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# Protamine Ratio as Predictor of the Fertility Potential of Sperm by Couple Undergoing ICSI

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**Abstract**

**Objectives:** The present study aimed to measure the protamines P1 and P2 concentrations, standard sperm parameters, and also DNA fragmentation and condensation. In addition, the correlations between the above-mentioned variables and the P1/P2 ratio were explored in order to find out whether the ratio could be used as a biomarker for semen quality.

**Materials and Methods:** A total of 272 semen samples were divided into 3 P1/P2 ratio groups: G1 as low (<0.8), G2 as normal (0.8-1.2), and G3 as high (>1.2) ratios. DNA fragmentation and condensation were evaluated by TUNEL and CMA3 (chromomycin$A_3$) assays and protamine levels (P1 and P2) were measured using acid urea polyacrylamide gel (AU-PAGE) electrophoresis and western blot (WB).

**Results**: In G1, CMA3 positive and DNA fragmentation index (DFI) were negatively correlated (r = -0.371, $P = 0.001$). In addition, P1/P2 ratio had a positive correlation with DFI (r = 0.652, $P = 0.001$);however it had a negative association with CMA3 (r = -0.623, $P = 0.001$). Moreover, G2 demonstrated positive correlations between sperm concentration and P1(r = 0.257, $P = 0.011$) and P2 (r = 0.277, $P = 0.006$), progressive motility, and P1 (r = 0.352, $P = 0.001$). The P2 was positively correlated with progressive motility (r = 0.380, $P < 0.01$). Besides, a positive relationship was found between P1/P2 ratio and CMA3 (r = 0.333, $P = 0.001$). In contrast, normal morphology showed a negative association ($P < 0.05$) with P1 (r = -0.206) and P2 (r = -0.208).Meanwhile, there was a negative correlation between P1 and DFI (r = -0.207, $P = 0.041$) as well. Finally, in G3, negative correlations were also observed between P1 and normal morphology (r = -.283, $P = .027$) and also P1/P2 ratio and P2 (r = -0.372, $P = 0.003$) while DFI showed a positive correlation with CMA3 (r = 0.299, $P = 0.019$).

**Conclusions**: The results revealed that P1/P2 ratio affected DNA integrity. Therefore, it was found that it could play a crucial role in human sperm quality and function and thus might be used as a predictor of fertility in assisted reproductive technology (ART) treatments.

**Keywords:** Infertility, Sperm DNA, TUNEL, CMA3, Protamine

## Introduction

Infertility is defined as the inability of a healthy couple to conceive after 12 months of regular, unprotected intercourse (1). Male infertility accounts for 40%–50% of infertility and may be caused by numerous factors including genetic causes, poor semen quality, medical disease, hormone aberrations, or it may be idiopathic (2).

Recently, a large number of cases have been overcome with assisted reproductive technologies (ARTs). Intrauterine insemination (IUI) is usually used as a first choice for the treatment of ovulatory dysfunction, minimal endometriosis, unexplained subfertility, and milder forms of male subfertility. Following a mild controlled ovarian stimulation, prepared semen is deposited into the woman's uterus. In vitro fertilization (IVF) is a second procedure where oocytes are fertilized by sperm in vitro (3). Two to 5 days later, the pre-embryo is placed in the woman's uterus. In intracytoplasmic sperm injection (ICSI), nearly the same principles are followed, but a single spermatozoon is selected and directly injected into cytoplasm of the oocyte.

Some cases are successful and lead to the birth of a healthy baby. In other cases, embryos may not develop (i.e., they are arrested) (4,5). There are many reasons and explanations for this failure including female inability to support the development, but it is clear that the normal constitution of the male genome, which is injected into the oocyte, is of crucial importance for development of a healthy embryo.

The sperm epigenome is unique and highly specialized because of the unique nature and function of the sperm and also diverse requirements for successful fertilization. Due to the need for better sperm quality, for example, motility, the sperm chromatin must be compacted and highly organized. During spermatogenesis, the chromatin in the sperm head is packaged tightly by the replacement of most histones with 2 types of protamines (i.e., 1 and 2).

Protamines which are small charged alkaline proteins, contain positively charged arginine amino acids that can

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bind to the negatively charged phosphorus in DNA (6). The interaction between positively charged arginine fragments and DNA backbone lead to tight coiling of the DNA, causing it to nearly appear hidden in the protamine; this structure is known as a toroid (6). Therefore, in humans, most of the sperm genome is packaged by protamines instead of histones (7). However, in fertile males, between 5% and 15% of the spermatozoa (SPZ) chromatin remains bound to histones rather than protamines (8-11).

It has been demonstrated that a relatively normal proportion of the 2 protamines in humans ranges between 0.80 and 1.20 (12). The protamine ratio can be decreased (<0.8) or increased (>1.2) in sub-fertile patients (12). Other researchers have found a high association between the presence of an altered protamine ratio and altered sperm parameters like sperm concentration, motility, and morphology (13,14).

