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ORIGINAL ARTICLE *Early pregnancy*

Unexplained recurrent miscarriages are associated with an aberrant sperm protamine mRNA content

Nina Rogenhofer[1], Johannes Ott[2,*], Adrian Pilatz[3], Julia Wolf[3], Christian J. Thaler[1], Lisa Windischbauer[2], Undraga Schagdarsurengin[3], Klaus Steger[3], and Viktoria von Schönfeldt[1]

[1]Division of Gynecological Endocrinology and Reproductive Medicine, Department of Gynecology and Obstetrics, Ludwig-Maximilians-University (LMU) Munich, Campus Grosshadern, Marchioninistrasse 15, 81377 Munich, Germany [2]Clinical Division of Gynecologic Endocrinology and Reproductive Medicine, Department of Obstetrics and Gynecology, Medical University of Vienna, Waehringer Guertel 18-20, 1090 Vienna, Austria [3]Department of Urology, Pediatric Urology and Andrology, Justus Liebig University Giessen, Schubertstraße 81, 35392 Giessen, Germany

*Correspondence address. Clinical Division of Gynecologic Endocrinology and Reproductive Medicine, Department of Obstetrics and Gynecology, Medical University of Vienna, Waehringer Guertel 18-20, 1090 Vienna, Austria. Tel: +43-14-04-00-28160; Fax: +43-14-04-00-28170; Email: Johannes.ott@meduniwien.ac.at

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STUDY QUESTION: Are unexplained recurrent miscarriages associated with abnormal protamine-1 and protamine-2 mRNA levels in spermatozoa?

SUMMARY ANSWER: Both protamine-1 and protamine-2 mRNA levels as well as the protamine-1 to protamine-2 mRNA ratio in spermatozoa from men whose female partners experienced two or more consecutive miscarriages were significantly different compared to those from both healthy control men and subfertile couples undergoing IVF/ICSI.

WHAT IS KNOWN ALREADY: Aberrant sperm protamine ratios are known to be associated with male-factor infertility. Data from this study suggest that the protamine mRNA ratio may additionally affect early embryo development.

STUDY DESIGN, SIZE, DURATION: The study population was recruited from men whose female partners presented with two or more consecutive unexplained miscarriages in a consultation for recurrent pregnancy loss between 2014 and 2016. At the research laboratory of the Urological Clinic of the University Giessen, spermatozoa from cases and controls were subjected to reverse transcription quantitative PCR (RTqPCR) using specific primer pairs for protamine-1 and protamine-2.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Protamine-1 and protamine-2 mRNA levels were analysed in semen samples from 25 men whose female partners experienced at least two consecutive idiopathic miscarriages before the 20th week of gestation. The couples were recruited during consultation at the Fertility Center of the LMU Munich, Germany, and at the Clinical Division of Gynecologic Endocrinology and Reproductive Medicine of the Medical University of Vienna, Austria. Results were compared with those from 32 healthy donors (WHO, 2010) recruited at the Department of Urology, Pediatric Urology and Andrology, Giessen, Germany, and 107 men whose partners participated in an IVF/ICSI program at the Fertility Center of the LMU Munich, Germany.

MAIN RESULTS AND THE ROLE OF CHANCE: Protamine-1 and protamine-2 mRNA levels as well as the protamine mRNA ratio and all routine semen parameters revealed significant differences between recurrent miscarriage couples and healthy volunteers ($P < 0.01$). When comparing recurrent miscarriage couples with couples undergoing IVF/ICSI, Ct-values of protamine-1 and protamine-2 mRNAs were significantly higher and the protamine mRNA ratio was significantly lower in RM couples ($P < 0.01$). When comparing protamine mRNA levels and the protamine mRNA ratio with routine semen parameters, a significant negative correlation was evident between progressive motility and the protamine-2 mRNA level ($P = 0.015$), as well as between non-progressive motility and the protamine mRNA ratio ($P = 0.023$).

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LIMITATIONS REASONS FOR CAUTION: Although our data demonstrate significant abnormalities in RM, larger sample sizes will be needed to confirm our results. Larger sample sizes should also balance the fact that we had to focus mainly on median protamine mRNA levels. Finally, men in the healthy control group were younger in age than those in the case group, which might have introduced some bias, at least concerning the classic semen parameters. Moreover, only protamine mRNA instead of protein levels could be measured.

WIDER IMPLICATIONS OF THE FINDINGS: Although the exact mechanism remains to be elucidated, our data suggest that protamine mRNA levels in spermatozoa are not only important for successful fertilization, but also for proper development of the early embryo.

