Infertility

The sperm protamine mRNA ratio as a clinical parameter to estimate the fertilizing potential of men taking part in an ART programme


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STUDY QUESTION: Could the protamine-1 to protamine-2 mRNA ratio serve as a biomarker to estimate the fertilizing capacity of sperm from men taking part in an IVF/ICSI programme?

SUMMARY ANSWER: The protamine mRNA ratio clearly discriminates between fertile and subfertile men and sperm with a normal protamine mRNA ratio exhibit a higher fertilizing capacity in IVF/ICSI.

WHAT IS KNOWN ALREADY: Aberrant sperm protamine ratios are associated with male factor infertility and mRNA ratio is comparable with protein ratio (due to transcriptional stop in elongating spermatids).

STUDY DESIGN, SIZE, DURATION: The study population was drawn from subfertile men, whose female partners participated in IVF or ICSI programmes between September 2010 and February 2012. Normozoospermic healthy volunteers served as controls. Sperm cells were lysed, mRNA extracted, reverse transcribed and subjected to real-time quantitative PCR using specific primer pairs for protamine-1 and protamine-2. Relative protamine-1 and protamine-2 mRNA levels were analysed with the Mann–Whitney U-test (two-tailed).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Quantitative RT–PCR for protamines 1 and 2 has been performed in ejaculates from 32 normozoospermic volunteers (control, University Clinic Giessen, Germany) and 306 patients, whose female partners took part in an IVF (n = 76; University Clinic Hamburg, Germany and Shanghai Jiaotong University, China) or an ICSI (n = 230; University Clinic Munich, Germany and Kinderwunschzentrum Wiesbaden, Germany) programme.

MAIN RESULTS AND THE ROLE OF CHANCE: The sperm protamine mRNA ratio in normozoospermic men (0.98 ± 0.3) differed significantly from that of ICSI patients (Munich 0.81 ± 0.1; Wiesbaden 0.78 ± 0.2; P < 0.001), while processed samples obtained from IVF patients revealed a normal protamine mRNA ratio (Hamburg 1.0 ± 0.07; Shanghai 1.0 ± 0.54). Normal protamine mRNA ratios were associated with a significantly higher total motile sperm count and a significantly higher percentage of progressively motile sperm. Sperm with a normal protamine mRNA ratio revealed a higher fertilization capacity (fc) in both IVF (53.6% of patients with fc > 80%; P = 0.017) and ICSI (65.1% of patients with fc > 70%; P = 0.028).

† These authors contributed equally to the manuscript.

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Introduction

For successful fertilization, fusion of sperm and oocyte must be followed by a cascade of events resulting in complete embryo development. Fertilization failure, in contrast, may be due to reduced oocyte and/or sperm quality. Production of competent sperm requires replacement of DNA-binding histones by protamines (Steger et al., 2011). In a comparative study analysing various mammalian species, the protamine-1 to protamine-2 protein ratio varied between different species, but was constant within a specific species. In any case, aberrant protamine protein ratios were related to male infertility (Corzett et al., 2002). Data are corroborated by functional studies in knockout mice demonstrating that deletion of only one of the two protamine alleles—mimicking an aberrant protamine ratio—is sufficient to cause male infertility (Cho et al., 2001). In men, histone to protamine exchange is only ~85% complete (Tanphaichitr et al., 1978) and several studies reported an abnormal persistence of histones in spermatozoa of infertile men (Silvestroni et al., 1976; Blanchard et al., 1990; Hofmann and Hilscher, 1991; Foresta et al., 1992; Zhang et al., 2006).