Furthermore, a number of studies have demonstrated that protamine deficiency could also deteriorate chromatin tight packaging and increase susceptibility to external stress, which can cause a high risk of elevated sperm DNA damage (15,16). There is also conflicting evidence concerning the relationship between sperm DNA fragmentation and fertilization rates following IVF and ICSI. Thus, the aims of the present study were to determine protamine 1 and 2 values, P1/P2 ratio, standard sperm parameters, chromatin condensation, and DNA integrity; the correlation between the P1/P2 ratio and the other investigated parameters were also focused on. Finally, it was aimed to determine whether the P1/P2 ratio could be used as a predictor of sperm quality.

## Materials and Methods

### Study Population

Semen samples (N = 270) were randomly collected from male partners of the couples undergoing ICSI treatment at the reproduction and andrology laboratory at the Department of Obstetrics and Gynaecology, University of Saarland, Homburg/Saar, Germany.

The inclusion criteria of the patients were as follows: males who did not have cryptorchidism, present or past cancer treatment, genetic abnormalities such as Klinfelter syndrome or Y-chromosome microdeletion, hypogonadotrophic hypogonadism, drug abuse, varicocele, and/or recent fever episode, and female partners without any history of female-related cause of subfertility (endometriosis, tubal occlusion, or ovulatory disturbance), and no surgical or medical infertility treatment in the last 3 months before undergoing ICSI.

### Sperm Collection and Processing

All the semen samples were obtained from the participants through masturbation and then they were collected into sterile containers after at least 3 days of sexual abstinence. The samples were allowed to liquefy for 30 minutes and then were immediately processed. Next, the semen samples were analysed for primary semen parameters such as volume, pH, viscosity, sperm concentration, agglutination, motility, viability, and morphology according to World Health Organization (WHO) guidelines (17).

Sperm morphology was classified according to the strict criteria described by Kruger et al (18). Additionally, seminal smears were stained with Papanicolaou test and analysed accordingly. A total of 100 SPZ were examined per slide using bright field illumination with a magnification of 100× with oil immersion. The rest of the semen samples were stored at -20°C until the assay was performed (within 3 months).

### Sperm Chromatin Condensation ($CMA_3$ Assay)

Sperm chromatin condensation was assessed using the $CMA_3$ assay, as described by Hammadeh et al (19), with some modifications. Semen sample smears were prepared using 10 µL of sperm suspension on microscope slides and allowed to air dry. The smears were fixed in 3:1 methanol–glacial acetic acid ratio at 4°C for 30 minutes and were allowed to air dry at room temperature (RT). Then, 50 µL of $CMA_3$ solution (0.25 mg $CMA_3$ [Sigma-Aldrich, Darmstadt, Germany] in 1 ml phosphate-buffered saline (PBS)) was added to each slide and the slides were coated with cover slips before being incubated in the dark for 30 minutes at RT. The slides were rinsed in PBS buffer and mounted with 1:1 (v/v) PBS/glycerol ratio and allowed to air dry for 1 hour.

A total of 200 SPZ were analysed on each slide using the fluorescence microscope BH2-RFCA (Olympus, Japan) with a green fluorescence filter to distinguish the SPZ that stain bright green ($CMA_3$-positive) from those that stain a dull green ($CMA_3$-negative).

### Sperm DNA Integrity TUNEL Assay

The terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay was performed using an *in situ* cell death detection kit according to the manufacturer's instructions (Sigma-Aldrich) with some modifications as described by Borini et al (20). The smears were fixed with 4% paraformaldehyde-PBS, pH = 7.4, at RT for 2 hours and washed with PBS. For sperm permeabilisation, the smears were incubated with 0.1% Triton X-100 in 0.1% sodium citrate, pH = 6.0, for 15 minutes at RT. Then, 50 µL of the TdT-labelled nucleotide mixture (50 µL of enzyme and 450 µL of label solutions) was added to each slide and incubated in a humidified chamber at 37°C in the dark overnight. Then, the slides were rinsed twice with PBS buffer and 25 µL of 5 µg/mL 4', and 6-diamidino-2-phenylindole (DAPI) stain was added to each slide as a counter stain. For evaluation, a total of 200 SPZ were analysed on each slide by distinguishing SPZ stained green (TUNEL-positive with fragmented DNA) from those stained blue (TUNEL-negative with intact DNA). A fluorescence microscope (Olympus BX-61, Japan), DAPI and FITEC filters, and Meta Systems

Isis software were used for fluorochrome evaluation via a combination of exciter dichromic barrier filter of BP (band pass) (436/10:FT, 580:LP 470). A negative control was performed for each sample using fluorescent isothiocyanate-labelled dUTP without enzyme.