STUDY FUNDING/COMPETING INTEREST(S): Grant from the University Clinic Giessen and Marburg (UKGM 29/2015GI). There are no competing interests.

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Key words: pregnancy loss / recurrent miscarriages / male infertility / protamine ratio

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Introduction

Recurrent miscarriage (RM) is defined as two or more consecutive pregnancy losses with the same partner before the completed 20th week of gestation (American College of Obstetricians and Gynecologists: Practice Committee of the American Society for Reproductive Medicine, ACOG, 2002). Reflecting the emotional distress of the affected couples, this definition has been increasingly applied in recent years, even though the World Health Organization (WHO) definition of RM is: 'three or more consecutive miscarriages before the 20th week of gestation' (WHO, 1977). Despite efforts undertaken to understand its possible causes, up to 50% of the cases remain without clearly defined etiology (Puscheck and Jeyendran, 2007).

Apart from established pathophysiological factors (i.e. genetic, endocrine, anatomical, immunological, thrombophilic and environmental factors), the impact of a possible male contribution has entered the discussion about RM in the last decades (Bareh *et al.*, 2016). Many studies, including several recent ones, have evaluated the influence of sperm DNA fragmentation, integrity and aneuploidy on RM (Robinson *et al.*, 2012; Ruixue *et al.*, 2013; Coughlan *et al.*, 2015; Ramasamy *et al.*, 2015; Zidi-Jrah *et al.*, 2016), and another focus for many years has been on sperm morphology (Hill *et al.*, 1994; Gopalkrishnan *et al.*, 2000; Gil-Villa *et al.*, 2010; Brahem *et al.*, 2011). The majority of these studies have suggested an association between RM and abnormal results of sperm testing (Rogenhofer *et al.*, 2012a). In other words, it is likely that a male factor contributes to the development of RM, further to the one already identified in our studies on paternal thrombophilic association with RM (Rogenhofer *et al.*, 2012b). This seems to be of clinical relevance. On the one hand, RM is associated with impaired quality of life (Mevorach-Zussman *et al.*, 2012a, b) and an explanation to offer to affected couples might lighten some of their burden. On the other hand, exact knowledge of the etiology of male factors might offer new treatment strategies.

In recent years, the sperm protamine-1 to protamine-2 mRNA ratio has been identified and evaluated as a new parameter of sperm functionality (Steger *et al.*, 2008; Nanassy *et al.*, 2011; Rogenhofer *et al.*, 2013). Recently, it has been proven to be a reliable prognostic factor for fertilization outcome in IVF and ICSI treatments (Rogenhofer *et al.*, 2013). An analysis of the sperm protamine-1 to protamine-2 mRNA ratio allows couples to be counselled regarding their chances of conceiving using assisted reproductive techniques and patients can be assigned to treatment strategies including ICSI only, if this is associated with an additional benefit regarding the expected outcome.

To date, the sperm protamine-1 to protamine-2 mRNA ratio has not been evaluated in couples with RM. In seeking further clarification of the male role in RM, the present study was undertaken to analyze the protamine ratio in spermatozoa of couples suffering from idiopathic consecutive RM.

Materials and Methods

RTqPCR for protamine-1 and protamine-2 was performed on semen samples from males whose female partners had conceived naturally but had experienced at least two consecutive idiopathic miscarriages. The results were compared with those of semen samples from healthy volunteers and subfertile couples taking part in an IVF/ICSI program. An overview of the study and population is shown in Fig. 1.

Study population with RMs

We analyzed ejaculates from 25 men whose female partners underwent two or more consecutive miscarriages before the 20th week of gestation, following the definition of the American College of Obstetricians and Gynecologists (Practice Committee of the American Society for Reproductive Medicine, ACOG, 2002). The couples sought advice between March 2014 and March 2016 at the Recurrent Miscarriages Consultation at the Fertility Center of the LMU Munich, Germany, and at the Clinical Division of Gynecologic Endocrinology and Reproductive Medicine of the Medical University Vienna, Austria. Time to conception for all pregnancies was no more than 1 year and all couples conceived naturally, without reproductive therapies. The criteria for strengthening the reporting of observational studies in epidemiology were applied as far as possible. All females had undergone extensive diagnostics. Potential causes of RM were analyzed and excluded, resulting in RM being classified as unexplained (Li *et al.*, 2002; Jeve and Davies, 2014). In detail, the following parameters were determined and excluded in the same manner in both centers: endocrinological dysfunction, such as polycystic ovary syndrome, according to the Rotterdam criteria (Rotterdam ESHRE, 2004), hyperprolactinemia, hyperandrogenemia (testosterone, androstendione, dehydroepiandrosterone), hypo-/hyperthyroidism (thyreotropine, thyroxine, tri-iodothyronine) and thyroid autoantibodies (thyroid peroxidase antibodies, thyreoglobuline antibodies); fetal and parental chromosomal disorders (karyogram); inherited thrombophilias, such as factor V-Leiden mutation (FVL), prothrombin 20210G > A substitution, 5,10-methylenetetrahydrofolate reductase (MTHFR) 677C > T exchange and deficiencies of natural anticoagulants protein C, protein S, antithrombin,