The relative proportion of protamine-1 to protamine-2 protein is regulated approximately at a 1:1 ratio (Balhorn et al., 1988). Recently, a reference range from 0.5 to 1.4 has been reported in normozoospermic men (Nanassy et al., 2011). Despite this rather wide normal range, numerous studies reported a clear correlation between an aberrant protamine mRNA/protein ratio and male infertility (Balhorn et al., 1988; Belokopytova et al., 1993; de Yebra et al., 1998; Carrell and Liu, 2001; Mengual et al., 2003; Nasr-Esfahani et al., 2004; Aoki et al., 2005a, 2006c; de Mateo et al., 2009; Hammoud et al., 2009; Depa-Martynow et al., 2012). As a consequence, the protamine mRNA/protein ratio has been suggested as a suitable biomarker for the success of IVF (Carrell and Liu, 2001; Aoki et al., 2006c; Depa-Martynow et al., 2012). In the case of ICSI, the prognostic value may be limited due to the fact that protamine levels may vary between individual spermatozoa within one ejaculate (Aoki et al., 2006a). Available data, however, are insufficient to draw definitive conclusions concerning the prognostic value for ICSI.

We therefore performed a multicentre study to analyse prognostic value and clinical utility of the protamine mRNA ratio in ejaculated spermatozoa for the discrimination between fertile and subfertile men. In patients, it should be further investigated whether the protamine mRNA ratio correlates with fertilization and pregnancy rates of infertile couples undergoing IVF or ICSI treatment.

Materials and Methods

Study design

This non-interventional, prospective, observational, multicentre cohort study was conducted in couples treated for infertility at two private (Hamburg, Wiesbaden) and two university (Munich, Shanghai) IVF centres between September 2010 and February 2012.

Participants

A total of 306 subfertile couples undergoing assisted reproduction (IVF or ICSI) with controlled ovarian hyperstimulation and fresh embryo transfer participated in the IVF/ICSI arm of the study. The age of the female partner ranged from 24 to 45 years (IVF: 34.5 ± 4.4, 10th percentile 28; ICSI: 35.6 ± 4.6, 10th percentile 30). The primary or combined indications for fertility treatment were male subfertility, tubal pathology, endometriosis, polycystic ovary syndrome and other causes. Couples with no mature oocytes on the day of ovum pick-up, no fertilized oocytes on the day of pronuclear evaluation or couples assigned to ‘freeze all’ approaches due to ovarian hyperstimulation syndrome or a rise of serum progesterone ≥ 1.5 ng/ml on the day of ovulation induction were excluded from the study.

Male factor parameters are summarized in Table I. A complete list of all available parameters of subfertile couples enrolled in the study is provided in Supplementary data, Table S1.

The study was approved by the Ethics Committee of the Medical Faculty of the Justus-Liebig-University (approval 95/04 from 22 July 2004, confirmed on 10 February 2009).

Assisted reproductive technology procedures

Controlled ovarian hyperstimulation was performed with GnRH agonist or GnRH antagonist protocols combined with gonadotrophins. Starting gonadotrophin dosage was individualized for each patient by a combination of factors, including age, serum level of anti-Müllerian hormone (AMH), antral follicle count, previous stimulation responses and BMI. Dosages were adjusted, according to individual ovarian response, as monitored by vaginal ultrasonographic scans and serum concentrations of estradiol (E2). 10 000 IU of hCG (Prelalon™, MSD Sharp and Dohme, Haar, Germany or Brevactid™, Ferring, Kiel, Germany) were administered subcutaneously when at least three follicles reached a mean diameter of 18 mm (hyperstimulation with GnRH antagonist) and oocytes were retrieved by transvaginal ultrasound-guided aspiration 34–36 h later.

All mature oocytes (metaphase II) were inseminated via IVF or ICSI 4–6 h after ovum pick-up. The fertilizing capacity was calculated as the percentage of the number of fertilized oocytes divided by the number of treated oocytes. Fertilization was assessed 16–20 h post-insemination.
and zygotes were assigned to extended embryo culture for 5 days in patients ≤42 years, who have ≥5 2-pronuclear stage oocytes (PNs) on post-retrieval Day 1 or ≥4 2-PNs (age < 42 years). Patients lacking these criteria received a Day 3 embryo transfer (ET). Embryos were cultured routinely up to Day 3 or Day 5 of embryonic development. On the day of ET, cleavage state embryos were graded, according to Scott (2000), and blastocysts, according to Gardner and Schoolcraft (1999), 1 h prior to transfer. All patients received a maximum of two embryos (average 1.8). Micronized progesterone (Utrogest, Dr Kade/Besins Pharma, Berlin, Germany) at a dose of 600 mg/day was applied vaginally and E2 (Tradelia TTS, Dr Wolff Arzneimittel, Bielefeld, Germany) at a dose of 100 μg within 3 days transdermally for luteal support for at least 14 days following oocyte retrieval. On Days 14–16 after ovulation induction, whole blood was collected between 8:00 and 10:00 AM for the measurement of β-hCG to assess early pregnancy outcome. Clinical pregnancy rate (fetal heart action per transferred patient) and miscarriage rate (loss of entire pregnancy ≤24 weeks per transferred patient) were assessed.