### Sperm Protamine Extraction

As previously explained, sperm nuclear protamines were extracted from all the studied semen samples (19). The sperm pellets with determined sperm concentration were removed from the storage at -20°C and used for protamine extraction. The pellets were washed in 1ml of washing buffer I containing 1 mM (mmol/L) of phenylmethylsulfonyl fluoride in distilled water (PMSF) and centrifuged at 250 ×g for 5 minutes at RT. Then, the pellet was re-suspended in 100 µL of wash buffer 2 containing 20 mM EDTA (ethylenediaminetetraacetic acid) and PMSF (1 mmol, pH = 8.0) and vortexed for 15 seconds. Then, 100 µL of decondensation buffer 1 (6 M guanidine hydrochloride and 575 mM dithiothreitol) was added and vortexed for 15 seconds followed by the addition of 200 µL of decondensation buffer 2 (522 mM sodium iodoacetate) and vortexing for 30 seconds.

The component was then incubated in the dark at RT for 30 minutes and 1 mL of cold absolute ethanol was added; the sample was then mixed and incubated at -20°C for 1 minute. The mixture was centrifuged at 1000 × *g* for 10 minutes at 4°C and the supernatant discarded (this step was repeated once). The pellet was re-suspended in 80 µL of denaturing solution (0.5 M HCl), mixed, and incubated for 15 minutes at 37°C. The sample was then centrifuged at 1000 × *g* for 10 minutes at 4°C. Next, the supernatant was transferred to another tube containing 200 µl of precipitating solution (100% trichloroacetic acid). The mixture was incubated on ice for 3 minutes and centrifuged at 1000 × g for 10 minutes at 4°C. The supernatant was removed carefully. Then, the precipitate was washed in 1 mL of wash buffer 3 (1 % β-mercapto-ethanol in 100% acetone) and vortexed for 15 seconds. The preparation was centrifuged at 1000 × *g* for 8 minutes at 4°C and the supernatant was removed. The final pellet was dried at 4°C overnight and stored at -80°C until further analysis.

### Preparation of the Human Protamine Standard

A human protamine standard was prepared according to what has been previously described (19). Sperm samples of 20 fertile donors were pooled in order to extract and estimate sperm protamines. The semen samples were centrifuged at 250 × g for 10 minutes at RT to remove the seminal plasma, then they were washed with PBS and centrifuged at 250 × g for 10 minutes at RT. The supernatant was discarded. The pellet of each sample was re-suspended in .5 ml of the denaturing solution (0.5 M HCl) and incubated at 37°C for 15 minutes. The samples were then centrifuged at 250 × g for 5 minutes at RT and the supernatant was also removed. The pellets were washed with 0.25 ml of washing buffer 2 (20 mM EDTA & 1 mM PMSF in 0.1 M Tris, pH = 8.0) and centrifuged at 250 × g for 5 minutes at RT and the supernatant was discarded as well. Nuclear proteins were extracted as described above. The final protein concentration was determined using the RCDC (educing agent compatible-detergent compatible) protein assay kit (BioRad Laboratories, Hercules, CA, USA) and an Ultrospec 2100 pro UV/Visible spectrophotometer (Amersham Biosciences Inc., Cambridge, UK). A regression curve was obtained from the four different concentrations of protamine standard (1.5, 1, 0.5, and 0.25 µg/µL) included in each gel; the intensity of their bands were also obtained in order to calculate the amount of P1 and P2 in each sample. The value of the regression curve ($R^2$) was ≥0.98 for each gel.

### Extraction of Protamine from Control Samples

Semen samples were pooled from 10 fertile men and treated as in the standard preparation.

In total, according to the procedure explained above, aliquots of 40 × $10^6$ sperm were prepared and stored at -80°C. One aliquot was extracted in tandem with test samples for every run. The sperm protamine extraction protocol, as described above, was followed.

### Quantification of P1 and P2 With Acetic AU-PAGE and WB

Protamine extracts were analysed, as previously described, using the AU-PAGE method (19). Extracted nucleoproteins were dissolved in 80 mL of loading buffer (0.375 M potassium acetate, pH = 4.0, 15% sucrose, and 0.05% methyl green). The stacking gel was prepared with 7.5% (w/v) acrylamide, .8% (w/v) N,N′-methylenebisacrylamide solution, and 0.375 M potassium acetate (pH = 4.0). The separating gel was prepared with 20% (w/v) acrylamide and 0.8% (w/v) N,N′-methylenebisacrylamide solution. For both gels, 2.5 M urea and 43% acetic acid were used. In addition, for polymerization, 1.6% (w/v) ammonium persulfate was used for both gels and 0.5 and 2% (w/v) N,N,N′,N′-tetramethylendiamine for resolving and stacking the gels, respectively. Then, the gels were pre-run at 200 V and 40 mA for 1.5 hours. Nucleoprotein samples were loaded on to the gel and run at 200 V and 80 mA for 5–6 hours.

