict the response to FSH treatment in men with idiopathic infertility.

**STUDY FUNDING/COMPETING INTEREST(S):** The study was supported by an unrestricted grant to M.S. and H.M.B. from Merck Serono that provided the drug used in the study. M.S received additional grants from Merck Serono and IBSA as well as honoraria from Merck Serono. The remaining authors declare that no conflicts of interest are present.

**TRIAL REGISTRATION NUMBER:** EudraCT number 2010-020240-35.

**Key words:** FSH / male infertility / FSH receptor / FSHR pN680S / FSHB -211G/T / sperm DNA fragmentation index

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**Introduction**

Male factor infertility is involved in ~50% of cases of couple infertility, although its pathogenesis remains largely unknown (Kumar and Singh, 2015). In most cases, the treatment of male infertility relies on assisted reproductive technology (ART), which is expensive and relatively inefficient and represents a remarkable example of gender inequity (Chansel et al., 2015).)

Several clinical trials were conducted to evaluate the efficacy of FSH treatment of the male improves the couples’ pregnancy rate for their partner, both spontaneous and after ART (Santi et al., 2015). However, the response to FSH treatment is variable and unpredictable, mainly because the factors influencing the response to FSH are poorly understood (Santi et al., 2015).

The statistical power of the study is 86.9% with alpha error 0.05. This is the first pharmacogenetic study suggesting that FSH treatment induces a significant improvement of total DFI in men carriers of the p.N680S homozygous FSHR; however, the results need to be confirmed in larger studies using a personalized FSH dosage and treatment duration.

The evaluation of sperm DFI as a surrogate marker of sperm quality, and of the FSHR SNP rs6166 (p.N680S), might be useful to predict the response to FSH treatment in men with idiopathic infertility.

The study was supported by an unrestricted grant to M.S. and H.M.B. from Merck Serono that provided the drug used in the study. M.S received additional grants from Merck Serono and IBSA as well as honoraria from Merck Serono. The remaining authors declare that no conflicts of interest are present.

Despite the possible FSHR genotype influence on spermatogenesis, this effect is not easily measurable considering the wide variability in male fertility-related parameters. Conventional semen analysis describes only visible features of spermatozoa and does not allow any prediction of the genetic constitution of the male gamete. The sperm DNA fragmentation index (DFI) was recognized as a predictor of the probability to conceive (Muratori et al., 2015a,b), and FSH treatment seems to improve this parameter in infertile men (Colacurci et al., 2012). The introduction and validation of the TUNEL/propidium iodide (PI) method allowed distinguishing between the brighter sperm fraction (containing viable sperm) and the dimmer fraction (where sperm are all dead) (Muratori et al., 2008, 2015a,b). The brighter DFI fraction predicts natural conception,
independently of age and semen parameters (Muratori et al., 2015a,b).

Therefore, DFI in the brighter and dimmer fraction represents a promising pharmacodynamic parameter of FSH action.

Overall, it appears that FSH treatment improves pregnancy rate in couples, in which the male partner presented idiopathic infertility but the response to FSH is variable and unpredictable. The main objective of this study is the evaluation of whether FSH is differently effective on sperm DFI in men with idiopathic infertility depending on the FSHR p.N680S polymorphism.

Materials and Methods

Study design

A Phase IIb, multicenter, prospective, open-label, one arm, clinical trial, stratified according to the patient’s FSHR genotype was carried out (trial registration number: EuadrCT 2010-020240-35). The centers participating in the study were: Unit of Endocrinology, Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Italy; Department of Gynecology, Humanitas Fertility Center, Research Hospital, Rozzano (Milan), Italy; Center for Reproductive Medicine and Andrology, Martin Luther University Halle-Wittenberg, Germany; Sexual Medicine and Andrology Unit, University of Florence, Italy; Department of Experimental Medicine, University of Rome ‘La Sapienza’, Italy; Unit of Andrology and Reproductive Medicine, University of Padova, Italy.

The study compared the response with the same FSH regimen in terms of changes in sperm DFI. Only FSHR p.N680S N and S homozygous men were included in the study. The FSHR p.N680S genotype was analyzed in one laboratory and the treating physician received only the information on whether the patient was eligible for entering the trial (i.e., homozygous) but both the physician and the patient remained blind to the genotype.