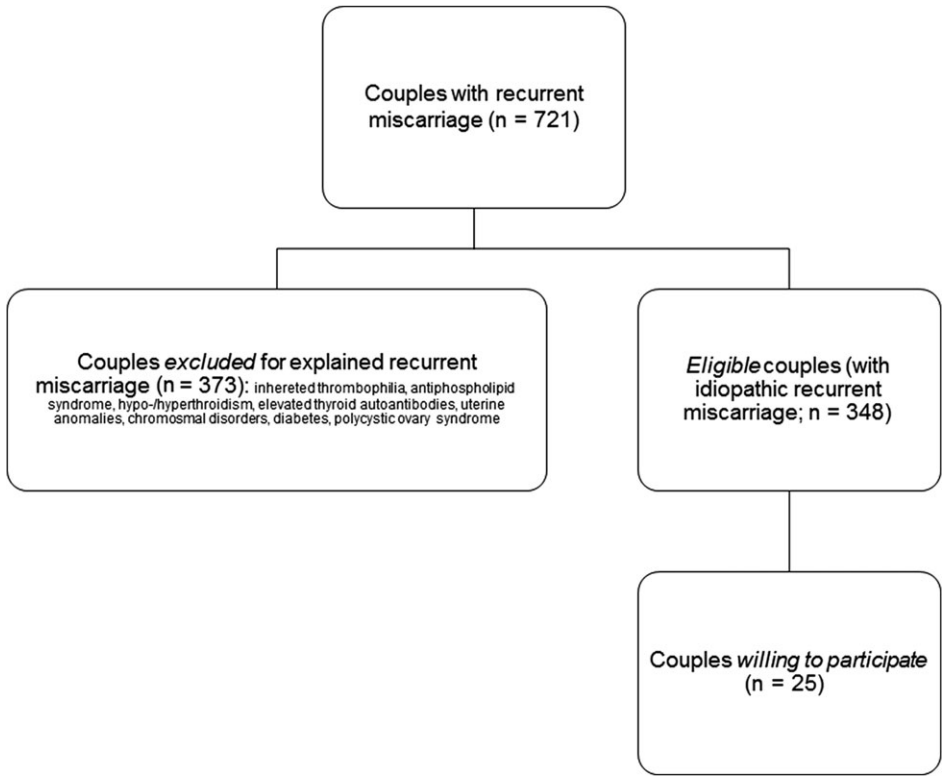

Figure 1 Study flow chart.

as well as auto-immunological disorders (antinuclear antibodies >1:240, anticardiolipin antibodies IgG and IgM, anti-ß2-glycoprotein antibodies IgG and IgM, lupus anticoagulant). Antiphospholipid syndrome was also excluded, according to the international consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (Miyakis *et al.*, 2006). Uterine anomalies were examined by vaginal ultrasonography and, in case of conspicuousness, by hysteroscopy and laparoscopy. The laboratory testing was performed in both centers at least two months after the last miscarriage in order to prevent any interference with gestation. Sperm analysis was also implemented within the same period after the latest miscarriage. Of 348 couples presenting with idiopathic RM within the period, 25 (7.2%) signed informed consent forms to take part in the protamine analysis for scientific purposes without compensation.

Control population of healthy volunteers

As a control population, 32 healthy volunteers exhibiting normozoospermia (WHO, 2010) were recruited at the Department of Urology, Pediatric Urology and Andrology, Giessen, Germany. All volunteers were informed about the nature of the study and provided written informed consent. Semen characteristics from these men were considered surrogate parameters reflecting normal fertility.

Control population of couples undergoing IVF/ICSI

Since the healthy volunteers were currently not interested in procreation, we aimed to compare the study population with RM with couples undergoing IVF/ICSI owing to couple infertility (longer than 12 months) in order to investigate the impact of protamines on oocyte fertilization as well as on live birth. For this issue, the data were obtained from our previous study from the Fertility Center of the LMU Munich (Rogenhofer *et al.*, 2013), but were updated with new cases since 2013, as well as the IVF/ICSI outcome. The indications for IVF/ICSI were primary or combined male subfertility, tubal pathology, endometriosis, polycystic ovary syndrome and other causes. Of the 192 available couples, 107 received complete follow-up after IVF/ICSI and the patients approved protamine analysis for scientific purposes.