**Seminal measurements**

To determine serum AMH levels of female partners, whole blood was drawn on Days 3–5 of menstrual cycle and serum was assessed with a Beckmann Coulter enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer’s instructions (Active MIS/AMH ELISA kit, Ref. no DSL-10-14400, DSL, Webster, TX, USA). Detection range of the kit is between 0.05 and 15 ng/ml.

**Semen samples**

Ejaculates were obtained from 306 patients, whose female partners took part in an IVF (n = 76) or an ICSI (n = 230) programme. The control group comprised 32 healthy volunteers revealing normozoosperma, according to current World Health Organisation (WHO) reference values (Cooper et al., 2010; WHO, 2010); hence, semen characteristics from these men are considered as surrogate parameters reflecting normal fertility. In addition, ejaculates from three normozoospermic volunteers were used to demonstrate specificity, efficiency and linearity of the protamine-1 and protamine-2 quantitative RT–PCR (qRT–PCR). All semen samples were analysed, according to recommendations of the WHO (1999, 2010), where available, sperm morphology was evaluated according to strict criteria. For classification of semen quality, the ‘total motile sperm count (TMSC)’ was calculated as the product of volume, concentration and progressive motility (category ‘PR’), equivalent to previous categories ‘A + B’ of native semen (van Voorhis et al., 2001). Aliquots of individual ejaculates were transferred into RNA later™ (Ambion, Heppenheim, Germany) and stored in liquid nitrogen until further processing (Munich, Wiesbaden). In the centre in Hamburg, 1 ml of ejaculate was pelletted by centrifugation (400g for 10 min), washed once in phosphate-buffered saline, and pellets frozen at −80°C. In Shanghai, 1 ml of ejaculate was separated by density-gradient centrifugation (300g for 20 min). The sperm fraction was transferred into 5 ml modified human tubal fluid (mHTF) medium (Irvin Scientific, Newtownmountkennedy, Ireland), with gentamicin, centrifuged (200g for 8 min), resuspended in 1 ml mHTF medium and incubated for 30 min at 37°C.

**Table I Characteristics of healthy male controls and subfertile patients whose female partners underwent IVF or ICSI.**

<table>
<thead>
<tr>
<th>Control</th>
<th>IVF programme</th>
<th>ICSI programme</th>
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<tbody>
<tr>
<td>Giessen</td>
<td>Hamburg</td>
<td>Shanghai</td>
</tr>
<tr>
<td>Munich</td>
<td>Wiesbaden</td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>Age (years)</td>
<td>Sperm concentration (Mio/ml)</td>
</tr>
<tr>
<td>32</td>
<td>26.0 ± 4.0</td>
<td>80.45 ± 41.2</td>
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<tr>
<td>56</td>
<td>36.1 ± 5.0</td>
<td>42.56 ± 48.3</td>
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<tr>
<td>20</td>
<td>32.1 ± 3.0</td>
<td>23.8 ± 11.5</td>
</tr>
<tr>
<td>129</td>
<td>39.5 ± 6.0</td>
<td>25.75 ± 38.1</td>
</tr>
<tr>
<td>101</td>
<td>39.7 ± 5.0</td>
<td>44.8 ± 28.4</td>
</tr>
</tbody>
</table>

Mean values ± standard deviation.

Further, intron spanning primers were employed (Eurofins, Ebersberg, Germany): Protamine-1: 5′-aagtggcagaggaagg-3′ (forward primer bp 63–81) and 5′-atctggcctctcgcc-3′ (reverse primer bp 123–142) resulting in a 79 bp amplification product.

