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# Sperm DNA damage and disturbed chromatin condensation indexes (DFI and CMA3) in normozoospermic men with unexplained infertility problem

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**ABSTRACT**

**Purpose:** The quantitatively measured sperm DNA damage and disturbed chromatin condensation indexes (%DNA fragmentation index [DFI%] and %high DNA staining index [%HDS]) and their relationships with sperm quality in normospermic men with unexplained infertility were investigated. The aim was also highlighting the impact of age on both DFI and CMA3 staining and on sperm quality, and their associations with male infertility.

**Methods:** In this retrospective study, conventional semen tests, including sperm motility and morphological evaluations and DFI and disturbed chromatin condensation indexes (DFI, CMA3) were performed according to the World Health Organization (WHO) 2021 criteria. DFI and CMA3 were evaluated using sperm chromatin dispersion (SCD) and chromomycin A3 (CMA3) staining assays and then correlation and regression analysis were done.

**Results:** By analyzing SCD and CMA3 results, notable differences were found in sperm parameters among different DFI and CMA3 groups (all $p < 0.05$). It was found that in the male fertility quality sperm concentration, progressive (PR)/non-progressive (NP) motility, immobility, and morphology were significantly associated with sperm DFI and CMA3, but not with age ($p < 0.05$).

**Conclusions:** Sperm molecular index DFI and CMA3 negatively affect male fertility quality through semen parameters (sperm concentration, motility, and morphology). SCD and CMA3 indexes show a significant negative correlation with sperm quality in normospermic males, which highlights its role in the assessment of male fertility potential and molecular evaluation of infertility treatment.

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## Introduction

### *Background*

Semen with normal parameters may not have fertility quality; many factors affect male fertility including genetic, endocrine, and immune factors, and varicocele disease [1–3]. Studies report that the integrity of sperm DNA correlates with fertility outcomes and miscarriage after IVF. In contrast, sperm quality parameters may have little or no effect on fertility quality and outcomes [4,5].

Accordingly, extrinsic and intrinsic factors may affect male fertility which includes aging processes, immune modulatory reactions, oxidative stress (OS), and sedentary work. Infections lead to immune modulatory reactions and increase the production of reactive oxidative species (ROS). Inflammatory cytokines in seminal plasma decrease sperm quality possibly through damaging sperm DNA [5,6]. Nutritional factors and vitamins (in particular vitamins E, A, D, and F), play important roles in sexual health and semen quality [7–11].

In addition, air pollution and occupational exposures affect sperm quality and can cover the effects of age and genetics. Sperm maturation is highly sensitive to these intrinsic and extrinsic factors that cause defects in the transition of histone-to-protamine (CMA3) in the DNA of sperm, DNA damage, and incomplete maturities of chromatin compaction. Defect in sperm DNA compaction by histone-protamine conversion increases the risk of DNA damage that

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can be quantified by DNA damage indexes (%DFI and %CMA3) [2,4,6].

Over the last decades, several new biochemical parameters including semen OS and protamine-DNA complexes have been introduced that affect male fertility quality and may cause unexplained infertility [1,2].

For example, pollution has a marked impact on the reproductive quality of males and influences sperm morphology, number, and motility, by causing oxidative damage to spermatozoa DNA. Spermatozoa are particularly sensitive to the pro-oxidant effects of environmental pollutants particularly because of the small volume of sperm cytoplasmic space and reduced levels of the antioxidant defenses and membrane lipids that are rich in polyunsaturated fatty acids (PUFA; the preferential target of ROS) [1,2,12].

In addition, histopathological studies on male reproductive tissues and molecular analysis of the semen have demonstrated that viral infections (such as Human Papillomavirus [HPV]) can irreversibly disrupt normal testicular function and spermatogenesis, causing poor semen quality [13].

Molecular analysis like sperm DNA damage assay (SCD index) and test disturbed chromatin condensation (CMA3 index, an indicative of protamine deficiency in sperm) can be potential biomarkers for male infertility and IVF unwanted outcomes [3,14]. According to the reports, the TUNEL assay can detect types of DNA damage caused by oxidative agents in sperm, however, all types of DNA damage can be also detected by the results of SCD assay, which may correlate with sperm chromatin structure disturbances (CMA3 staining index) [15].

Studies report that infertile males have damages in their sperm DNA that have a negative effect on their sperm quality and fertility [3,14]. Sperm microscopic analysis of DNA damage quantifies DNA fragmentation (DFI) and high staining (CMA3) as indicators of DNA damage and uncondensed chromatin where histone-protamine conversion occurs [16,17]. Assessment of DNA damages may play an appropriate role in better diagnosis and management of infertility in males or unwanted miscarriage after IVF [1,2,5,16].

In studies from 2020 to 2023, there are controversies and consensuses on indications for sperm DFI (SDF) testing in male infertility [1–3,18,19]. Evidence demonstrates diverse practices and there are not enough clinical recommendations or evidence on the indications for SDF testing in the case of infertile males.

In a survey by Agarwal et al., 436 experts from 55 countries participated, and 39% of them ordered SDF routinely in the review of recurrent pregnancy loss (RPL), and 62.2% investigated SDF in the case of smokers [18].

Here, about 70% would assess SDF for certain conditions, such as recurrent assisted reproductive technologies (ART).

In a recent study by Liu et al. in males with unexplained infertility, DFI had significant effects on sperm survival rate, sperm concentration, and progressive rates while there were no significant effects on semen volume, age, percentage of normal sperm, and *in vitro* fertilization rate, and clinical pregnancy rate [19].

### Objectives

The objective of this study was to highlight the impact of both DFI and CMA3 staining on sperm quality, their associations with male infertility, and their importance in assessing male fertility [16]. Thereby, samples according to DFI ($<20$, $20 \geq \leq 30$, and $>30\%$), CMA3 staining (CMA3) ($<20$, $20 \geq \leq 30$, $30 > \leq 45$, and $>45\%$), and age ($<35$, $35 \geq \leq 45$, and $>45$ years), were divided into three or four groups, respectively, and statistical analysis were done. According to our analysis, in normozoospermic men with unexplained infertility problems, DNA integrity levels have a strong effect on spermatozoa development and fertility quality.

## Methods

### Study design

This was a retrospective cross-sectional study of normozoospermic men with unexplained infertility problems. Selection of the semen samples and normospermia were in accordance with the World Health Organization (WHO) 2010 criteria [11,20].