The horseradish peroxidase-conjugated goat anti-mouse IgG (Dianova, Germany) antibody was diluted 1:10 000 ratio and incubated for 1 hour at RT. Protamines were detected using the lumi-light chemiluminescence kit (Roche, Germany). Negative immunoblot controls were performed, as above, without the primary antibody. Protamine bands were visualized using the enhance chemiluminescence system (Bio-Rad, Germany). The intensity of the bands corresponding to P1 and P2 were quantified. The P1 and P2 concentrations were calculated from the standard curve generated from the human

protamine standard as described above. The P1/P2 ratio of each sample was calculated and the mean values were reported as well. All the samples were tested in duplicate.

### Statistical Analysis

Analysis was performed at the Institute of Medical Biometry and Medical Information, University of the Saarland, using SPSS (statistical package for the social sciences) software, version 23. The obtained data were provided as the median ± standard deviation and the different correlations were described according to the correlation coefficient "r" Spearman. Comparison of the medians was determined using the independent sample *t* test (Mann–Whitney U test).

## Results

Following quantification of protamine P1 and P2, the P1/P2 ratio was determined and patients were divided into 3 groups according to P1/P2 values: G1 (n = 11), low P1/P2 ratio (<0.80); G2 (n = 98), normal P1/P2 ratio (0.8–1.20); and G3 (n = 61), high P1/P2 ratio (>1.20).

The means for age, sperm concentration, progressive motility, and normal morphology of all the investigated patients were 33.53 ± 7.36 years, 64.81 ± 39.66 × $10^6$/mL, 35.90 ± 18.64%, and 29.65 ± 23.46%, respectively. In addition, the medians of the DFI measured by TUNEL staining and chromatin condensation (protamination) measured by $CMA_3$ staining were 14.60±8.58% and 34.30±15.97%, respectively. Besides, concentrations of protamine P1 and P2 were 432.35±124.14 and 397.85±125.19 ng/$10^6$ SPZ, respectively, and the P1/P2 ratio was 0.83± 0.49 (Table 1).

**Table 1.** Median, Standard Deviation of Semen Parameters, DFI, CMA3 Positive and Protamine's Measurement by All Investigated Samples (N = 272)

| Parameters | Median ± SD |
|---|---|
| Age (y) | 33.53±7.36 |
| Concentration (x$10^6$/mL) | 64.81±39.66 |
| Progressive motility (%) | 35.90±18.64 |
| Normal morphology (%) | 29.65±23.46 |
| DFI (%) | 14.60±8.58 |
| CMA positive (%) | 34.30±15.97 |
| Protamine 1 (ng/$10^6$ SPZ) | 432.35±124.14 |
| Protamine 2 (ng/$10^6$ SPZ) | 397.85±125.19 |
| P1/P2 ratio | 0.83±.49 |

Abbreviations: SPZ, spermatozoa; SD, standard deviation

### Patients (G1) With Low P1/P2 Ratio (<0.80)

A positive correlation was observed between the P1/P2 ratio and the age of males (r = 0.354, *P* = .001) . The P1/P2 ratio showed highly significant negative correlations with sperm concentration (r = 0.465, *P* = 0.001), progressive motility (r = 0.381; *P* = 0.001), and normal morphology (r = 0.765, *P* = 0.001) (Table 2).

The DFI demonstrated a significantly positive correlation with the P1/P2 ratio (r = 0.652, *P* = 0.001) and chromatin condensation was found to have a significantly negative correlation with the P1/P2 ratio (r = 0.623, *P* = 0.001) (Table 3). The P1 and P2 values were positively associated (r = 0.746, *P* = 0.001) (Table 3), but neither of them was correlated with P1/P2 ratio.

### Patients (G2) With Normal P1/P2 Ratio (0.80 ≤ ratio ≤ 1.20)

In contrast to G1, participants' age did not correlate with the studied parameters (Table 4). However, sperm concentration was positively correlated with P1 (r = 0.257,

**Table 2.** Correlations Between Different Parameters Measured by Patients With Low P1/P2 Ratio (Ratio <0.8)

| Parameters | | Age (y) | Concentration ($10^6$ spz/mL) | Progressive Motility (%) | Normal Morphology (%) |
|---|---|---|---|---|---|
| Age (y) | *r* | 1.000 | -0.288** | -0.196* | -0.407** |
| | *P* | - | 0.002 | 0.038 | 0.001 |
| Concentration ($10^6$ spz/mL) | *r* | -0.288** | 1.000 | 0.196* | 0.512** |
| | *P* | 0.002 | - | 0.038 | 0.001 |
| Progressive motility (%) | *r* | -0.196* | 0.196* | 1.000 | 0.411** |
| | *P* | 0.038 | 0.038 | - | 0.001 |
| Normal morphology (%) | *r* | -0.407** | 0.512** | 0.411** | 1.000 |
| | *P* | 0.001 | 0.001 | 0.001 | - |
| DFI (%) | *r* | 0.225* | -0.156 | -0.407** | -0.306** |
| | *P* | 0.017 | 0.098 | 0.001 | 0.001 |
| CMA3 positive (%) | *r* | -0.336** | 0.273** | 0.345** | 0.675** |
| | *P* | 0.001 | 0.001 | 0.001 | 0.001 |
| P1 ($ng/10^6$ spz) | *r* | -0.054 | 0.136 | -0.086 | -0.065 |
| | *P* | 0.567 | 0.152 | 0.365 | 0.496 |
| P2 ($ng/10^6$ spz) | *r* | -0.070 | 0.069 | -0.004 | -0.050 |
| | *P* | 0.463 | 0.468 | 0.966 | 0.602 |
| P1/P2 ratio | *r* | 0.354** | -0.465** | -0.381** | -0.765** |
| | *P* | 0.001 | 0.001 | 0.001 | 0.001 |

Abbreviations: SPZ, spermatozoa.