Protamine-2: 5′-aagtgcggctctggcaac-3′ (forward primer bp 234–253) and 5′-gcgtcgctctctctct-3′ (reverse primer bp 284–305) resulting in a 71 bp amplification product. Negative controls included samples lacking reverse transcriptase. Furthermore, intron spanning primers were applied to avoid amplification of genomic DNA. All PCR amplifications were carried out in duplicate and mean values were calculated.

Values recorded for quantification were the fractional cycle numbers (Ct) where the background corrected amplification curves crossed a threshold value. The threshold value was set within the log-linear phase of the amplification curves.

Efficiency of protamine-1 and protamine-2 qRT–PCR was derived from a standard curve of known quantities of protamine-1 and protamine-2

**RNA extraction and first strand cDNA synthesis**

RNA extraction was conducted with RNeasy Mini kit and RNeasy Plus Micro kit (Qiagen, Hilden, Germany) to eliminate the problem of excessive genomic DNA (Cappallo-Obermann et al., 2011). First strand cDNA synthesis was performed with Omniscript™, according to the manufacturer’s protocol (Qiagen, Hilden, Germany).

**Real-time quantitative reverse transcription polymerase chain reaction**

Real-time qRT–PCR was performed using iQ™ SYBR Green Supermix and iCycler (BioRad, Munich, Germany). Per sample, 5 μl cDNA were used for amplification of protamine-1 and protamine-2. Cycling conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 30 s. Primers were generated using protamine-1 and protamine-2 gene sequence (GenBank, Accession Z46940). The following primers were employed (Eurofins, Ebersberg, Germany):

Protamine-1: 5′-aagtggcagaggaagg-3′ (forward primer bp 63–81) and 5′-atctggcctctcgcc-3′ (reverse primer bp 123–142) resulting in a 79 bp amplification product.

Protamine-2: 5′-aagtgcggctctggcaac-3′ (forward primer bp 234–253) and 5′-gcgtcgctctctctct-3′ (reverse primer bp 284–305) resulting in a 71 bp amplification product. Negative controls included samples lacking reverse transcriptase. Furthermore, intron spanning primers were applied to avoid amplification of genomic DNA. All PCR amplifications were carried out in duplicate and mean values were calculated.
templates. Reliability of protamine-1 to protamine-2 mRNA method was tested on ejaculates at room temperature for 72 h.

Statistical analysis
Data were analysed using SPSS 20 (IBM, Düsseldorf, Germany). Ct values of technical replicates were averaged and averages were used for further calculations. Relative expression levels were reflected by ΔCt values which represent a measure of the log-ratio of the transcript abundances in the samples. Relative protamine-1 and protamine-2 levels were calculated as relative to maximal values of protamine-1 and protamine-2 Ct transformed to log scale using GenEx software (Multid Analyses AB, Gothenburg, Sweden). The normal distribution of Ct and ΔCt values was checked with normal-QQ plots. The categorized protamine-1 to protamine-2 ratios (0.88–1.15 for IVF; 0.85–1.30 for ICSI) were analysed using the Bonferroni-adjusted Mann–Whitney U-test. For this comparison, the non-adjusted statistical level of significance of \( P < 0.05 \) among the four tested categories (sperm concentration, TMSC, progressive sperm motility, fertilization rate) for IVF and ICSI patients corresponds to a Bonferroni-adjusted statistical significance of \( P < 0.0125 \). Fisher’s exact test was used to assess the statistical correlation of categorized protamine-1 to protamine-2 ratios with clinical variables. All statistical tests were two-sided.

Results
Specificity, efficiency and linearity of the protamine-1 and protamine-2 qRT–PCR
Single product-specific melting temperatures (protamine-1, 88°C; protamine-2, 86°C) and high resolution gel electrophoresis displaying distinct bands of expected size (protamine-1, 79 bp; protamine-2, 71 bp) demonstrated high specificity of selected primer pairs. The qRT–PCR efficiency of one cycle in the exponential phase was calculated, according to the equation PCR efficiency = \( (10^{\frac{1}{slopes}} - 1) \times 100 \). Efficiency was 101% for protamine-1 and 99.4% for protamine-2 in a dilution range from 0.01 to 10 ng \((n = 3)\). Pearson correlation coefficient for linearity was 0.995 for protamine-1 and 0.996 for protamine-2 (Supplementary data, Fig. S1).