### Setting

Recorded data were from samples with unexplained infertility (having sperm concentrations 15–120 $*10^6$ spermatozoa/mL), who were referred to clinics of infertility in the Andrology Laboratory of the Reproductive Medicine Centers of Shahid Sadouqhi University of Medical Sciences, from March 2020 to October 2022. The Ethics Committee of Shahid Sadouqhi University of Medical Sciences (IR.SSU.SRH.REC.1401.011) approved the study.

### Participants

Samples of 1992 men with unexplained infertility who had normal sperm count (aged 20–70 years) were

included for analysis. Exclusion criteria included the use of any drugs, such as antibiotics and anabolic hormones, history of genetic and systemic disorders, and/or having any conditions, such as varicocele, cryptorchidism, testicular trauma, testicular or other cancer, previous chemotherapy and/or radiotherapy, endocrine diseases, renal and urinary tract infections (current or previous infections affecting sperm count), and other abnormalities that affecting semen parameters [11,20].

## Variables

After liquefaction, each semen sample was graded for sperm motility (progressive motility [PR], non-progressive motility [NP], and immotility [IM]), concentration, and normal morphology (NM), according to 2010 WHO guidelines.

The quantitatively measured sperm DNA damage indexes ([DFI%] and %high DNA staining index [%HDS] CMA3) and their relationships with sperm quality in normospermic men with unexplained infertility were investigated. The data were divided into three to four categories according to the %DFI ($<20$, $20\geq \leq 30$, and $>30\%$), age ($<35$, $35\geq \leq 45$, and $>45$ years), and % CMA3 staining ($<20$, $20\geq \leq 30$, $30> \leq 45$, and $>45\%$), according to references [1–3,16], to assess the relationship between sperm parameters and these indexes in normospermic men with infertility problem.

## Data sources/measurements

### Sperm kinetics and morphology

Andrology Laboratory using appropriate methods and instruments performed semen analysis. Semen sample collections were made between 2 and 5 d after the last ejaculation and then analyzed following 2010 WHO criteria. By using phase-contrast microscopy (Zeiss, Axiostar Plus, Aalen, Germany), sperm kinetics and morphology were analyzed (with X400 magnification), by rendering at least 200 sperms and examining at least 5 random views. The percentage of sperm movements was assessed as follows; PR = PR sperms/total count; NP = NP sperms/total count.

PR: stands for sperms with forward movement, NP: stands for sperms with nonforward movement, and IM: stands for sperms with immobility.

### Assaying DNA fragmentation by chromatin dispersion (SCD) method

To determine DNA strand breaks in sperms and calculate the DFI%), a chromatin dispersion (SCD) assay was used according to the WHO protocol (2010). Briefly, the sperm samples were mixed with 1% low-melting-point aqueous agarose (to obtain a 0.7% final agarose concentration) (37 °C), pipetted 50 μL of the mixtures onto glass slides precoated with 0.65% standard agarose dried at 80 °C. Then, the preparations were covered with coverslips, put the slide horizontally on a cold glass surface, and placed in the fridge at 4 °C for 5 min, to allow the agarose to solidify. The coverslips were removed and the slides were immediately immersed horizontally in a tray with fresh acid denaturation solution (0.08 N HCl), for 7 min in the dark (22 °C). Slides were put in the neutralizing and lysing solution (0.4 M Tris, 0.8 M DTT, 1% SDS, and 50 mM EDTA, pH 7.5) for 10 min at RT. Then, the slides were incubated in a second neutralizing and lysing solution (0.4 M Tris, 2 M NaCl, and 1% SDS, pH 7.5) for 5 min, washed with Tris-borate-EDTA buffer (pH 7.5) for 2 min, dehydrated using sequential baths of ethanol at 70%, 90%, and 100%, for 2 min each, and let to dry horizontally, at RT. Dried microgels were covered with a layer of fresh dye solution (Wright solution with DPBS (1:1)), for 10–15 min, washed in tap water, and air-dried. The slides were mounted and then examined under a light microscope using a 100× immersion oil objective (about 500 sperms were evaluated and analyzed). DFI was determined by the presence of a shadow around the nucleus. The spermatozoa with DFI (with a small/without shadow around the nucleus) and spermatozoa without DFI (with a large and medium shadow around the nucleus) were reported. DFI: sperm counts with a small/without shadow/total sperm counts [3,21].

### Chromatin condensation assay with chromomycin A3 (CMA3) staining

DFI with %HDS with fluorescent stain Chromomycin A3 (CMA3) (as an indicator of uncondensed), quantify DNA damage index (DFI) and protamine deficiency (CMA3 staining) in the chromatin of sperms. Sperm smears were prepared according to the WHO protocol (2010), to evaluate sperm chromatin quality. CMA3+ technique was performed to detect indirectly the amount of protamine in sperm nuclear DNA. CMA3 competes with protamine for binding to the minor groove of the DNA helix. About $1 \times 10^6$ spermatozoa were fixed in 50 μL of 4% paraformaldehyde (final concentration: 400,000 spermatozoa/10 μL) for 30 min and then were centrifuged at 300 *g* for 7 min at RT. After removing the supernatants, pellets were washed in DPBS, incubated in 100 μL CMA3 solution (0.25 mg/mL), for 20 min at RT, and centrifuged. The pellets were suspended in 10 μL McIlvaine buffer, placed on

slides, and let to be air-dried. A drop of DPBS was added and a coverslip was placed over the slides and observed under a fluorescence microscope (excitation wavelength: 445 nm and emission wavelength: 575 nm), using 1000× magnification (oil immersion) objective. At least 200 spermatozoa were evaluated and analyzed. The percentage of stained spermatozoa was calculated according to CMA3-positive sperm cells counted. CMA3+ staining or CMA3 can indirectly measure the amount of protamine in sperm nuclear DNA and %chromatin condensation. CMA3 preferentially stains histone-DNA complexes rather than the protamine-DNA complexes and produces a high DNA staining region used to calculate %CMA3+. Shiny yellow CMA3 fluorescence shows the less protamine-DNA complex degree in sperm nuclei [16,21].

### Bias

This was a retrospective cross-sectional study of recorded data from normozoospermic men with unexplained infertility problems. Selection of the semen samples and normospermia were in accordance with the WHO 2010 criteria. There were no efforts for potential sources of bias.

### Study size

Data included in this study were from 1992 men with unexplained infertility (having normal seminal fluid and sperm concentration $>$ 15–120 * $10^6$ spermatozoa/mL), who were referred to clinics of infertility in the Andrology Laboratory of the Reproductive Medicine Centers of Shahid Sadouqhi University of Medical Sciences, from March 2020 to October 2022.