Semen analysis

Semen samples were collected by masturbation into a sterile container at the clinic. The men had been asked to refrain from sexual activity for 2–7 days. Analysis was performed within one hour of collection in a blinded fashion, according to WHO recommendations (WHO, 2010), apart from morphology criteria which were assessed according to the Quality Control program of the German Society of Andrology (QuaDeGa, 2017), with all the three laboratories undergoing regular quality control programs (Giessen, Munich: German Society of Andrology, twice a year; Vienna: Austrian Federal Office for Safety in Health Care and Food Safety, once a year). Details about testing are provided in checklists for semen analysis according to Björndahl *et al.* (2016) Aliquots of individual ejaculates were stored in 'RNA later' (Ambion, Heppenheim, Germany) for shipping to the Department of Urology, Pediatric Urology and Andrology of the Justus Liebig University Giessen, where the analyses of the protamine-1 and protamine-2 mRNA levels were carried out. For patient details, see Supplementary Tables SI and SII. For an overview on semen analyses and laboratory performance at all centers see Fig. 2.

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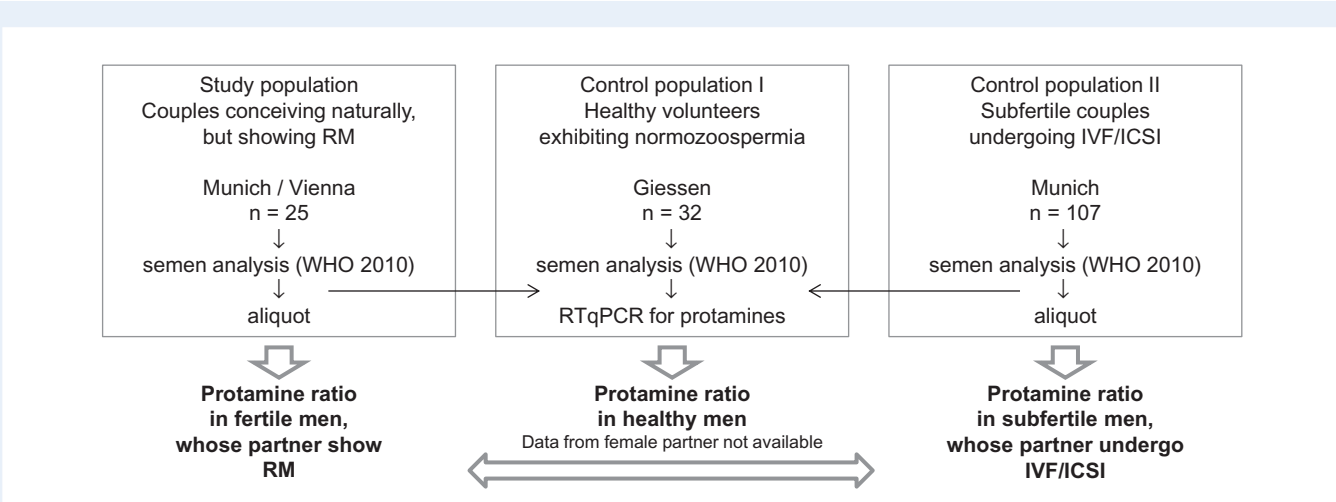

Figure 2 Overview of semen analysis and laboratory performance at all centers.

RTqPCR

RNA extraction was performed with the RNeasy Mini Kit and first-strand cDNA synthesis with Omniscript, according to the manufacturer's protocols (Qiagen, Hilden, Germany). Equal mRNA concentrations were used for RTqPCR in the different groups. For RTqPCR, iQ SYBR Green Supermix and iCycler (BioRad, Munich, Germany) were applied, as previously described (Rogenhofer *et al.*, 2013). In brief, cycling conditions for the amplification of protamine-1 and protamine-2 were: 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 58°C for 30 s and 73°C for 30 s. Primer sequences (Eurofins, Ebersberg, Germany) for protamines (GenBank, Accession Z46940) were protamine-1:5′aagtcgcagacgaaggagg3′ (forward primer bp 63–81) and 5′ atctcggtctgtacctgggg3′ (reverse primer bp 123–142), resulting in a 79 bp amplification product, and protamine-2:5′aagacgctcctgcaggcac3′ (forward primer bp 234–253) and 5′gccttctgcatgttctcttcct3′ (reverse primer bp 284–305), resulting in a 71 bp amplification product. Negative controls included samples lacking reverse transcriptase. All PCR amplifications were carried out in duplicate and mean values were calculated.