Reliability of mRNA-based calculation of the protamine ratio in sperm was validated by testing different mRNA concentrations and ejaculates stored at room temperature for up to 96 h with and without RNA later\textsuperscript{TM} (Ambion, Heppenheim, Germany). mRNA quality is a restricting factor for the mRNA-based protamine-1 to protamine-2 test. In preliminary tests, these restrictions were avoidable by using RNA later. The protamine ratio was not affected even after 4 days storage of ejaculates at room temperature (Supplementary data, Fig. S2).

The protamine mRNA ratio discriminates between fertile and subfertile men
Patients whose female partners underwent IVF cycles displayed a normal protamine mRNA ratio (Hamburg, semen samples after simple washing, median 1.0 ± 0.075; Shanghai, sperm after density-gradient centrifugation, median 1.0 ± 0.54; Fig. 1A). With regard to protamine mRNA ratios in processed samples from Hamburg compared with those in native semen from Shanghai marked differences in semen quality should be noted (Table I). Moreover, there is a significant difference in the protamine mRNA ratio of sperm obtained from Shanghai IVF patients before and after work-up (median 0.64 ± 0.17, \( P < 0.001 \)). After preparation, sperm protamine mRNA ratio is similar to that of the Hamburg and the control group demonstrating the functional integrity of these spermatozoa (Fig. 1A). In contrast, the protamine mRNA ratio of normozoospermic healthy volunteers (median 0.989 ± 0.317) is significantly different from that of patients involved in an ICSI programme in Munich or Wiesbaden (median 0.812 ± 0.17 and 0.78 ± 0.2, respectively; \( P < 0.001 \); Fig. 1B). No substantial effect on statistical significance could be observed when excluding patients with sperm concentration > 15 Mio/ml (Fig. 1C) and progressive sperm motility > 32% (Fig. 1D), thus considering the lower reference values for basic semen parameters recommended by the WHO (Cooper et al., 2010; WHO, 2010).

Ejaculate with normal protamine mRNA ratio contains higher TMSCs and higher percentage of progressively motile sperm
In ejaculates obtained from patients in both IVF and ICSI programmes, a normal protamine mRNA ratio was associated with a significantly higher TMSC compared with semen samples with an aberrant protamine mRNA ratio (IVF: 41 × 10⁶ ± 13 × 10⁶ versus 16 × 10⁶ ± 52 × 10⁶; \( P = 0.004 \); ICSI: 35 × 10⁶ ± 54 × 10⁶ versus 23 × 10⁶ ± 32 × 10⁶; \( P = 0.008 \); Fig. 2A and B). Analysing single read-out parameters reflecting sperm quality in ejaculates from IVF patients, 60.0 ± 13.8% of sperm with a normal protamine mRNA ratio (0.88–1.15) were progressively motile, while the respective proportion of sperm with an aberrant protamine mRNA ratio was only 33.0 ± 18.0% (\( P < 0.001 \); Fig. 2C). A smaller, but still significant difference in the proportion of progressively motile sperm could be observed in semen samples from ICSI patients (normal versus aberrant protamine mRNA ratio: 44.5 ± 16.6 versus 39.0 ± 17.4%, respectively; \( P = 0.012 \); Fig. 2D). Sperm morphology data according to strict criteria were available for ejaculates obtained from ICSI programmes (Munich, Wiesbaden), but after Bonferroni correction showed no significant differences (8.0 ± 20.0 versus 8.0 ± 9.7%; \( P = 0.041 \); Fig. 2E). Moreover, no correlation could be found with sperm concentration as single surrogate parameter (data not shown).