### Quantitative variables

The percentage of sperm movements was assessed as follows; PR = PR sperms/total count; NP = NP sperms/total count.

The percentage of DFI and %HDS CMA3 were calculated according to references [21,22]. The data were divided into three to four categories according to the % DFI ($<$20, 20$\geq \leq$30, and $>$30%), age ($<$35, 35$\geq \leq$45, and $>$45 years), and % CMA3 staining ($<$20, 20$\geq \leq$30, 30$> \leq$45, and $>$45%).

### Statistical analysis

Normal distribution of variables was tested (the skewness and kurtosis indices between 1 and −1 were considered normal) and then the mean value + standard deviation (SD) was calculated. By One-way ANOVA (SPSS software version 21; IBM Corp., Armonk, NY), the groups were compared for each variable. Family of hypotheses was also performed by multivariate analysis of variance (MANOVA) procedure to compare groups for multiple dependent variables, simultaneously and appropriate corrected $p$ values were calculated.

The correlation of different sperm variables with age, %DFI, and %CMA3 was calculated using Pearson's correlation test.

Univariate logistic regression analysis was also performed to identify the risk factors for male infertility, and calculated the odds ratio (OR) and confidence interval (CI).

The cut-off value of DFI ($>$20%), CMA3 ($>$30%), NM ($>$4%), and PR ($>$32%), was coded 1 and assumed as a dependent variable then its association with independent parameters was analyzed by univariate logistic regression analysis, respectively. Each parameter at its cut-off value can cause male infertility [4,6,16,17].

By microscopic analysis of semen, DFI% (as an indicator of DNA damage), %chromatin high staining by CMA3 (CMA3%, as an indicator of uncondensed chromatin), % NM, and %PR of sperms were determined in each sample. The $p$ values $<$ 0.05 were accepted as statistical significance level.

## Results

### Participants

The experiment included 1992 semen samples with unexplained infertility (the mean age ± SD ∼37.23 ± 6.03 years; ranging from 21 to 68 years). Samples with incomplete data were not included in the study. According to the % DFI ($<$20, 20$\geq \leq$30, and $>$30%), % CMA3+ ($<$20, 20$\geq \leq$30, 30$> \leq$45, and $>$45%), and age ($<$35, 35$\geq \leq$45, and $>$45 years), data were divided into 3–4 groups.

### Descriptive data

Subgroups, according to %DFI, %CMA3, and age, were compared, and then their association with sperm concentration, NM, progressive-movement (PR), non-progressive-movement (NP), and immobility (IM) were statistically analyzed (Tables 1–5).

### Outcome data

Increased DFI and CMA3 were significantly associated with downward trends in sperm concentration, NM,

and PR. The percentage of immotile and NP sperms showed upward trends with the increase of DFI and CMA3. However, there were no significant differences in sperm quality among different age groups (< 35, 35–45, and >45 years).

In fact, DNA-strand breaks reduce the level of chromatin condensation and sperm quality where resulting in abnormality of sperm morphology, motility, and progressive movement. These parameters are essential for successful fertilization.

In the fragmentation of DNA, histone-DNA complexes preferentially form chromatin structure rather than protamine-DNA complexes, and this parameter causes the genome of the sperm to be closed improperly [16,21]. However, the average age of patients exhibited no significant effect on the sperm characteristics and its quality for fertilization.

The aging had no considerable impact on the change of morphology, motility, and progressive movement of sperms.

## Main results

### Effect of %DFI on sperm quality

Table 1 and Figure 1 show that the sperm quality is significantly affected by DFI groups. Increased DFI is significantly associated with a downward trend of sperm concentration, NM, and PR. The percentage of immotile and NP sperms show an upward trend with the increase of DFI. Also, the frequency of CMA3 was greater than DFI in the groups (mean; CMA3 ~ 29.6 *vs.* DFI ~17.19).

In infertile normospermic males, different SCD groups have different averages for each of the sperm parameters (*F*-test (2, 1988) = 3.084–248.699, $p < 0.05$) (Table 1). Sheffe's *post hoc* analysis showed that the SCD1 group (DFI%: 8–19%) has a significantly different average of sperm concentration and sperm NP parameters compared to group SCD2 (DFI: 20–30%) and SCD3 (DFI: 31–69%), but there is no significant difference in the averages of these parameters ($p = 0.553$ and 0.808, respectively) between two groups SCD2 and SCD3. The obtained data show that the SCD1 group had the lowest mean value for sperm NP (mean = 10.44) and the highest mean value for sperm concentration (mean = 66.02), in infertile normospermic males (Table 1).

Here, the concentration and motility of sperm in patients with DFI < 20% were between the normal ranges, but the numbers of immotile sperms with abnormal morphology and frequency of CMA3 (mean value = 29.6%) were significantly high.

Tables 4 and 5 show that sperm DFI has a positive correlation with CMA3+ staining and %HSD of chromatin which in turn increases the risk of sperms with abnormal morphology, IM, and NP (all $p < 0.01$).

### Effect of %CMA3 staining on sperm quality

CMA3 staining is used to quantify the fractions of DNA with an uncondensed structure and high staining feature (CMA3 index). CMA3 stains histone-DNA complexes more preferentially than protamine-DNA complexes, and this parameter helps to identify fractions of DNA in sperm that are uncondensed [16,21].

The obtained data show that in infertile normospermic males, different CMA3 groups have different averages for all sperm parameters (*F*-test (3, 1987) = 1.83–127.7, $p < 0.01$), an exception is the NP ($p = 0.061$) and age ($p = 0.139$). Sheffe's *post hoc* analysis showed that the CMA3-1 (CMA3: 9–19%) and CMA3-2 (CMA3: 20–30%) groups have no significant differences in the average of sperm parameters: SCD ($p = 0.559$), NM ($p = 0.568$), PR ($p = 0.651$), concentration ($p = 0.966$), and IM ($p = 0.713$). However, in the average of sperm parameters, there is a significant difference between CMA3-1 and CMA3-2 with CMA3-3 (CMA3: 31–45%)

**Table 1.** Comparison of SCD groups: (SCD1: 8–19% (DFI < 20%), SCD2: 20–30% (DFI, 20≥<30%), SCD3: 31–69% (DFI > 30%)), and relationships with sperm parameters, in normospermic men with infertility problem.

| SCD | Numbers | Age | CMA3 | Normal morphology | Sperm concentration | Immotile | Non progressive | Progressive | Corrected *p* value*** |
|---|---|---|---|---|---|---|---|---|---|
| SCD1 | 618 | 36.2 | 29.6 | 3.81 | 66.02 | 49.56 | 10.44 | 39.99 | <0.001 |
| SCD2 | 1002 | 37.2 | 40.76 | 3.33 | 58.09 | 51.79 | 10.72 | 37.48 | |
| SCD3 | 371 | 38.6 | 47.37 | 3.09 | 55.91 | 54.08 | 10.84 | 35.07 | |
| *F*-test | – | 15.780 | 248.699 | 42.392 | 14.876 | 30.924 | 3.084 | 33.362 | |
| *p* Value* | – | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.050 | <0.001 | |
| Corrected *p* value** | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.05 | <0.001 | |

*Each variable was compared between SCD groups for difference by one-way ANOVA. Sheffe's *post-hoc* analysis was performed to compare groups one-by-one.