Statistical analysis

Protamine levels and semen parameters of patients and controls were calculated as median (range). Statistical analysis was performed to detect possible differences between patients and controls regarding protamine levels and semen parameters. For all metric variables, the Mann–Whitney *U* test was used to investigate differences between the two groups. Spearman's rank correlation was used to investigate possible associations between protamines and semen parameters in patients and controls. When investigating the impact of protamines on the time point of abortion, Spearman's rank correlation was used. In patients undergoing IVF/ICSI, the role of protamines and semen parameters on the fertilization rate of oocytes was investigated in a univariate model using the Mann–Whitney *U* test. In addition, a linear regression model with stepwise entering was used to investigate the parameters in a multivariate model. Finally, a possible association between protamines and semen parameters on IVF/ICSI outcome (birth) was calculated using the Mann–Whitney *U* test, as well as binary logistic regression analysis with the method enter. A value of $P < 0.05$ was considered statistically significant in all calculations. Statistical analyses were performed using IBM SPSS Statistics 22 for Windows (IBM GmbH, Ehningen, Germany).

Ethical approval

This study was approved by the local institutional review board of the Medical University of Vienna (ethical vote number: 1540/2013). Written informed consent was obtained from all participants.

Results

Both protamine-1 and protamine-2 mRNA levels, as well as the protamine mRNA ratio and all routine semen parameters revealed highly significant differences between RM couples and healthy volunteers. Ct-values of both protamine-1 and protamine-2 mRNAs were significantly higher in men whose female partners experienced RM when compared with healthy volunteers (in all cases $P < 0.01$). In comparison with healthy volunteers, IVF/ICSI patients also exhibited significantly decreased routine semen parameters (in all cases $P < 0.01$, Table I).

When comparing RM couples with couples undergoing IVF/ICSI, Ct-values of both protamine-1 and protamine-2 mRNAs were significantly higher in the RM couples, and the protamine mRNA ratio was significantly decreased (in all cases $P < 0.01$, Table I). Regarding routine semen parameters, higher sperm concentrations, higher total sperm counts and higher total motile sperm counts (TMSCs) were evident in couples with RM when compared with couples undergoing IVF/ICSI (in all cases $P < 0.01$, Table I).

Couples taking part in an IVF/ICSI program can be subcategorized into (i) those with no pregnancy, (ii) those with pregnancy followed by miscarriage and (iii) those with live birth. A subgroup analysis for the protamine levels is shown in Figure 3. While RM patients exhibited significantly increased Ct-values for both protamine-1 and protamine-2 mRNAs (in all cases $P < 0.01$), subfertile men from the IVF/ICSI group whose female partners became pregnant but suffered a miscarriage had significantly decreased Ct-values for both protamine mRNAs when compared with all other groups (in all cases $P < 0.01$) (Fig. 3).

When comparing protamine levels and the protamine ratio with routine semen parameters in the RM group and healthy volunteers, a significant negative correlation was evident between progressive motility and

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Table I Demographics, semen parameters and protamine levels in the study population with RM and control groups*.

	Couples with RM	Healthy volunteers	Couples with IVF/ICSI
	n = 25	*n* = 32	*n* = 107
Age Male (years)	35 [29–50][a]	26 [22–44][a, c]	39 [24–56][c]
Age Female (years)	34 [24–42]	n.a.	36 [24–45]
Protamine-1 (C_t)	34.0 [25.4–40.0][a, b]	27.9 [20.9–37.3][a]	25.3 [17.6–34.8][b]
Protamine-2 (C_t)	34.7 [23.9–40.0][a, b]	28.0 [20.0–36.5][a, c]	24.9 [14.2–36.7][b, c]
Protamine 1/2 ratio	0.61 [0.13–5.12][a, b]	0.99 [0–1.74][a, c]	0.81 [0.25–2.67][b, c]
Semen parameters			
Semen volume (ml)	2.5 [1.1–8.1]	3.7 [1.5–6.5]	2.9 [0.5–12.6]
Sperm concentration (10^6 per ml)	66.2 [2.2–306.0][b]	72.0 [7.3–163.8][c]	13.1 [0.8–310.0][b, c]
Total sperm number (10^6 per ejaculate)	152.9 [15.5–1193.4][b]	249.2 [16.5–790.0][c]	45.0 [2.4–682.0][b, c]
Total motile sperm count (10^6 per ejaculate)	82.6 [4.4–751.8][a, b]	182.0 [18.1–537.2][a, c]	16.2 [0.2–307.0][b, c]
Progressive motility (PR, %)	53 [28–63][a]	70 [35–84][a, c]	46 [5–66][c]
Non-progressive motility (NP, %)	20 [4–28][a]	14 [4–22][a, c]	21 [6–47][c]
Immotile spermatozoa (IM, %)	29 [20–52][a]	17 [7–69][a, c]	31 [13–85][c]