Subsequently, the response to the same FSH regimen was evaluated considering also the FSHB -211G>T genotype.

All subjects included in the study underwent a treatment phase of 12 weeks, receiving recombinant FSH therapy ( follitropin alpha: Gonal-f) 150 IU s.c. every other day. The follow-up phase continued for a further 12 weeks after the end of the treatment.

Subjects

Inclusion criteria were: age 20–50 years, idiopathic male factor infertility for at least 1 year, homozygous FSHR p.N680S N or S genotype, sperm DFI > 15%, normal serum FSH levels (<8 IU/I), normal serum LH, testosterone, prolactin and estradiol levels, as measured at the recruiting centers and considering the reference range of the method used in each center. A normal ovulatory female partner was required. Exclusion criteria were azoospermia, all known etiologies of male infertility, such as endocrine disorders, varicocele, cryptorchidism, infections, immunological infertility and obstructive infertility, and genetic disorders causing infertility, such as chromosome abnormalities, congenital bilateral absence of the vas deferens and microdeletions within the AZF regions of the Y chromosome. (Esteves and Chan, 2015). Moreover, all known etiologies of female infertility in the partner (such as tubal occlusion, endometriosis, endocrine abnormalities including anovulation and polycystic ovariies, anatomical abnormalities, infections), heterozygous FSH-R p.N680S genotype, drug abuse, major systemic diseases and primary testicular failure were reasons for exclusion. ART could be planned during the study, although it was neither an inclusion nor an exclusion criterion.

Each center involved in the study evaluated, enrolled, treated and followed up the patients. Written informed consent was obtained from all participants, and the Institutional review Board approval for the study was obtained.

Methods and patient allocation

Randomization was not applicable as all men included in this study were treated with the same dose of FSH. A blood EDTA sample for DNA analysis was sent at room temperature to the Department of Endocrinology, Modena, Italy for analysis of FSHR genotype. The investigators at the study sites as well as the patients were only informed whether the patient was homozygous for the FSHR gene at codon 680 or heterozygous. All persons involved in analysis of the FSHR genotype were not involved in the collection of any other study data. All persons involved in data collection as well as the patients remained blinded up to final database closure.

Semen analysis and basal hormone determination to assess eligibility criteria were performed at the study sites (WHO, 2010). A blood sample was collected at each visit and serum was stored at −20°C at the sites until the end of the study, when sera were shipped to the coordinating center in Modena for hormone analysis. For DFI determination, sperm were fixed and labeled using TUNEL at the study sites and shipped to the Andrology Laboratory of Florence for flow cytometric analysis within 1 week from collection.

At each study visit, a serum sample was prepared and stored at −20°C until the shipment to the Central Laboratory in Modena.

The study was independently approved by the Ethics Committee of each participating center (file Number of the coordinating center: 114/10). Each participant gave written informed consent.

Hormone assays

At the end of the study, serum total testosterone was measured by Chemiluminescent Microparticle Immunoassay (Architect, Abbott, Dundee, UK), with inter- and intra-assay coefficients of variation (CV) of 5.2 and 5.1%, respectively. Serum total estradiol was measured by Chemiluminescent Microparticle Immunoassay (Architect, Abbott, Longford, Ireland) with inter- and intra-assay CV of 7.4 and 6.4%, respectively. The analytical sensitivity was ≤10 pg/ml, linearity to 1000 pg/ml. The cross-reactivity with estrone and with 17β-estradiol 3-sulfate was 0.7 and 0.1%, respectively. FSH and LH were measured by Chemiluminescent Microparticle Immunoassay (Architect, Abbott, Longford, Ireland) with inter- and intra-assay CV of 4.1 and 3.1% for LH, and 4.6 and 4.2% for FSH, respectively. Inhibin B was measured by enzyme-linked immunosorbent assay (Inhibin B Gen II ELISA, Beckman Coulter, Germany) with inter- and intra-assay coefficients of variation of 4 and 6%, respectively. Anti-Müllerian hormone (AMH) was measured by a paramagnetic particle chemiluminescent immunoassay (Access Beckman Coulter; Germany) with inter- and intra-assay CV of 4 and 5.8%, respectively.