**Different variables were compared between SCD groups simultaneously by multivariate analysis of variance analysis (MANOVA) and appropriate corrected *p* value was obtained for each variable.

***Effect of the independent variable SCD on sperm parameters was simultaneously analyzed by MANOVA. The *p* values < 0.05 were accepted as statistical significance level.

Normal distribution of variables in groups was tested with the skewness and kurtosis analysis and indices from 1 to −1 were considered normal.

SCD: sperm chromatin damage % (DFI: DNA fragmentation index); CMA3: Chromomycin A3 staining% (CMA3: high DNA staining). Concentration ($\times 10^6$/mL); Progressive motility (%); non-progressive motility (%); immotile (%); normal morphology (%).

**Table 2.** Comparison of CMA3 (high DNA staining (CMA3)) groups: (CMA3–1: 9–19% (CMA3 < 20%), CMA3–2: 20–30% (CMA3, 20> <30%), CMA3–3: 31–45%: (CMA3, 30><45%), CMA3–4: 46–96%, (CMA3 > 45%), and relationships with sperm parameters, in normospermic men with infertility problem.

| CMA3 | Numbers | Age | SCD | Normal morphology | Sperm concentration | Immotile | Non progressive | Progressive | Corrected *p* value*** |
|---|---|---|---|---|---|---|---|---|---|
| CMA3–1 | 154 | 36.61 | 19.93 | 3.84 | 69.14 | 49.31 | 10.33 | 40.36 | <0.001 |
| CMA3–2 | 503 | 36.84 | 20.91 | 3.66 | 67.60 | 50.26 | 10.48 | 39.25 | |
| CMA3–3 | 747 | 37.43 | 24.72 | 3.38 | 58.78 | 51.76 | 10.84 | 37.39 | |
| CMA3–4 | 587 | 37.50 | 28.90 | 3.14 | 53.28 | 52.82 | 10.87 | 36.48 | |
| *F*-test | – | 1.83 | 127.7 | 16.9 | 22.04 | 11.021 | 2.462 | 12.235 | |
| *p* Value* | – | <0.140 | <0.001 | <0.001 | <0.001 | <0.001 | >0.060 | <0.001 | |
| Corrected *p* value** | >0.130 | <0.001 | <0.001 | <0.001 | <0.001 | >0.06 | <0.001 | | |

*Each variable was compared between CMA3 groups for difference by one-way ANOVA. Sheffe's *post-hoc* analysis was performed to compare groups one-by-one.
**Different variables were compared between CMA3 groups simultaneously by multivariate analysis of variance analysis (MANOVA) and appropriate corrected *p* value was obtained for each variable.
***Effect of the independent variable CMA3 on sperm parameters was simultaneously analyzed by MANOVA. The *p* values < 0.05 were accepted as statistical significance level.
Normal distribution of variables in groups was tested with the skewness and kurtosis analysis and indices from 1 to −1 were considered normal.
SCD: Sperm chromatin damage % (DFI: DNA fragmentation index); CMA3: Chromomycin A3 staining % (CMA3: high DNA staining). Concentration (×106/mL); progressive motility (%); non-progressive motility (%); immotile (%); normal morphology (%).

**Table 3.** Comparison of the age groups: Age 1 (old: 21–34 years), Age 2 (old: 35–45 years), Age 3 (old: 46–68 years), and their relationships with sperm quality parameters, in normospermic men with infertility problem.

| Age | Numbers | CMA3 | SCD | Normal morphology | Sperm concentration | Immotile | Non progressive | Progressive | Corrected *p* value*** |
|---|---|---|---|---|---|---|---|---|---|
| Age 1 | 799 | 37.63 | 23.54 | 3.48 | 59.94 | 50.8 | 10.67 | 38.52 | <0.001 |
| Age 2 | 1049 | 38.93 | 24.91 | 3.46 | 60.36 | 51.83 | 10.75 | 37.54 | |
| Age 3 | 143 | 41.33 | 27.56 | 3.34 | 60.30 | 52.8 | 10.88 | 36.31 | |
| *F*-test | – | 4.206 | 21.344 | 2.967 | 0.048 | 5.105 | 0.924 | 5.014 | |
| *p* Value* | – | >0.01 | <0.01 | >0.050 | >0.950 | <0.01 | >0.395 | <0.01 | |
| Corrected *p* value** | <0.05 | <0.001 | >0.050 | >0.950 | <0.01 | >0.390 | <0.01 | | |

*Each variable was compared between age groups for difference by one-way ANOVA. Sheffe's *post-hoc* analysis was performed to compare groups one-by-one.
**Different variables were compared between age groups simultaneously by multivariate analysis of variance analysis (MANOVA) and appropriate corrected *p* value was obtained for each variable.
***Effect of the independent variable age on sperm parameters was simultaneously analyzed by MANOVA. The *p* values < 0.05 were accepted as statistical significance level.
Normal distribution of variables in groups was tested with the skewness and kurtosis analysis and indices from 1 to −1 were considered normal.
SCD: Sperm chromatin damage % (DFI: DNA fragmentation index); CMA3:= Chromomycin A3 staining % (CMA3: high DNA staining). Concentration ($\times 10^6$/mL); progressive motility (%); non-progressive motility (%); immotile (%); normal morphology (%).