*Data are displayed in median [range], Mann–Whitney *U* test, $P < 0.01$.
[a]Couples with RM vs healthy volunteers.
[b]Couples with RM vs couples with IVF/ICSI.
[c]Healthy volunteers vs couples with IVF/ICSI, n.a. not available.

protamine-2 mRNA ($P = 0.015$), as well as non-progressive motility and the protamine mRNA ratio ($P = 0.023$) (Table II).

When analyzing protamine mRNA data from couples undergoing IVF/ICSI in relation to the fertilization rate of oocytes, no significant association between fertilization and Ct values of protamine mRNAs could be observed, in either a univariate or a multivariate model (for both $P > 0.1$). Only male age was significantly associated with the fertilization outcome (univariate model: $P = 0.007$; multivariate model: $P = 0.023$) (Table III).

Finally, the impact of protamines and routine semen parameters were evaluated after successful fertilization (IVF/ICSI) and embryo transfer for the final outcome birth. While the TMSC was significantly associated with live birth in a univariate model, no significant parameter estimate was evident in a multivariate model (Table IV).

Discussion

As ~50% of RMs remain idiopathic, a possible contribution of the male factor has been discussed over the last 20 years (Hill *et al.*, 1994; Gopalkrishnan *et al.*, 2000; Gil-Villa *et al.*, 2010; Brahem *et al.*, 2011; Robinson *et al.*, 2012; Rogenhofer *et al.*, 2012a; Ruixue *et al.*, 2013; Coughlan *et al.*, 2015; Ramasamy *et al.*, 2015; Bareh *et al.*, 2016; Zidi-Jrah *et al.*, 2016). To the best of our knowledge, the present study is the first to investigate the possible role of sperm protamine mRNA levels in couples with idiopathic RM. The major finding is that spermatozoa from men whose female partners had undergone RM contained significantly higher protamine-1 ($P < 0.001$) and protamine-2 ($P = 0.001$) mRNA levels compared to spermatozoa from healthy volunteers and couples taking part in an IVF/ICSI program. The protamine mRNA ratio was lower in the case group (median: 0.61) when compared with the healthy control group (median 0.99), although six patients of the RM group revealed a protamine mRNA ratio above the normal range. Interestingly, one patient (No. 10, Supplementary Table SI) showed no amplification product, neither for protamine-1, nor for protamine-2, within the 40 PCR cycles, and his female partner had experienced five RMs.

These data added to previous reports from our group (Steger *et al.*, 2000, 2001, 2003, 2008; Rogenhofer *et al.*, 2013) and others (Nanassy *et al.* 2011) suggesting that RTqPCR for sperm protamine mRNA content represents a suitable tool for estimating the fertilizing capacity of sperm. The finding that an aberrant protamine mRNA ratio is also associated with RM is new and suggests that protamines are not only important for fertilization, but may additionally have an impact on the correct initiation of gene expression in the early embryo. Negative effects on embryo development may be caused by abnormal epigenetic signatures, as incorrect histone to protamine exchange results in prolonged presence of histones, and retained sperm histones are well known to exhibit a variety of epigenetic modifications that are transmitted in fertilization (Bao and Bedford, 2016; Luense *et al.*, 2016; Schagdarsurengin and Steger, 2016). However, there may also be a direct effect, as protamines have been demonstrated to exhibit epigenetic modifications, the functions of which are still unknown (Brunner *et al.*, 2014).

Besides the protamine mRNA ratio, highly significant differences in all routine semen parameters were found between the tested groups, with significantly higher sperm quality in the healthy volunteer group. The fact that male partners of women with RM exhibit decreased sperm morphology and sperm motility has already been known (Hill *et al.*, 1994; Gopalkrishnan *et al.*, 2000; Gil-Villa *et al.*, 2010; Brahem *et al.*, 2011; Rogenhofer *et al.*, 2012a). Furthermore, an association between sperm motility and sperm protamine mRNA ratio has previously been reported by Rogenhofer *et al.* (2013). These data are in line with observations of the present study demonstrating a significant negative correlation between sperm motility and protamine-2 mRNA levels. Interestingly, the TMSC of RM couples was found to be significantly lower than in the