Genetic analyses

Genomic DNA was purified from whole peripheral blood with the automatic nucleic acids extractor EZ1 Advanced XL (Qiagen, Hilden, Germany) using the EZ1 DNA Blood Kit (Qiagen, Hilden, Germany). DNA concentration was measured with the UV spectrophotometer UV–1601 (Shimadzu, Milan, Italy). The single nucleotide polymorphisms (SNPs) rs6166 (NM_000145.3:c.2039G>A, NP_000136.2:p.S680N) and rs10835638 (NG_008144.1: g.4790G>T) were genotyped with the high-resolution melting technology, on a CFX96 Real-Time PCR detection system (Bio-Rad, Laboratories, Hercules, CA, USA), using the Eva Green Supermix (Bio-Rad), according to the manufacturers’ protocol. This system is subject to regular annual checks and calibrations and quality reports are released, in accordance with the lab accreditation program of the region Emilia Romagna (http://www.cgr.unimore.it/cgi-bin/cgr/gruppi.pl/View/doc=endolab.html). Primers used to genotype rs6166 and rs10835638 (328-FSH-R-Fw 5′-AAACCCCCATCCAAGGAT-3′, 329-FSH-R-Rev 5′-ATGACTTGGGGAACAAGG-3′; 638-FSH-B-Fw 5′-GGGGCATCACTGTATC-3′; 639-FSH-B-Rev 5′-AATGTTACTAGATGATGATT-3′, respectively)
were designed with the software Beacon Designer 7 (Bio-Rad). A number (10%) of samples, randomly chosen, were further confirmed through direct sequencing on a 4-capillary ABI-Prism 3130 Genetic Analyzer instrument (Applied Biosystems, Foster City, CA, USA).

Sperm DFI analyses
Sperm DNA fragmentation was determined by the TUNEL/PI method (Muratori et al., 2008, 2010). Briefly, after steps of washing, fixation (4% paraformaldehyde for 30 min at room temperature) and permeabilization (0.1% sodium citrate/0.1% Triton X-100, 4 min in ice), samples were labeled for TUNEL using the ‘In Situ Cell Death Detection Kit, fluorescein’ (Roche Molecular Biochemicals, Milan, Italy). Samples were shipped in the dark at 4°C to the Laboratory of Andrology of the University of Florence within 7 days from collection (Muratori et al., 2010). This protocol allows the determination of the percentage of DNA fragmented spermatozoa in neat semen eliminating all the interference present, such as leucocytes, immature germ cells and apoptotic bodies (Muratori et al., 2008, 2010). At the same time, this method quantifies DNA fragmentation in two separate cytometric populations of spermatozoa, dimmer DFI and brighter DFI spermatozoa, characterized by different extents of DNA fragmentation as well as different associations with semen parameters (Muratori et al., 2015a,b). In addition, the amount of apoptotic bodies (Marchiani et al., 2007) was evaluated in all samples (Lotti et al., 2012; Tamburrino et al., 2012).

Statistical analysis
Statistical analysis was performed using the Statistical Package for the Social Sciences software for Macintosh (version 21.0; SPSS, Inc., Chicago, IL, USA) through the ‘intention-to-treat’ method (Hollis and Campbell, 1999).

Patients were first divided according to the genotype of the rs6166 in the FSHR, forming two groups: homozygous FSHR p.N680S N and homozygous FSHR p.N680S S men, respectively. In addition, patients were stratified considering the FSHR p.N680S polymorphism together with sperm parameter classification. Furthermore, considering the effects of FSHB-211G>T SNP on serum FSH action (Grigorova et al., 2008) which were described in detail only after this study began (Grigorova et al., 2010, 2011; Tuttelmann et al., 2012), patients were further stratified according to the FSHR p.N680S polymorphism together with FSHB-211G>T SNP.