**Table 4.** Correlation analysis of sperm DNA damage assay (DFI and CMA3) with sperm parameters and age, in normospermic infertile men.

| | Age (years) | | DFI% | | CMA3% | |
|---|---|---|---|---|---|---|
| | *r** | *p* Value | *r** | *p* Value | *r** | *p* Value |
| Age | – | – | 0.144 | <0.001 | 0.055 | 0.014 |
| CMA3 | 0.055 | 0.014 | 0.417 | <0.001 | – | – |
| Normal morphology | −0.048 | 0.031 | −0.214 | <0.001 | −0.159 | <0.001 |
| Sperm concentration | 0.022 | 0.335 | −0.127 | <0.001 | −0.190 | <0.001 |
| Immotile | 0.067 | 0.003 | 0.220 | <0.001 | 0.123 | <0.001 |
| Non progressive | <0.001 | 0.985 | 0.034 | 0.125 | 0.049 | 0.030 |
| Progressive | −0.063 | 0.005 | −0.219 | <0.001 | −0.131 | <0.001 |

*The correlation of different sperm variables with age, %DFI, and %CMA3 was calculated using Pearson's correlation test. Correlation coefficient (*r*) of associations was calculated.
Normal distribution of variables was tested with the skewness and kurtosis analysis and indices from 1 to −1 were considered normal.
DFI: DNA fragmentation index; CMA3: Chromomycin A3 staining % (CMA3: high DNA staining). Concentration ($\times 10^6$/mL); progressive motility (%); non-progressive motility (%); immotile (%); normal morphology (%).

**Table 5A.** Univariate logistic regression analysis of the associations between sperm DNA fragmentation index (DFI > 20%) with age and CMA3, in normospermic infertile men.

| | | | | 95% CI for EXP(*B*) | | |
|---|---|---|---|---|---|---|
| DFI% | *B* | *p* Value | Exp(B)(OR)* | Lower | Upper | Nagelkerke $R^{2**}$ |
| Age | 0.038 | 0.000 | 1.040 | 1.018 | 1.060 | 0.067 |
| CMA3 | 0.091 | 0.000 | 1.096 | 1.084 | 1.108 | 0.265 |

**Table 5B.** Univariate logistic regression analysis of the associations between sperm high DNA staining (CMA3+ > 30%) with age and SCD, in normospermic infertile men.

| | | | | 95% CI for EXP(*B*) | | |
|---|---|---|---|---|---|---|
| CMA3+% | *B* | *p* Value | Exp(B)(OR)* | Lower | Upper | Nagelkerke $R^{2**}$ |
| Age | −0.001 | 0.910 | 0.999 | 0.982 | 1.017 | 0.051 |
| SCD | 0.139 | 0.000 | 1.149 | 1.127 | 1.171 | 0.213 |

and CMA3-4 (CMA3: 46–96%) groups and between groups CMA3–3 and CMA3–4, in turn ($p < 0.05$). CMA3-1 and CMA3-2 groups had the lowest average values (mean = 10.33 and 10.48, respectively) for sperm NP and the highest average values (mean = 40.36 and 39.25, respectively) for sperm PR parameters, compared to the two other groups CMA3-3 and CMA3-4 (Table 2). Table 2 here shows that the average age of the groups with high frequency of CMA3 (30–96%) was not significantly different from the groups with low frequency of CMA3 (<30%).

**Table 5C.** Univariate logistic regression analysis of the associations between sperm normal morphology (NM > 4%) with age, CMA3, and SCD, in normospermic infertile men.

| | | | | 95% CI for EXP(*B*) | | |
|---|---|---|---|---|---|---|
| NM% | *B* | Sig. | Exp(B)(OR)* | Lower | Upper | Nagelkerke $R^{2**}$ |
| Age | 0.012 | 0.219 | 1.012 | 0.993 | 1.032 | 0.197 |
| CMA3 | −0.012 | 00.011 | 0.988 | 0.979 | 0.997 | 0.201 |
| SCD | −0.041 | .000 | 0.960 | 0.942 | 0.978 | 0.219 |

**Table 5D.** Univariate logistic regression analysis of the associations between sperm progressive motility (PR > 32%), with age, CMA3, and SCD, in normospermic infertile men.

| | | | | 95% CI for EXP(*B*) | | |
|---|---|---|---|---|---|---|
| PR% | *B* | Sig. | Exp(B)(OR)* | Lower | Upper | Nagelkerke $R^{2**}$ |
| Age | −0.022 | 0.018 | 0.979 | 0.961 | 0.996 | 0.010 |
| CMA3 | −0.009 | 0.025 | 0.991 | 0.983 | 0.999 | 0.115 |
| SCD | −0.050 | 0.000 | 0.941 | 0.938 | 0.964 | 0.35 |

*Exp(B): odd ratios (OR) were calculated by univariate logistic regression analysis with 95% CI and $p$ values < 0.05.
**Reporting the goodness-of-fit measure for each model by the Nagelkerke $R^2$.
Univariate logistic regression model was used to identify the risk factors for male infertility, and calculating odds ratio (OR) and confidence interval (CI). Cut-off value of DFI (>20%), CMA3 (>30%), NM (>4%), and PR (>32%), was coded 1, and assumed as a dependent variable then its associations with independent parameters were analyzed.
Each parameter at its cut-off value can cause male infertility cut-offs as dependent variables: (A) DNA fragmentation index, DFI > 20%; (B) high DNA staining, CMA3/CMA3+ >30%; (C) normal morphology, NM > 4%; (D) progressive motility, PR > 32%, and age. Odds ratios (OR)(Exp (*B*)) and 95% confidence interval (CI) were used to measure the strength of association. For all tests, a two-tailed $\alpha$ below 0.05 was considered statistically significant ($p$ value).
SCD: sperm chromatin damage; (DFI): DNA fragmentation index; NM: sperm normal morphology; PR: sperm progressive motility; CMA3+: Chromomycin A3 staining %; CMA3: high DNA staining

In other words, sperms from young and old patients may show similar frequencies of CMA3 with a subsequent decrease in NM and PR and an increase in IM and NP (all $p < 0.05$; Table 2 and Figure 2). There were statistically significant correlations between CMA3 and DFI with all other sperm-measured parameters (kinetics and morphology) (Table 4, $p < 0.05$). Tables 4 and 5 show that increased CMA3 increases the risk of abnormal morphology, IM, and NP of sperms in males ($p < 0.05$).

### *Other analyses*

#### *Effect of age on sperm quality*

Aging has been reported to have a negative effect on seminal characteristics [3,16]. In this regard, there were significant differences in %CMA3 and %DFI%, IM, non-progressive, and PR of sperms among different age groups (21–34, 35–45, and 46–68 years) ($p < 0.05$). However, sperm NM and concentration were not statistically different (Table 3, Figure 3). Different age groups show different averages for sperm parameters ($F$-test (2, 1988) = 0.048–21.344, $p < 0.05$), but exceptions are sperm concentration ($p = 0.953$), NM ($p = 0.052$), and NP ($p = 0.397$).