**Correlation is significant at the 0.01 level (*P*<0.01); *Correlation is significant at the 0.05 level (*P*<0.05).

**Table 3**. Correlations Between Different Parameters Measured by Patients With Low P1/P2 Ratio (Ratio <0.8)

| Parameters | | DFI (%) | CMA3 Positive (%) | P1 (ng/$10^6$ spz) | P2 (ng/$10^6$ spz) | (P1/P2) Ratio |
|---|---|---|---|---|---|---|
| DFI (%) | *r* | 1.000 | -0.371** | -0.028 | -0.058 | 0.652** |
| | *P* | - | 0.001 | 0.772 | 0.544 | 0.001 |
| CMA3 positive (%) | *r* | -0.371** | 1.000 | -0.117 | 0.051 | -0.623** |
| | *P* | 0.001 | - | 0.216 | 0.594 | 0.001 |
| P1 (ng/$10^6$ spz) | *r* | -0.028 | -0.117 | 1.000 | 0.746** | 0.062 |
| | *P* | 0.772 | 0.216 | - | 0.001 | 0.514 |
| P2 (ng/$10^6$ spz) | *r* | -0.058 | 0.051 | 0.746** | 1.000 | 0.068 |
| | *P* | 0.544 | 0.594 | 0.001 | - | 0.477 |
| P1/P2 ratio | *r* | 0.652** | -0.623** | 0.062 | 0.068 | 1.000 |
| | *P* | 0.001 | 0.001 | 0.514 | 0.477 | - |

Abbreviations: SPZ, spermatozoa.
**Correlation is significant at the 0.01 level (*P*<0.01); *Correlation is significant at the 0.05 level (*P*<0.05).

**Table 4.** Correlations Between Different Parameters Measured by Patients With Normal P1/P2 Ratio (0.8≤ Ratio ≤1.2)

| Parameters | | Age (y) | Concentration ($10^6$ spz/mL) | Progressive Motility (%) | Normal Morphology (%) |
|---|---|---|---|---|---|
| Age (y) | *r* | 1.000 | -0.007 | -0.037 | -0.096 |
| | *P* | | 0.947 | 0.720 | 0.348 |
| Concentration ($10^6$ spz/mL) | *r* | -0.007 | 1.000 | 0.577** | 0.003 |
| | *P* | 0.947 | | 0.001 | 0.980 |
| Progressive motility (%) | *r* | -0.037 | 0.577** | 1.000 | -0.039 |
| | *P* | 0.720 | 0.001 | - | 0.702 |
| Normal morphology (%) | *r* | -0.096 | 0.003 | -0.039 | 10.000 |
| | *P* | 0.348 | 0.980 | 0.702 | - |
| DFI (%) | *r* | 0.102 | -0.203* | -0.304** | -0.029 |
| | *P* | 0.315 | 0.044 | 0.002 | 0.780 |
| CMA3 positive (%) | *r* | -0.028 | -0.410** | -0.238* | -0.217* |
| | *P* | 0.787 | 0.001 | 0.018 | 0.032 |
| P1 (ng/$10^6$ spz) | *r* | 0.053 | 0.257* | 0.352** | -0.206* |
| | *P* | 0.604 | 0.011 | 0.001 | 0.041 |
| P2 (ng/$10^6$ spz) | *r* | 0.061 | 0.277** | 0.380** | -0.208* |
| | *P* | 0.553 | 0.006 | 0.001 | 0.040 |
| P1/P2 ratio | *r* | 0.012 | -0.143 | -0.091 | 0.034 |
| | *P* | 0.906 | 0.160 | 0.374 | 0.738 |

Abbreviations: SPZ, spermatozoa.
**Correlation is significant at the 0.01 level (*P*<0.01); *Correlation is significant at the 0.05 level (*P*<0.05).

*P*=0.011) and P2 (r=0.277, *P*=0.006). The mean percentage of morphologically normal SPZ demonstrated a significant negative correlation with P1 (r=-0.206, *P*=0.041) and P2 (r=-0.208, *P*=0.040) (Table 4). The DFI showed no correlation with the P1/P2 ratio. Similarly, no association was observed between the P1/P2 ratio and chromatin condensation (r=0.333, *P*=0.001) (Table 5).