Comparison of variables among different groups was performed by univariate analysis of variance when they showed a normal distribution, whereas Mann–Whitney or Kruskal–Wallis tests were applied for not normally distributed parameters. Post hoc tests were performed by Dunnet test. Parameters were compared through Spearman’s correlation coefficient.

For all comparisons, *P* < 0.05 was considered statistically significant. The statistical power of the study, considering the primary end-point variation, was 0.87, with an *α* error = 0.05.

Results
Of the 163 patients screened, 89 were enrolled, 66 Caucasian men completed the first analysis (38 p.N680S N homozygous and 28 p.N680S S homozygous, respectively) and 55 completed the study (Fig. 1). Median age of the 55 patients who completed the study was 37.0 years (interquartile range: 25% 33.5; 50% 37.0; 75% 39.0 years; min 26.0; max 48.0 years). A total of 11 drop-outs were registered during the study, 5 from the p.N680S N homozygous group and 6 from the p.N680S S homozygous group (Fig. 1). No drug-related adverse events were registered and dropping out was entirely the patient’s decision.

No significant differences were found at Visit 1 (baseline) between the two groups in hormonal and semen parameters analyzed (Table I).
Table I  Variables at each study visit, dividing idiopathic infertile men according to FSH receptor (FSHR) p.N680S polymorphism.