Sheffe's *post hoc* analysis showed that the Age 1 group (Old: 21–34 years) has a significant difference in sperm parameters: SCD, CMA3 staining, IM, and progressive mobility with the Age 3 group (Old: 46–68 years) ($p < 0.05$). There is a significant difference between all three age groups in the DNA damage parameter, SCD ($p < 0.01$) (Table 3). The Age 1 group had the lowest average value (mean = 23.54) for sperm DNA damage (DFI%).

There was a positive correlation between the age and sperm DFI ($r \sim 0.144$, $p < 0.01$, Table 4). Herein, aged patients may have an increased risk of increased DFI and CMA3 in their sperms.

## Discussion

### *Key results*

In this study, recorded data from normospermic patients with unexplained infertility problems were analyzed. Semen parameters and laboratory results were studied and quantified to perform a valuable statistical analysis of the relationship between DFI and CMA3 with sperm parameters. The aim was to confirm the association between DFI and CMA3 with the problem of unexplained infertility in men, whereby a molecular model and better management of the problem will be established in the future. Conventional parameters of semen and DFI and CMA3 were investigated at the same time in patients of the same age.

There was a negative association between sperm parameters with DFI and CMA3 in males with unexplained fertility.

Here, various physiological and pathophysiological events by intrinsic and extrinsic factors may be involved where they disrupt genome structure and impair sperm quality for male fertility.

In the testis, the seminiferous tubules are the place where sperm is produced and stored in the epididymis. In the spermatozoa nucleus, the genome is tightly packed and condensed with protamine to protect DNA as well as possible from intrinsic- and extrinsic-damaging factors. However, some conditions may disturb chromatin integrity and gradually lead to accumulating DNA damage [3,5,23].

Sperm quality is influenced by many factors, including genetic background (chromosomal microdeletions), endocrine function (such as androgen levels), immunity reactions (such as cytokines), and environment, such as temperature and radiation. Physiological conditions, such as testicular function and a large

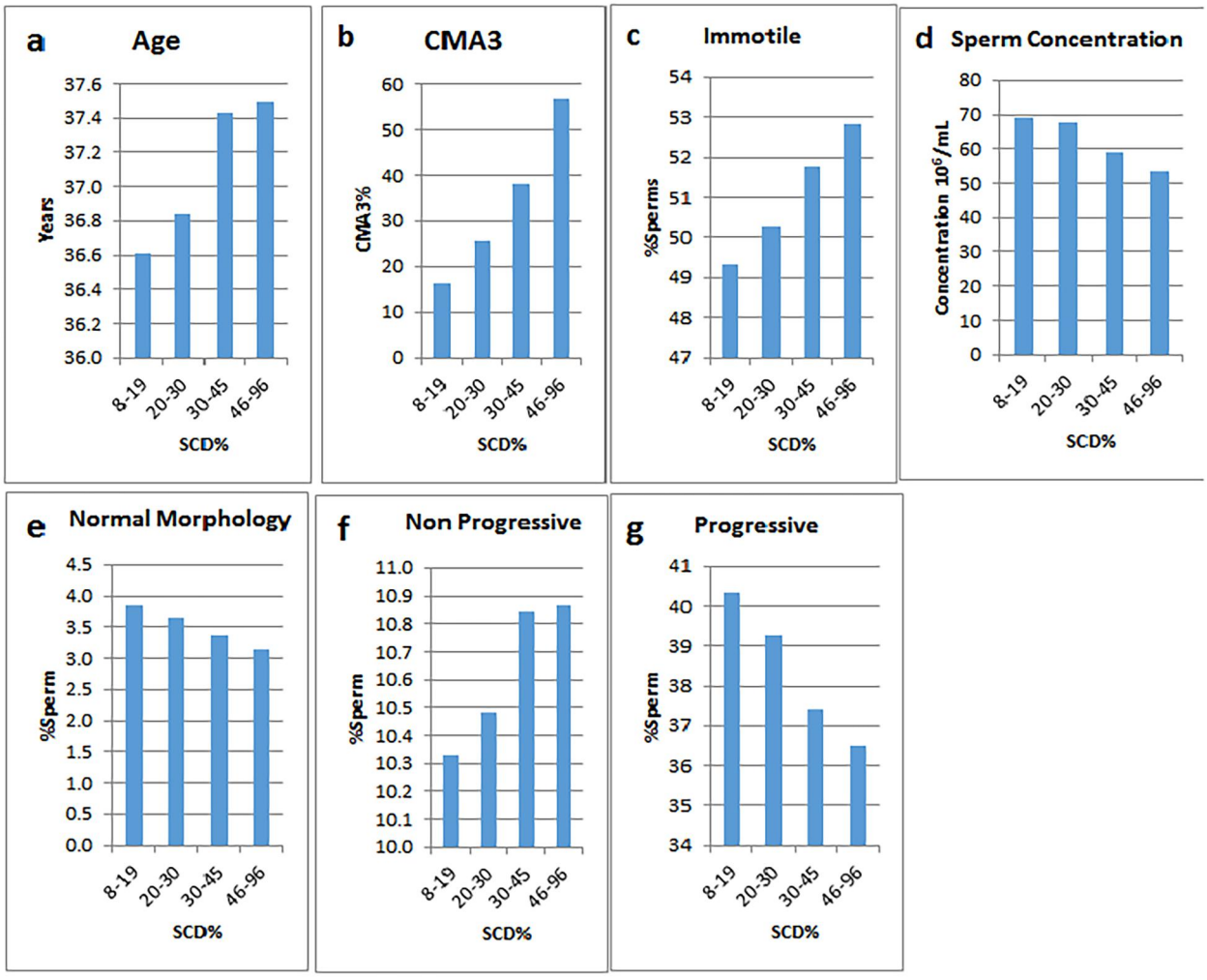


**Figure 1.** The relationship between sperm quality and DNA fragmentation index (DFI%), in normospermic men with infertility problems. (a) The relationship between the age and DFI %, (b) the relationship between CMA3 staining (CMA3%) and SCD (DFI%), (c) the relationship between sperm immotility and DFI%, (d) the relationship between the sperm concentration and DFI%, (e) the relationship between the sperm normal morphology and DFI%, (f) the relationship between the sperm non-progressive motility and DFI%, and (g) the relationship between the sperm non-progressive motility and DFI %.

amount production of reactive oxygen species are other influencing important factors [3,23].