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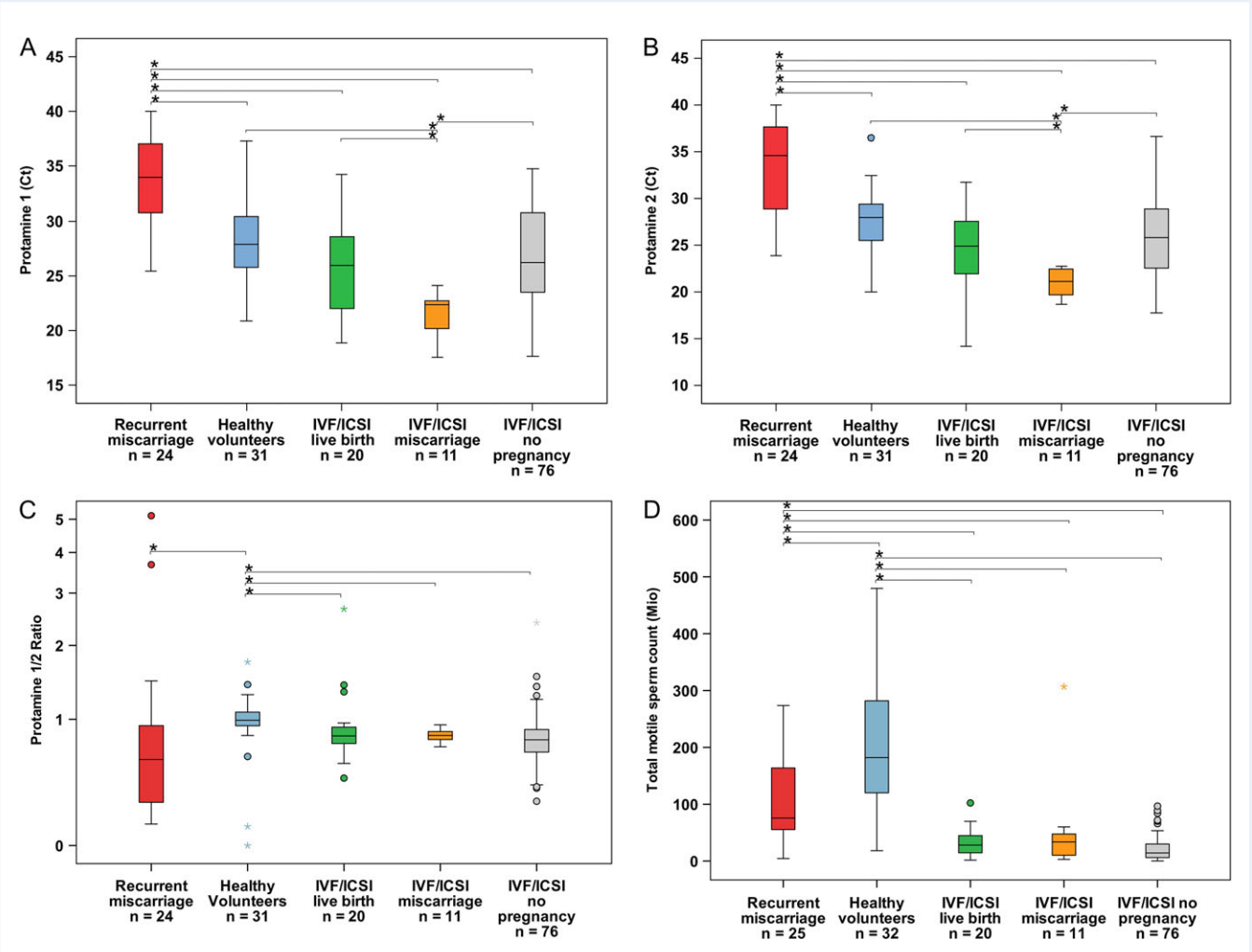

Figure 3 Illustration of protamine-1 levels (A), protamine-2 levels (B), protamine 1/2 ratios (C) and total motile sperm count (D) in couples with recurrent miscarriage, healthy volunteers and couples undergoing IVF/ICSI grouped according to outcome: live birth, miscarriage or no pregnancy. Box plots display median and interquartile range. Significant differences indicated with *$P < 0.01$ in all cases, Mann–Whitney *U* test. Error bars are the 95% confidence limits and outliers are marked with circles.

Table II Correlation between protamine levels and semen parameters in patients with RM and healthy volunteers.