<table>
<thead>
<tr>
<th></th>
<th>Visit 1</th>
<th>Visit 2</th>
<th>Visit 3</th>
<th>P-Value</th>
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<tbody>
<tr>
<td>Total testosterone (ng/ml)</td>
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<tr>
<td>p.N680S S homozygous</td>
<td>5.48 ± 2.16</td>
<td>5.52 ± 2.16</td>
<td>5.79 ± 2.30</td>
<td>0.779</td>
</tr>
<tr>
<td>p.N680S N homozygous</td>
<td>5.40 ± 1.61</td>
<td>5.68 ± 2.03</td>
<td>5.69 ± 1.87</td>
<td>0.833</td>
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<tr>
<td>Estradiol (pg/ml)</td>
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<tr>
<td>p.N680S S homozygous</td>
<td>32.96 ± 9.26</td>
<td>32.60 ± 9.44</td>
<td>31.95 ± 8.78</td>
<td>0.926</td>
</tr>
<tr>
<td>p.N680S N homozygous</td>
<td>33.26 ± 8.65</td>
<td>29.68 ± 5.92</td>
<td>30.90 ± 7.05</td>
<td>0.275</td>
</tr>
<tr>
<td>FSH (IU/l)</td>
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<tr>
<td>p.N680S S homozygous</td>
<td>3.55 ± 1.53</td>
<td>5.08 ± 2.34</td>
<td>3.63 ± 1.42</td>
<td>0.018</td>
</tr>
<tr>
<td>p.N680S N homozygous</td>
<td>3.43 ± 1.70</td>
<td>4.08 ± 1.82</td>
<td>3.24 ± 1.48</td>
<td>0.120</td>
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<tr>
<td>LH (IU/l)</td>
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<tr>
<td>p.N680S S homozygous</td>
<td>2.74 ± 1.02</td>
<td>2.88 ± 1.09</td>
<td>2.84 ± 1.26</td>
<td>0.811</td>
</tr>
<tr>
<td>p.N680S N homozygous</td>
<td>2.89 ± 1.08</td>
<td>2.76 ± 1.14</td>
<td>3.00 ± 1.19</td>
<td>0.831</td>
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<tr>
<td>AMH (ng/ml)</td>
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<tr>
<td>p.N680S N homozygous</td>
<td>5.50 ± 3.19</td>
<td>5.84 ± 3.42</td>
<td>5.09 ± 2.63</td>
<td>0.767</td>
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<tr>
<td>Inhibin B (pg/ml)</td>
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<tr>
<td>p.N680S S homozygous</td>
<td>160.81 ± 53.05</td>
<td>176.80 ± 60.93</td>
<td>181.16 ± 66.66</td>
<td>0.475</td>
</tr>
<tr>
<td>p.N680S N homozygous</td>
<td>194.49 ± 76.27</td>
<td>214.18 ± 119.65</td>
<td>210.63 ± 108.89</td>
<td>0.943</td>
</tr>
<tr>
<td>Total DFI (%)</td>
<td></td>
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<tr>
<td>p.N680S S homozygous</td>
<td>57.79 ± 17.70</td>
<td>58.59 ± 19.70</td>
<td>62.11 ± 21.64</td>
<td>0.817</td>
</tr>
<tr>
<td>p.N680S N homozygous</td>
<td>57.92 ± 16.98</td>
<td>47.28 ± 16.72</td>
<td>43.68 ± 11.99</td>
<td>0.013</td>
</tr>
<tr>
<td>Brighter DFI (%)</td>
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<tr>
<td>p.N680S N homozygous</td>
<td>34.19 ± 15.46</td>
<td>25.48 ± 10.58</td>
<td>22.42 ± 9.34</td>
<td>0.004</td>
</tr>
<tr>
<td>Dimmer DFI (%)</td>
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<tr>
<td>p.N680S S homozygous</td>
<td>28.64 ± 13.64</td>
<td>26.59 ± 11.85</td>
<td>37.44 ± 19.90</td>
<td>0.221</td>
</tr>
<tr>
<td>Apoptotic bodies (%)</td>
<td></td>
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<tr>
<td>p.N680S S homozygous</td>
<td>34.17 ± 22.74</td>
<td>33.61 ± 22.93</td>
<td>31.67 ± 23.18</td>
<td>0.970</td>
</tr>
<tr>
<td>p.N680S N homozygous</td>
<td>26.54 ± 16.98</td>
<td>26.61 ± 21.61</td>
<td>29.21 ± 23.17</td>
<td>0.831</td>
</tr>
<tr>
<td>Testicular volume (ml)</td>
<td></td>
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<tr>
<td>p.N680S S homozygous</td>
<td>31.53 ± 10.12</td>
<td>32.36 ± 10.66</td>
<td>32.19 ± 11.09</td>
<td>0.908</td>
</tr>
<tr>
<td>Total spermatozoa number (million)</td>
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</tr>
<tr>
<td>p.N680S S homozygous</td>
<td>80.10 ± 68.98</td>
<td>72.84 ± 84.10</td>
<td>93.62 ± 92.81</td>
<td>0.351</td>
</tr>
<tr>
<td>p.N680S N homozygous</td>
<td>84.71 ± 133.93</td>
<td>75.33 ± 109.76</td>
<td>83.82 ± 104.63</td>
<td>0.993</td>
</tr>
<tr>
<td>Concentration of spermatozoa (million/ml)</td>
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<td></td>
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</tr>
<tr>
<td>p.N680S S homozygous</td>
<td>24.64 ± 20.10</td>
<td>20.76 ± 21.00</td>
<td>25.53 ± 23.04</td>
<td>0.345</td>
</tr>
<tr>
<td>p.N680S N homozygous</td>
<td>24.64 ± 27.90</td>
<td>21.85 ± 26.56</td>
<td>25.32 ± 29.97</td>
<td>0.975</td>
</tr>
<tr>
<td>Progressive motility (million)</td>
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<tr>
<td>p.N680S S homozygous</td>
<td>18.49 ± 22.23</td>
<td>13.88 ± 23.42</td>
<td>18.28 ± 25.50</td>
<td>0.218</td>
</tr>
<tr>
<td>p.N680S N homozygous</td>
<td>30.30 ± 64.72</td>
<td>17.73 ± 26.00</td>
<td>16.35 ± 21.58</td>
<td>0.903</td>
</tr>
<tr>
<td>Non progressive motility (million)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>p.N680S S homozygous</td>
<td>9.68 ± 11.25</td>
<td>10.76 ± 14.63</td>
<td>13.00 ± 16.34</td>
<td>0.656</td>
</tr>
<tr>
<td>p.N680S N homozygous</td>
<td>12.04 ± 24.23</td>
<td>10.08 ± 18.03</td>
<td>8.50 ± 10.45</td>
<td>0.982</td>
</tr>
</tbody>
</table>

Variables are expressed as mean ± SD. The P values indicate the statistical difference among the three visits through Kruskal–Wallis test. Bold/italic values show significant results.