Sperm with normal protamine mRNA ratio reveal higher fertilization capacity in both IVF and ICSI
53.6% of IVF patients with a normal protamine mRNA ratio exhibited a fertilization rate of > 80%, while 78.9% with an aberrant protamine mRNA ratio displayed a fertilization rate of < 80% (\( P = 0.017 \); Fig. 3A). In ICSI patients, 65.1% with a normal protamine mRNA ratio reveal a fertilization rate of > 70%, whereas 56.0% with an aberrant protamine mRNA ratio display a fertilization rate of < 70% (\( P = 0.028 \); Fig. 3B). No significant correlation could be demonstrated between protamine mRNA ratio and pregnancy rates in IVF and ICSI patients (data not shown). However, a significant correlation could be observed between pregnancy rate and both age (\( P < 0.004 \)) and serum AMH concentration (\( P < 0.015 \)) of the female partner participating in an ICSI programme. The age of the male partner was not related to protamine ratio and pregnancy rate.
Discussion

Since the first report on an aberrant protamine-1 to protamine-2 protein ratio in sperm of infertile patients (Balhorn et al., 1988), there has been a discussion whether the protamine ratio may serve as a clinical parameter to discriminate fertile from subfertile men and to predict fertilization and pregnancy rates in subfertile couples undergoing IVF or ICSI. Although several studies on this topic have been published during the last 20 years, these questions could still not completely be answered mainly due to the low number of patients analysed. We, therefore, performed a multicentre study enabling the analysis of 338 samples, including 32 normozoospermic healthy volunteers serving as control (Giessen), two IVF groups (Hamburg, Shanghai) containing 76 individuals and two ICSI groups (Munich, Wiesbaden) comprising 230 individuals.

Real-time qRT–PCR on protamine mRNA has been demonstrated to represent an excellent tool for the identification of testicular spermatozoa which subsequently may be used for ICSI (Steger et al., 2000). As protamine genes are solely transcribed in round spermatids (Steger et al., 2000) and stored as silent mRNAs for later translation in transcriptionally silent elongated spermatids and spermatozoa (Steger et al., 2011), there is a link between mRNA levels in testicular spermatids and protein levels in ejaculated spermatozoa, which has been demonstrated by Aoki et al. (2006b) and Depa-Martynow et al. (2012). Own studies demonstrated aberrant protamine mRNA ratios in subfertile men applying in situ hybridization on testicular mRNA levels.
Figure 2 Correlation of protamine mRNA ratio (in range versus out of range) with sperm quality criteria in patients involved in IVF (A and C) or ICSI (B, D and E) programmes shown as box-and-Whisker plots. Sperm quality criteria examined were TMSC (A and B), progressive sperm motility (C and D) and sperm morphology according to strict criteria (E). The IVF in range group contained 56 samples, the out of range group 19 probes. The ICSI in range group comprises 56 samples, the out of range group 122 probes. *P < 0.05, **P < 0.001 (Mann–Whitney U test).
Figure 3 Correlation between protamine mRNA ratio and oocyte fertilization. Sperm fertilizing capacity was calculated as the percentage of the number of fertilized oocytes divided by the number of treated oocytes. Comparison between men exhibiting a normal (in range: 0.88–1.15 for IVF, cut-off 80%; 0.85–1.30 for ICSI, cut-off 70%; B) and an aberrant (out of range) protamine ratio. Different cut-offs for IVF and ICSI are due to optimal association with progressive sperm motility and fertilization rate. $P = 0.017$ (IVF), $P = 0.028$ (ICSI; Fishers exact test).
biopsies (Steger et al., 2001, n = 65; Mitchell et al., 2005, n = 41), RT–PCR on testicular biopsies (Steger et al., 2003, n = 57; Steger et al., 2008, n = 74) and RT–PCR on ejaculates (Steger et al., 2008, n = 95). As real-time qRT–PCR is both highly specific and sensitive (Steger et al., 2000), it is of interest for clinical use. In addition, this method is both time- and cost-effective, as 24 patients can be analysed simultaneously in a 96-well plate thermocycler.

In the present study, we confirmed both specificity and efficiency of the protamine-1 and protamine-2 qRT–PCR prior to patient analysis applying agarose gel electrophoresis and melting point analysis. Both primer pairs exhibited similar efficiencies and revealed specific and stable binding in a dilution range from 0.01 to 10 ng. Furthermore, we demonstrated that mRNA-based calculation of the protamine ratio in human sperm is reliable for up to 2 days without RNA later or up to 4 days with RNA later when stored at room temperature.