Besides, nutrition, air pollution, viral infections, and occupational exposures all affect sperm quality whereby may cover the effects of age and genetics. Sperm maturation is highly sensitive to intrinsic and extrinsic factors whereby cause defects in the histone-to-protamine transition (CMA3+), chromatin damage and immaturities. Defect in sperm DNA compaction by histone-protamine conversion increases the risk of DNA damage that can be quantified by DFI and CMA3 [4,6,14].

Regarding the identification of the relationship between DNA integrity and unexplained infertility in normospermic men, our results confirmed that there is a notable correlation between DNA structure integrity and sperm fertility quality (concentration, progressive/NP, and NM). The fraction of non-motile and non-progressive sperm increases with increasing DFI and CMA3 (Tables 4 and 5).

In identifying the effect of age on infertility, patients of the same age showed different frequencies of DFI and CMA3 and were included in different subsets of SCD and CMA3. The analysis showed that the sperm quality of different age groups was not affected by age, but was controlled by DFI and CMA3. For example, young patients in age groups less than 45 years and with DFI and CMA3 $>$ 20% did not show a higher frequency of sperm quality than elderly patients in age groups above 45 years.

According to reports, sperm abnormalities and damage in DNA integrity may be associated with a possible decrease in testosterone levels and sexual performance, causing delayed, or inappropriate sperm

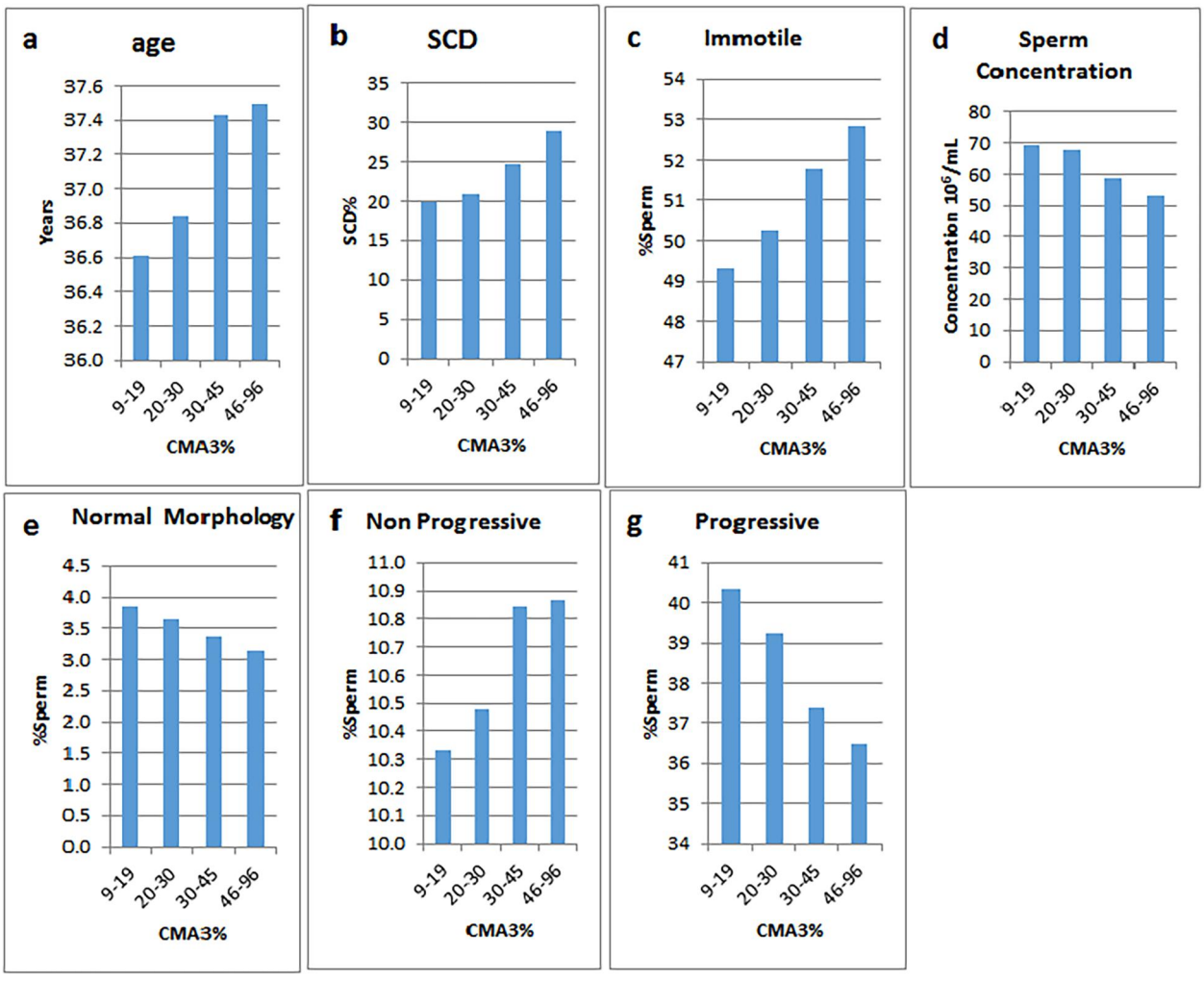


**Figure 2.** The relationship between sperm quality and high DNA staining (CMA3 positive) (CMA3%), in normospermic men with infertility problems. (a) The relationship between the age and CMA3%, (b) the relationship between DNA damage and CMA3%, (c) the relationship between sperm immotility and CMA3%, (d) the relationship between the sperm concentration and CMA3%, (e) the relationship between the sperm normal morphology and CMA3%, (f) the relationship between the sperm progressive motility and CMA3%, and (g) the relationship between the sperm progressive motility and CMA3%.

maturation and prolonged storage in the epididymis and excessive exposure to ROS [5,6,16]. With the increase in the time of sexual performance, the damage induced by the external matters to the sperm in the epididymis would increase. Here, an excessive level of ROS with low levels of antioxidant capacity and redox defense would lead to sperm abnormality [2,3,14]. NRF2 controls ROS levels in human cells by its key downstream genes (such as SOD2, CAT, or GSTM1). In patients with sperm abnormalities, NRF2 is in particular, under-expressed and is significantly associated with sperm quality parameters [24].