AMH, anti-Müllerian hormone; DFI, DNA fragmentation index.
particular, serum FSH levels, sperm concentration/number and sperm DFI did not differ between the two groups (Table I). Since inclusion criteria were independent of these parameters, sperm concentration/number was highly heterogeneous and both groups included men ranging from severe oligozoosperma to normozoosperma.

FSH administration resulted in a limited increase in serum FSH as measured at the end of the treatment phase (Visit 2). Serum FSH significantly differed from baseline only in the p.N680S S homozygous group (Table I). This reflects the fact that the patients were not requested to come to Visit 2 immediately after the last injection. The significant increase of serum FSH levels at Visit 2 in one group and the improvement in DFI as a treatment effect; however, suggests a good compliance to the therapy. None of the other hormones (LH, testosterone, estradiol, inhibin B and AMH) and semen parameters changed among the visits (Table I).

Twelve pregnancies (both spontaneous and after ART), biochemically evaluated by serum hCG measurement (higher than 10 mIU/ml), were observed during and after conclusion of the trial, six (21.4%) in the p.N680S S homozygous and six (15.8%) in the p.N680S N homozygous group, without significant differences (P = 0.792).

As expected, DFI values in the three sperm fractions at Visit 1 were much higher compared with those found in a cohort of fertile men where DFI was evaluated with the same method (Muratori et al., 2015a). Total DFI decreased progressively and significantly from visit 1 (57.92 ± 16.98%) to Visit 3 (43.68 ± 11.99%) only in the p.N680S N homozygous group (P = 0.013) (Table I). The decrease of total DFI in FSHR p.N680S N homozygous men was entirely due to a significant decrease in the brighter sperm DFI fraction from visit 1 (34.19 ± 15.46%) to visit 3 (22.42 ± 9.34%, P = 0.004) (Table I) (Fig. 2), while the dimmer and bodies fractions, represented essentially by dead sperm and apoptotic bodies, respectively, did not change (Table I).

A significant correlation between sperm total DFI and serum testosterone levels was observed only in the p.N680S N homozygous men and only at Visit 2 (R = −0.418, P = 0.038) (Fig. 3). No other significant correlations were demonstrated among all other parameters (data not shown). In particular, no significant correlation was found between DFI values at Visit 1 in the three sperm fractions and patient age (data not shown), in contrast with previous results (Wyrobek et al., 2006; Lotti et al., 2012). Lack of correlation with age might be due to the lower number of subjects and the different age range of the present study.

Since we included both oligozoospermic and normozoospermic men, we analyzed whether the effect on the sperm DFI was related to sperm concentration rather than FSHR genotype. Patients were stratified as normo- (41 patients, 62.12%) and oligozoospermic (25 patients, 37.88%), according to baseline sperm number. The distribution of oligo- and normozoospermic was not significantly different between p.N680S N homozygous and p.N680S S homozygous men (P = 0.060). The beneficial FSH effect was confirmed only in the p.N680S N homozygous group (Fig. 4). In particular at Visit 3 brighter DFI decreased significantly in the p.N680S N homozygous, normozoospermic patients (P = 0.040) (Fig. 4A) and sperm total DFI decreased significantly in the p.N680S N homozygous, oligozoospermic men (P = 0.035) (Fig. 4B). No significant changes were observed in the p.N680S S homozygous patients, confirming that the improvement of sperm DNA fragmentation was mainly related to the genotype.