### Patients (G3) With High P1/P2 Ratio (>1.20)

In G3, there was no correlation between males' age and the investigated sperm parameters in the present study (Tables 6 and 7). The P1 demonstrated a significantly positive correlation with P2 (r=0.785, *P*=0.001) (Table 7) while P1 showed a significantly negative correlation (r=-0.299, *P*=0.027) with the mean percentage of morphologically normal SPZ (Table 6). The P1/P2 ratio in G3 was only significantly and negatively correlated with P2 (r=-0.372, *P*=0.003) (Table 7).

### Comparison of Studied Parameters Between Ratio Groups (Low, Normal, and High)

Table 8 illustrates the different studied parameters (as the median ± standard deviation) compared between all the 3 groups. The age and DFI were significantly higher in G3 compared with G1 and G2 (*P*=0.016 and *P*=0.001, respectively). In addition, the progressive motility and P2 values were significantly higher in G2 (*P*=0.001). Besides, the mean percentage of morphologically normal SPZ and $CMA_3$-positive values were significantly higher in G1 (*P*=0.001) while the P1 value was significantly lower in this group compared with the other groups (*P*=0.001).

## Discussion

Protamine ratio (P1/P2) at the level of 0.8–1.2 in semen

**Table 5**. Correlations Between Different Parameters Measured by Patients With Normal P1/P2 Ratio (0.8≤ Ratio ≤1.2)

| Parameters | | DFI (%) | CMA3 Positive (%) | P1 (ng/10$^6$ spz) | P2 (ng/10$^6$ spz) | (P1/P2) Ratio |
|---|---|---|---|---|---|---|
| DFI (%) | *r* | 1.000 | 0.143 | -0.207* | -0.198 | 0.028 |
| | *P* | - | 0.161 | 0.041 | 0.051 | 0.782 |
| CMA3 positive (%) | *r* | 0.143 | 1.000 | -0.016 | -0.064 | 0.333** |
| | *P* | 0.161 | - | 0.873 | 0.534 | 0.001 |
| P1 (ng/10$^6$ spz) | *r* | -0.207* | -0.016 | 1.000 | 0.969** | 0.136 |
| | *P* | 0.041 | 0.873 | - | 0.001 | 0.181 |
| P2 (ng/10$^6$ spz) | *r* | -0.198 | -0.064 | 0.969** | 1.000 | -0.054 |
| | *P* | 0.051 | 0.534 | 0.001 | - | 0.597 |
| P1/P2 ratio | *r* | 0.028 | 0.333** | 0.136 | -0.054 | 1.000 |
| | *P* | 0.782 | 0.001 | 0.181 | 0.597 | - |

Abbreviations: SPZ, spermatozoa.
**Correlation is significant at the 0.01 level (*P*<0.01); *Correlation is significant at the 0.05 level (*P*<0.05).

**Table 6.** Correlations Between Different Parameters Measured by Patients With High P1/P2 Ratio (Ratio >1.2)

| Parameters | | Age (y) | Concentration (10$^6$ spz/mL) | Progressive Motility (%) | Normal Morphology (%) |
|---|---|---|---|---|---|
| Age (y) | *r* | 1.000 | 0.055 | -0.211 | -0.160 |
| | *P* | - | 0.678 | 0.103 | 0.217 |
| Concentration (10$^6$ spz/mL) | *r* | 0.055 | 1.000 | 0.256* | -0.017 |
| | *P* | 0.678 | - | 0.048 | 0.899 |
| Progressive motility (%) | *r* | -0.211 | 0.256* | 1.000 | -0.221 |
| | *P* | 0.103 | 0.048 | - | 0.087 |
| Normal morphology (%) | *r* | -0.160 | -0.017 | -0.221 | 1000 |
| | *P* | 0.217 | 0.899 | 0.087 | - |
| DFI (%) | *r* | 0.103 | -0.165 | -0.454** | 0.441** |
| | *P* | 0.429 | 0.208 | 0.001 | 0.001 |
| CMA3 positive (%) | *r* | -0.168 | -0.628** | -0.178 | 0.123 |
| | *P* | 0.195 | 0.001 | 0.170 | 0.345 |
| P1 (ng/10$^6$ spz) | *r* | 0.166 | 0.037 | 0.173 | -0.283* |
| | *P* | 0.202 | 0.780 | 0.183 | 0.027 |
| P2 (ng/10$^6$ spz) | *r* | 0.105 | -0.021 | 0.131 | -0.107 |
| | *P* | 0.419 | 0.873 | 0.313 | 0.412 |
| P1/P2 ratio | *r* | 0.131 | 0.185 | -0.177 | -0.216 |
| | *P* | 0.315 | 0.158 | 0.171 | 0.095 |

Abbreviations: SPZ, spermatozoa.
**Correlation is significant at the 0.01 level (*P*<0.01); *Correlation is significant at the 0.05 level (*P*<0.05).