Normal protamine-1 to protamine-2 protein ratios in fertile men analysed by several groups result in very similar outcomes: 0.98 ± 0.12 (Balhorn et al., 1988; n = 17), 0.99 ± 0.06 (Belokopytova et al., 1993; n = 20), 0.83 ± 0.05 (Carrell and Liu, 2001; n = 50), 1.01 ± 0.15 (Mengual et al., 2003; n = 10), 1.06 ± 0.01 (Aoki et al., 2005a; n = 87), 1.30 ± 0.10 (De Mateo et al., 2009; n = 12) and 0.98 ± 0.02 (Nanassy et al., 2011; n = 77). The protamine mRNA ratio of 0.98 ± 0.31 emerging from this study (n = 32) is therefore in line with the protamine protein ratio of the above mentioned studies.

Although the protamine protein ratio in normozoospermic men is allowed to vary between 0.54 and 1.43, and hence from 1.2 to 2:1 (Nanassy et al., 2011), numerous studies demonstrated a significantly aberrant protamine protein ratio in infertile men (Carrell and Liu, 2001; Mengual et al., 2003; Nasr-Esfahani et al., 2004; Aoki et al., 2005a; De Mateo et al., 2009). Within a population of 144 men older than 18 years, 110 exhibited a normal, 4 a decreased and 30 an increased protamine protein ratio (Nanassy et al., 2011). In patients with a low protamine protein ratio, protamine-1 was underexpressed, while protamine-2 was overexpressed. In patients with a high protamine protein ratio, protamine-1 was normally expressed and protamine-2 was underexpressed. In subfertile patients with a normal protamine protein ratio, protamine-1 and protamine-2 were both underexpressed, but never in donors with proven fertility (Aoki et al., 2006c).

In this study, we demonstrated that the protamine mRNA ratio clearly discriminates between healthy normozoospermic men considered to be fertile according to reference data provided by the WHO (Cooper et al., 2010) and patients with an infertile relationship undergoing IVF (P < 0.001; native semen) or ICSI (P < 0.001). Interestingly, in patients from the SIA, a significant difference could be demonstrated between native semen and spermatozoa after work-up subjected to IVF (P < 0.001). In contrast, no significant differences could be observed between spermatozoa from control samples and those after preparation for IVF demonstrating a normal functional capacity of these cells.

Protamine mRNA ratio, in addition, was correlated with several semen quality criteria, such as the TMSC which was calculated by multiplying the ejaculate volume times the sperm concentration times the proportion of progressive motile sperm (van Voorhis et al., 2001). The TMSC was significantly higher in both IVF and ICSI patients exhibiting a normal protamine mRNA ratio when compared with semen samples revealing aberrant protamine mRNA ratios (IVF P = 0.004; ICSI P = 0.027). The predictive value of the TMSC has been shown for both natural conception as well as assisted reproductive technology (van Voorhis et al., 2001; Brandes et al., 2011). However, with regard to the overall impact of reduced semen quality on a subfertile couple’s chance to achieve a pregnancy, the complex interaction between male and female factors has to be considered (Eimers et al., 1994; Snick et al., 1997; Dunson et al., 2004; Cooper et al., 2010; van der Steeg et al., 2011). Moreover, the TMSC contains no information on sperm morphology. However, no significant correlation could be found between protamine mRNA ratio and sperm morphology according to strict criteria. In contrast, 43.5 ± 16.6% of sperm from ICSI patients with a normal protamine mRNA ratio exhibit progressive motility versus 38.0 ± 17.4% of sperm with an aberrant protamine mRNA ratio (P = 0.04). As expected, this effect is even more visible in IVF patients, where 58 ± 16% of sperm with a normal protamine mRNA ratio are progressive motile compared with only 33 ± 19% of sperm with an aberrant protamine mRNA ratio (P < 0.001). Our findings are in line with data from Depa-Martynow et al. (2012) demonstrating a significant correlation between protamine mRNA and protein levels and progressive sperm motility in 92 subfertile couples, but are in contrast to data from Mengual et al. (2003) reporting no significant differences between progressive sperm motility and protamine protein ratios within a group of 12 oligozoospermic men and a group of 13 asthenozoospermic men.