In the literature, several cohort studies demonstrated the relationship between FSH action and the -211G>T polymorphism in the FSHB gene (Grigorova et al., 2008, 2010, 2011; Tuttelmann et al., 2012), most of which were published after this trial was initiated. We, therefore, also investigated whether the observed FSH effect could be influenced by this SNP. We found 49 (75.7%) FSHB -211G>T homozygous G patients, 14 (21.2%) FSHB -211G>T heterozygous G/T patients and 2 (3.1%) FSHB -211G>T homozygous T patients. In one patient the evaluation
of FSHB -211G>T genotype was not possible. Patients were stratified in four groups according to the FSHR p.N680S and FSHB -211G>T genotype, the latter considering the homozygous T and the heterozygous G/T genotypes together (dominant model). A significant improvement in sperm total and brighter DFI was found only in carriers of the combination of FSHR p.N680S N homozygous and FSHB G homozygous genotypes (Fig. 5). Considering the FSHB -211G>T genotype alone, irrespective of the FSHR p.N680S genotype, brighter DFI was not different between FSHB -211G>T homozygous G (n = 50) and FSHB -211G>T homozygous T and heterozygous G/T genotypes together (n = 16). However, brighter DFI decreased significantly among visits only in the homozygous G group (P = 0.018). A post hoc test showed significantly lower levels at Visit 3 than Visit 1 (data not shown).

Taken together, these results demonstrate that FSH, at the dose and duration treatment employed, improves sperm DFI, particularly in the fraction containing viable spermatozoa, in patients who are carriers of the FSHR p.N680S N homozygous genotype. This effect is independent of sperm concentration/number and may be modulated by the FSHB -211G>T genotype.

Discussion

This study demonstrates that FSH administration to men with idiopathic infertility improves sperm quality, reducing, with the regimen used, sperm DNA fragmentation only in FSHR p.N680S N homozygous men, thereby identifying the FSHR genotype as a putative pharmacogenetic marker of FSH response in men. This finding is fully in line with our previous results obtained in women (Behre et al., 2005) and with the concept that the FSHR p.N680S N homozygous genotype is more sensitive to FSH both in vivo and in vitro (Simoni and Casarini, 2013; Casarini et al., 2014). The only other pharmacogenetic study published so far investigated standard semen parameters in idiopathic, oligozoospermic men and suggested that not FSHR p.N680S homozygous N men but rather p.N680S homozygous S men responded to FSH treatment by improving sperm concentration and total sperm number (Selice et al., 2011). Different patient selection criteria, study design and the well-known inter-laboratory variability in results of semen analysis (Cooper et al., 1999; Mallidis et al., 2012) could explain these conflicting results. Our results, obtained with a rigorous study design, can be fully explained by the current knowledge of pathophysiology of FSHR polymorphisms (Simoni and Casarini, 2013).
Sperm DFI represents a promising new diagnostic tool in infertile men, considering that high sperm DFI levels are associated with poor ART outcomes (Tamburino et al., 2012). In addition, sperm DFI appears to be the most stable, among the other seminal parameters, within the time of a spermatogenetic cycle (Muratori et al., 2015a). Here, we find that only the subgroup of FSHR p.N680S homozygous N men experience an improvement of sperm DFI with FSH treatment. Thus, FSHR p.N680S could be a predictive marker to recognize which men could respond better to FSH treatment. Remarkably, this beneficial effect is not evident immediately after the treatment phase but becomes significant after 6 months from study start, a period including 3 months of FSH administration and 3 months of treatment washout. This suggests that FSH acts probably at the early steps of spermatogenesis, improving sperm quality progressively. Thus, the treatment duration chosen might be too short to be effective in all men. We speculate that FSH administration, acting at the early germ cell stage of spermatogenesis, should cover at least two spermatogenetic cycles in order to produce sperm improvement in the majority of men, possibly extending its beneficial effects to FSH-R p.N680S 5 carriers. Therefore, future studies should consider extending the FSH treatment phase to at least 6 months.

Sperm DNA fragmentation originates mostly in the testis resulting from an abortive apoptotic mechanism or from oxidative stress during transit in the male genital tract (Muratori et al., 2015b). Since FSH is a germ cell pro-survival factor, altering the ratio of B-cell lymphoma 2 (BCl2) family protein members (Ruwanpura et al., 2008a,b), we speculate that FSH decreases sperm DFI by reducing apoptosis in the testis.

Stratifying the patients according to sperm concentration/number did not change the result that only FSHR p.N680S N homozygous men respond with an improvement of sperm DFI. Although the number of men studied is low, it seems that FSHR p.N680S N homozygosis/high basal levels of DFI is a better predictor of response to FSH treatment than basal semen parameters.