**Table 7**. Correlations Between Different Parameters Measured by Patients With Normal P1/P2 Ratio (0.8≤ Ratio ≤1.2)

| Parameters | | DFI (%) | CMA3 Positive (%) | P1 (ng/10$^6$ spz) | P2 (ng/10$^6$ spz) | (P1/P2) Ratio |
|---|---|---|---|---|---|---|
| DFI (%) | *r* | 1.000 | 0.299* | -0.078 | -0.040 | 0.179 |
| | *P* | 0. | 0.019 | 0.552 | 0.757 | 0.167 |
| CMA3 positive (%) | *r* | 0.299* | 1.000 | -0.082 | -0.028 | -0.138 |
| | *P* | 0.019 | - | 0.529 | 0.831 | 0.288 |
| P1 (ng/10$^6$ spz) | *r* | -0.078 | -0.082 | 1.000 | 0.785** | 0.095 |
| | *P* | 0.552 | 0.529 | - | 0.001 | 0.181 |
| P2 (ng/10$^6$ spz) | *r* | -0.040 | -0.028 | 0.785** | 1.000 | -0.372** |
| | *P* | 0.757 | 0.831 | 0.001 | - | 0.003 |
| P1/P2 ratio | *r* | 0.179 | -0.138 | 0.095 | -0.372** | 1.000 |
| | *P* | 0.167 | 0.288 | 0.181 | 0.003 | - |

Abbreviations: SPZ, spermatozoa.
**Correlation is significant at the 0.01 level (*P*<0.01); *Correlation is significant at the 0.05 level (*P*<0.05).

**Table 8.** Comparison of Sperm Parameters Between the 3 Groups of Protamine Ratio P1/P2

| Parameters | Age (y) | Concentration ($10^6$ spz/mL) | Progressive Motility (%) | Normal Morphology (%) |
|---|---|---|---|---|
| Age (y) | 32.10 ± 7.65 | 34.09 ± 6.84 | 35.26 ± 7.23 | 0.016* |
| | 66.67 ± 33.50 | 68.23 ± 45.40 | 55.70 ± 39.67 | 0.126 |
| Concentration ($10^6$ spz/mL) | 31.18 ± 15.17 | 43.19 ± 19.88 | 32.93 ± 19.06 | 0.001** |
| | 36.02 ± 17.37 | 21.74 ± 23.30 | 30.57 ± 29.43 | 0.001** |
| Progressive motility (%) | 12.02 ± 8.01 | 14.60 ± 7.12 | 19.44 ± 9.71 | 0.001** |
| | 39.84 ± 14.09 | 29.26 ± 14.36 | 32.13 ± 18.60 | 0.001** |
| Normal morphology (%) | 392.03 ± 115.94 | 460.84 ±123.73 | 461.27 ± 120.53 | 0.001** |
| | 372.69 ± 116.91 | 455.09 ± 123.23 | 352.52 ± 109.96 | 0.001** |
| DFI (%) | 0.39 ±0.25 | 1.01 ±.05 | 1.3513 ± 0.24 | 0.001** |
| | 0.429 | 0.208 | 0.001 | 0.001 |
| CMA3 positive (%) | -0.168 | -0.628** | -0.178 | 0.123 |
| | 0.195 | 0.001 | 0.170 | 0.345 |
| P1 ($ng/10^6$ spz) | 0.166 | 0.037 | 0.173 | -0.283* |
| | 0.202 | 0.780 | 0.183 | 0.027 |
| P2 ($ng/10^6$ spz) | 0.105 | -0.021 | 0.131 | -0.107 |
| | 0.419 | 0.873 | 0.313 | 0.412 |
| P1/P2 ratio | 0.131 | 0.185 | -0.177 | -0.216 |
| | 0.315 | 0.158 | 0.171 | 0.095 |

Abbreviations: SPZ, spermatozoa.
**Correlation is significant at the 0.01 level ($P$<0.01); *Correlation is significant at the 0.05 level ($P$<0.05).

has been associated with male infertility (12). This has generated significant interest in determining whether the P1/P2 ratio can be a useful biomarker for sperm of people undergoing IVF or ICSI therapy, especially given that classical semen analysis does not provide sufficient information with respect to the quality and function of the sperm.

In the present study, the P1 and P2 values in the first group (P1/P2 ratio <0.8) showed no correlation with the age of the males, standard sperm parameters, DFI, chromatin condensation ($CMA_3$), and the P1/P2 ratio' although higher positive correlations (r = 0.746, $P$ = 0.001) were confirmed between the P1 and P2 concentrations.

In G2, the protamines P1 and P2, in addition to their high positive correlation (r = 0.969, $P$ = 0.001), demonstrated similar correlations with other parameters in particular, sperm concentration and P1 (r = 0.257, $P$ = 0.011) and P2 (r = 0.277, $P$ = 0.006) values. Progressive motility had a significantly positive correlation with P1 (r = 0.352, $P$ = 0.001) and P2 (r = 0.380, $P$ = 0.001) values. The mean percentage of morphologically normal SPZ demonstrated a significantly negative correlation with P1 (r = -0.206, $P$ = 0.041) and P2 (r = -0.208, $P$ = 0.040) values.