	Protamine-1		Protamine-2		Protamine ratio	
	r	*P**	*r*	*P**	*r*	*P**
Sperm concentration (10^6 per ml)	−0.046	0.754	−0.117	0.423	0.085	0.567
Progressive motility (PR, %)	−0.262	0.053	−0.325	**0.015**	−0.022	0.873
Non-progressive motility (NP, %)	0.264	0.061	0.231	0.104	−0.321	**0.023**
Immotile spermatozoa (IM, %)	0.135	0.344	0.176	0.216	0.092	0.524

*Spearman's rank correlation.

healthy control group, but significantly higher than in the IVF/ICSI group. This result corroborates a previous study suggesting that the TMSC may represent a better indicator for the severity of male-factor infertility than the WHO classification system (Hamilton *et al.*, 2015).

Several study limitations need to be mentioned: only a minority of couples were willing to participate in this study, which is most likely because the evaluated parameters had no therapeutic consequences at this time. Thus, the study group with RM might not fully represent

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Table III Impact of protamine and other parameters on oocyte fertilization rate in 107 couples undergoing one cycle of IVF/ICSI.

Parameter	Univariate*		Multivariate#	
	r	*P*	beta	*P*
Protamine-1 (C_t)	−0.127	0.200	0.161	0.492
Protamine-2 (C_t)	−0.160	0.105	−0.306	0.204
Protamine ratio	−0.028	0.779	0.027	0.804
Age Male (years)	0.264	**0.007**	0.329	**0.023**
Age Female (years)	0.189	0.054	−0.037	0.797
Semen volume (ml)	0.012	0.903	0.144	0.307
Sperm concentration (10^6 per ml)	0.086	0.390	0.181	0.595
Total sperm number (10^6 per ejaculate)	0.103	0.289	−1.146	0.111
Total motile sperm count (10^6 per ejaculate)	0.108	0.276	1.064	0.112
Progressive motility (PR, %)	0.087	0.377	−0.463	0.743
Non-progressive motility (NP, %)	−0.053	0.595	−0.201	0.783
Immotile spermatozoa (IM, %)	−0.058	0.556	−0.325	0.818

*Mann–Whitney *U* test.
#Stepwise linear regression model, method: enter, $n = 102$.

Table IV Investigation of protamine and other parameters on live birth ($n = 20$) in 107 couples undergoing one cycle of IVF/ICSI

Parameter	Univariate*	Multivariate#
	P	*P*
Protamine-1 (C_t)	0.939	0.282
Protamine-2 (C_t)	0.879	0.235
Protamine ratio	0.168	0.209
Age Male (years)	0.076	0.859
Age Female (years)	0.085	0.192
Semen volume (ml)	0.223	0.854
Sperm concentration (10^6 per ml)	0.292	0.399
Total sperm number (10^6 per ejaculate)	0.059	0.669
Total motile sperm count (10^6 per ejaculate)	**0.038**	0.985
Progressive motility (PR, %)	0.445	1.000
Non-progressive motility (NP, %)	0.370	1.000
Immotile spermatozoa (IM, %)	0.104	1.000

[a]Mann–Whitney *U* test, $n = 107$.
[b]Logistic regression analysis, method: enter, $n = 106$.

the average patient population with idiopathic RM found in clinical routine. We consider this a minor study limitation. Moreover, although our data demonstrate significant sperm abnormalities in the RM group, larger sample sizes will be needed to confirm our results and to add to this first description of sperm protamines in RM. For this important clinical phenomenon, we believe that more light must be shed on the actual distribution of the sperm protamine ratio in the future. A larger sample size should then also balance the fact that we had to focus mainly on median protamine mRNA levels. Furthermore, men in the healthy control group were younger in age than those in the case group, which might have introduced some bias, at least concerning the classic semen parameters. Last not least, one might argue that measuring only protamine mRNA instead of protein levels must be seen as a limitation. This was the case since (i) subfertile patients revealed decreased sperm counts when compared with fertile volunteers and (ii) the only material which could be used for protamine diagnostics was that which would not be needed for IVF/ICSI and thus the remaining material was not sufficient for protein diagnostics.

In conclusion, spermatozoa from men whose female partners suffered from at least two miscarriages revealed abnormal median protamine-1 and protamine-2 mRNA levels and aberrant protamine ratio levels in comparison with healthy controls. Although the exact pathophysiological mechanisms are unclear at this time, these results underline the importance of a male factor in early miscarriage and should open a new field of research in RM.

Supplementary data

Supplementary data is available at *Human Reproduction* online.

Acknowledgments

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Authors' roles

N.R., V.v.S. and J.O. designed the study, collected samples and drafted the article. K.S. analyzed samples and also worked on the article. A.P. and J.W. performed statistical analyses and interpreted data. C.J.T.,

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L.W. and U.S. were involved in interpreting the results. All authors were involved in writing the paper and approved the final submitted versions.

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Conflict of interest

The authors declare no conflict of interest.

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