Most interestingly, sperm with a normal protamine mRNA ratio reveal higher fertilization rates in both IVF and ICSI. While 33.6% of IVF patients exhibit a fertilization rate of ≥80, 78.9% with an aberrant protamine mRNA ratio reveal a fertilization rate of <80% (P = 0.017). 65.1% of ICSI patients display a fertilization rate of ≥70%, while 56.0% with an aberrant protamine mRNA ratio show a fertilization rate of <70% (P = 0.028). The effect is more prominent in IVF, as success of ICSI highly depends on the selection of a successful individual spermatozoan and sperm of a given semen sample are known to exhibit a significant intra-sample heterogeneity of the protamine content (Aoki et al., 2006a). Our results are in line with data from Nasr-Esfahani et al. (2004) reporting a negative correlation between fertilization and protamine protein ratio in 71 ICSI patients when using chromomycin A3 staining. In addition, Carrell and Liu (2001) were able to demonstrate that an abnormal protamine protein ratio was a common defect in IVF patients, where 13 out of 75 patients had no measurable protamine-2. Similarly, Aoki et al. (2006c) analysed 251 men with normal, 43 men with reduced and 121 men with elevated protamine protein ratios and found that, in IVF patients, fertilization and pregnancy rates were significantly reduced when the protamine protein ratio is abnormally low. Recently, Depa-Martynow et al. (2012) demonstrated a significant correlation of the protamine mRNA and protein levels with both fertilization and embryo quality in 92 couples undergoing IVF. Unlike de Mateo et al. (2009; n = 102), who reported that the protamine protein ratio is associated with pregnancy rates and proportion of embryos obtained by IVF, but not ICSI, we did not observe any significant correlation between the protamine mRNA ratio and the pregnancy rate in this study.

Several studies reported a correlation between the protamine protein ratio and the sperm DNA fragmentation. A first study (Aoki et al., 2005b) demonstrated a significantly increased sperm DNA...
fragmentation in men exhibiting a low protamine protein ratio. Similarly, Nasr-Esfahani et al. (2005) reported that sperm DNA fragmentation was more frequent in protamine deficient spermatozoa from ICSI patients. Tarozzi et al. (2009), in addition, demonstrated a significant negative correlation between abnormal protamination and sperm DNA fragmentation, as well as fertilization and pregnancy in IVF patients, but not in ICSI patients. Simon et al. (2011) found that both low and high protamine protein ratios were associated with increased sperm DNA fragmentation. While fertilization rate and embryo quality was associated with abnormal protamination and sperm DNA fragmentation, no significant correlation could be demonstrated for pregnancy outcomes, neither for IVF nor for ICSI.

In conclusion, we were able to demonstrate that the protamine mRNA ratio in ejaculated sperm clearly discriminates between fertile and subfertile men. Normal protamine mRNA ratios are associated with a higher TMSC, a higher percentage of progressively motile sperm and a higher fertilization rate when undergoing IVF or ICSI treatment. Our data demonstrate that the protamine mRNA ratio represents an excellent prognostic marker to estimate the sperm fertilizing potential in patients with an infertile relationship in addition to standard semen parameters.

Supplementary data
Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors’ roles
N.R., M.S., V.S., H.C.-O.: contribution of patient material/data, data analysis, manuscript drafting; T.D.: statistical analysis, manuscript drafting; A.S.: experiment performance, data analysis, manuscript drafting; H.W.: contribution of patient material/data, experiment performance, data analysis, manuscript drafting; V.B., B.C., C.J.T.: contribution of patient material/data, manuscript drafting; H.Y.: experiment performance, data analysis, manuscript drafting; T.D.: statistical analysis, manuscript drafting; A.P., W.W.: manuscript drafting; H.C.S.: data analysis, manuscript drafting.

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Conflict of interest
None of the authors have any financial or commercial interest to declare no any other relationships with pharmaceutical manufacturers.

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