In G3, the P1/P2 ratio demonstrated only one significantly negative correlation with P2 (r = -0.372, $P$ = 0.003). The P1 concentration was found to have a significantly positive association with P2 value (r = 0.785, $P$ = 0.001) and a significant negative correlation (r = -0.283, $P$ = 0.027) was also observed between the mean percentage of morphologically normal SPZ and P1 concentration.

It seems that the alterations in P1/P2 ratio affected the quality and function of SPZ. These results are in agreement with findings of previous studies (21,22) which showed that sperm concentration, motility, viability, and mean percentage of morphologically normal SPZ were decreased in patients with an abnormal P1/P2 ratio. These results are also in accordance with the results obtained by other studies demonstrating that protamine deficiency resulted in a severe disruption of spermatogenesis affecting male infertility (23,24). In addition, the protamine expression disorder resulted in a decrease in number, motility, and morphology of SPZ (25).

Similarly, Iranpour (25) found that patients presenting an abnormal P1/P2 ratio showed less sperm with normal heads and more tapered heads in comparison with patients with a normal protamine ratio. However, the differences were not significant in this study. This is in line with the result of the present study, which demonstrated a negative correlation with the mean percentage of morphologically normal SPZ at the level of the 3 groups for P1, P2, and P1/P2 ratio. Moreover, the results of the current study are in conformity with the findings of a study by Aoki et al (12), who found that alterations in P1/P2 ratio were associated with a reduction of progressive motility and morphology of sperm.

An increase in the expression of P2 precursors discovered among infertile individuals explained their low levels of P2, indicating that disorders might occur during the processing of P2 (27). The pre-P1/P2 ratio can therefore influence the P1/P2 ratio (28) leading to a defective compaction of sperm DNA and changing the sperm quality. In addition, alteration of the P1/P2 ratio can occur as a consequence of the replacement of histones during faulty spermiogenesis. Indeed, increased histone levels have been reported in the semen of infertile men compared with fertile controls (29,30).

Overall, alterations in the P1/P2 ratio were appeared to play a key role in male infertility. However, the exact mechanisms by which this may occur can differ between individuals, and the underlying mechanisms have not yet been elucidated. Moreover, protamines were found to play a critical role in sperm chromatin condensation and the protection of paternal genomic DNA from alterations (21,31,32). It has also been proposed that a deficiency in protamine may lead to the accumulation of lesions at the level of spermatic DNA (33,34), morphological abnormalities, the triggering of apoptotic pathways, mitochondrial inactivation, and consequently a decrease in sperm motility (35).

Thus, it would be useful to determine with certainty if a particular alteration of DNA is linked to protamination defects. Until recently, the most commonly used method to analyse the protamine deficiencies has been the $CMA_3$ method and, according to some studies, measurement of the P1/P2 ratio has been shown to be closely related to sperm DNA (15-17,21,36,37). Furthermore, in the present study, the association between protamine deficiencies and sperm DNA lesions was systematically analysed. It was found that in G1, the $CMA_3$ and DFI were negatively correlated ($r = -0.371$, $P = 0.001$). The P1/P2 ratio demonstrated a positive correlation with DFI ($r = 0.652$, $P = 0.001$) but a negative correlation with $CMA_3$ ($r = -0.623$, $P = 0.001$). In G2, the P1/P2 ratio showed a positive association ($r = 0.333$, $P = 0.001$) with $CMA_3$ while P1 had a negative correlation ($r = -0.207$, $P = 0.041$) with DFI. In contrast to G1, the DFI and $CMA_3$ were positively correlated ($r = 0.299$, $P = 0.019$) in G3.

It has been shown that DFI was significantly higher ($P = 0.001$) in G3; meanwhile, $CMA_3$ was found to be significantly higher ($P = 0.001$) in G1; these results contradict the findings of several previous studies (21,22,24,38).

However, the findings of the present study are in agreement with the results obtained by previous studies (19,21,39,40), in that, a positive correlation was found between an alteration in protamine ratio and the presence of DNA damage.

In a meta-analysis, Ni et al (40) analysed the results of 12 selected studies and determined that the deficiency in protamine measured by $CMA_3$ was significantly associated with DNA fragmentation of sperm whereas the P1/P2 ratio was not associated with DNA fragmentation ($P = 0.33$). By contrast, the present study demonstrated that the correlations between DFI, $CMA_3$, and the P1/P2 ratio were dependent on the values of the protamine ratio (<0.8; 0.8–1.2; >1.2).

## Conclusions

The results of the study revealed that the protamine ratio (P1/P2) had an effect on DNA integrity and played a crucial role in human sperm quality and function. As a result, it can be used as a biomarker in addition to standard sperm parameters for the selection of sperm in ART treatments.

## Conflict of Interests

Authors declare that they have no conflict of interests.

## Ethical Issues

This study was approved by the Institutional Ethics Committee of Saarland University (under the code of ethics PHRC/HC/13/14). All the subjects provided written consent before participation in this study.

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