The other patient stratification applied, according to the FSHB -211G>T polymorphism, confirmed the positive effect only in FSHR p.N680S N homozygous men and, in addition, identified the FSHB -211G>T homozygous genotype as the most responsive to FSH therapy. This result seems to be in contrast to the only previous study available in the literature (Ferlin et al., 2011). However, although the FSHB -211G>T analysis was applied a posteriori, and a selection bias exists, since only homozygous FSHR p.N680S were included in the study, this result is in line with the model proposed by Tuttelmann et al. (2012). In this cohort study, the authors proposed a ‘traffic light’ model in which FSHR p.N680S and FSHB -211G>T SNPs interacted influencing FSH action (Tuttelmann et al., 2012). Tuttelmann et al. proposed that those men with the genotype combination FSHR -2039A>G AA or AG and FSHB -211G>T GG in the ‘green’ area of the model in Fig. 2 in Tuttelmann et al., 2012) were carriers of the best combination for FSH action. In our study, patients with such genotype combination, corresponding to the ‘green’ area, are indeed the best responders to FSH, probably due to a more favorable genetic background of higher FSHR sensitivity and higher exposure to endogenous FSH. Considering the two polymorphisms together, total and brighter DFI improved in combined FSHR p.N680S N homozygous and FSHB -211G>T homozygous men. However, a larger sample size is needed to better understand the influence and the interaction of these two SNPs on the response to FSH treatment in men. Future studies should prospectively evaluate the effects of FSH in men stratified according to both genotypes and based on more personalized treatment regimens, i.e. longer treatment duration with higher FSH doses in men with less responsive FSHR/FSHB genotype combinations, possibly assessing ART outcome and/or spontaneous pregnancies.

We recently demonstrated the molecular basis for the different response to FSH of the two FSHR p.N680S genotypes in human granulosa-lutein cells (hGLC) (Casarini et al., 2014). In vitro, upon stimulation with recombinant FSH, FSHR p.N680S homozygous N hGLC show a faster
cyclic adenosine mono-phosphate (cAMP) and progesterone production rate and a higher phosphorylated extracellular signal-regulated kinase 1/2 and cAMP response element-binding protein CREB phosphorylation compared with the homozygous FSHR p.N680S N hGLC (Casarini et al., 2014). Therefore, the present results are completely consistent with the expected higher sensitivity of the FSHR p.N680S N genotype both in vivo and in vitro (Casarini et al., 2014; Simoni and Casarini, 2014).

Finally, we confirm that FSH treatment does not affect the hormonal status. In particular, no significant modifications were found in serum AMH, inhibin B, testosterone, estradiol and gonadotrophin levels. However, a significant and inverse relationship is found between total testosterone serum levels and sperm DFI only in FSHR p.N680S N homozygous men at the end of treatment phase. This result suggests a possible effect of FSH administration on testosterone in a specific subgroup of infertile men, mediated by FSH sensitivity. However, the low number of patients enrolled to this arm suggests caution in interpretation.

In conclusion, we demonstrate that FSH administration to infertile men is effective in reducing sperm DNA fragmentation when the FSHR p.N680S polymorphism is considered in selecting patients. In particular, the FSH regimen applied improves sperm quality when FSHR p.N680S N homozygosity is present. The effect is seen both in normo- and in oligozoospermic men and is associated with the FSHB -211G/T polymorphism. The relatively low number of subjects represents a limitation of this study but the rigorous design suggests that the highest beneficial effect of FSH is obtained in men carrying the combined FSHR p.N680S N homozygous and FSHB -211 G/T G homozygous genotypes.

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Authors’ roles
M.S. and H.M.B. designed the study. D.S. and F.L. collected and analyzed the data. M.S. and D.S. wrote the manuscript. L.N., I.H., Mari.Ma., T.G., F.L., F.P., C.K., A.F., R.B., P.E.L.S. and H.M.B. selected and treated the patients. Mo.Mu., El.Ba., M.C. and L.G. performed semen and DFI analysis. En.Ba, S.T., D.C. and Marc.Ma. performed hormone and genetic analyses. E.P. organized the study logistics. All authors contributed to the final version and approved the manuscript.

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Conflict of interest
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