Low levels of ROS are essential for normal growth and development of sperm and functions by acrosome [2,14]. ROS, along with environmental and genetic factors (such as the limited capacity of the DNA repair system), can cause damage to DNA integrity. For example, changes in the structure of palindromic repeats of the highly sensitive region of AZFc can occur and cause a wide range of infertility phenotypes, from normospermia to azoospermia [14,15,23]. Sperms are particularly vulnerable to oxidative stress and full of materials susceptible to ROS and free radical attack. For example, PUFA dominate the lipid profile in sperm where there is a limited level of antioxidant capacity and protection [15]. Oxidation of PUFA is particularly detrimental to sperm morphology and function [2,14,24].

Due to the limited amount of cytoplasm and consequently low antioxidant activity, oxidative stress can cause damage to different sperm structures such as cellular and acrosome membranes, mitochondria, and genomic DNA. Fertilizing sperm speeds its motility by enhancing oxidative phosphorylation (OXPHOS) and

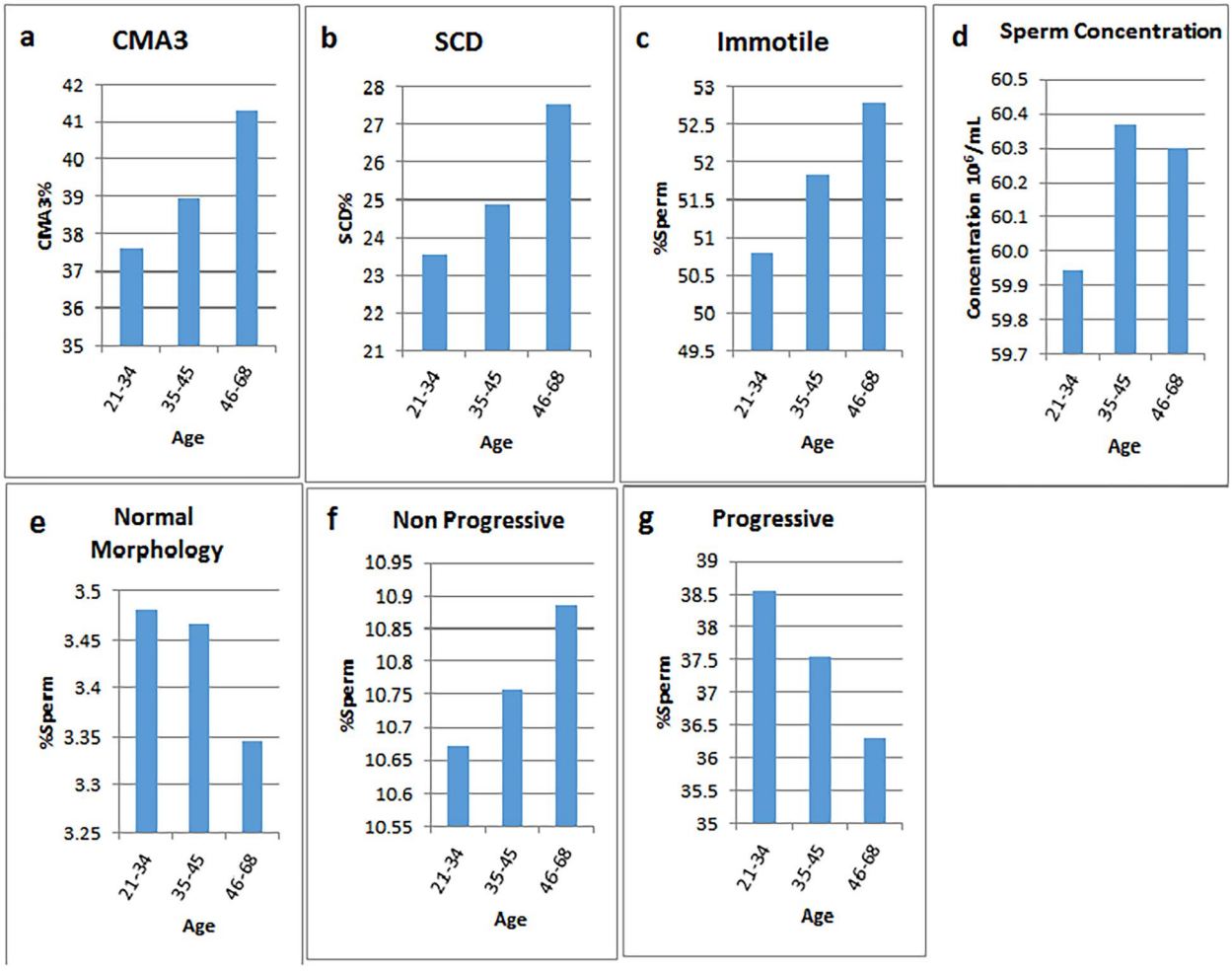


**Figure 3.** The relationship between sperm quality and age groups (years), in normospermic men with infertility problems. (a) The relationship between uncondensed chromatin and age, (b) the relationship between DNA damage and age, (c) the relationship between sperm immotility and age, (d) the relationship between the sperm concentration and age, (e) the relationship between the sperm normal morphology and age, (f) the relationship between the sperm progressive motility and age, and (g) the relationship between the sperm non-progressive motility and age.

exceeding ROS production where sacrificing mtDNA as well [23,25,26]. Here, increased copy numbers of mtDNA and their alterations negatively correlate with sperm chromatin integrity and normal parameters whereby affecting fertility quality and successive outcomes in this aspect [20]. ROS overproduction leads to the accumulation of oxidative stress products and DFI in the semen of patients with unexplained infertility and has been linked to low levels of vitamin A and E in the seminal plasma [7,11].

DNA repair system in human spermatozoa with a highly compacted genome possesses a weak capacity to repair DNA strand breaks in response to oxidative stress. Mature spermatozoa possess a modified version of the severely shortened base-excision repair pathway that is more readily an available DNA repair system in these cells [15].

Data exhibits a considerable association between %SDF and increased levels of sperm-borne miRNAs in the semen. SDF $>$ 2.9% increased the risk of obtaining a non-viable embryo by almost 4-fold. A high level of miRNAs is associated with a high level of SDF and increases the probability of obtaining non-viable embryos [27].

Several articles have also shown a correlation between sperm DFI and poor embryo quality [17,18,19,20]. Borges et al. found that SDF was significantly correlated with a slower rate of cleavage speed, poor embryo quality at day 3, poor blastocyst development, and implantation but did not identify any correlation with fertilization and pregnancy [19]. Our data also indicate that the fertilization rate depends on the couple's age and BMI but not on DNA damage. Indeed, a significant and positive association between

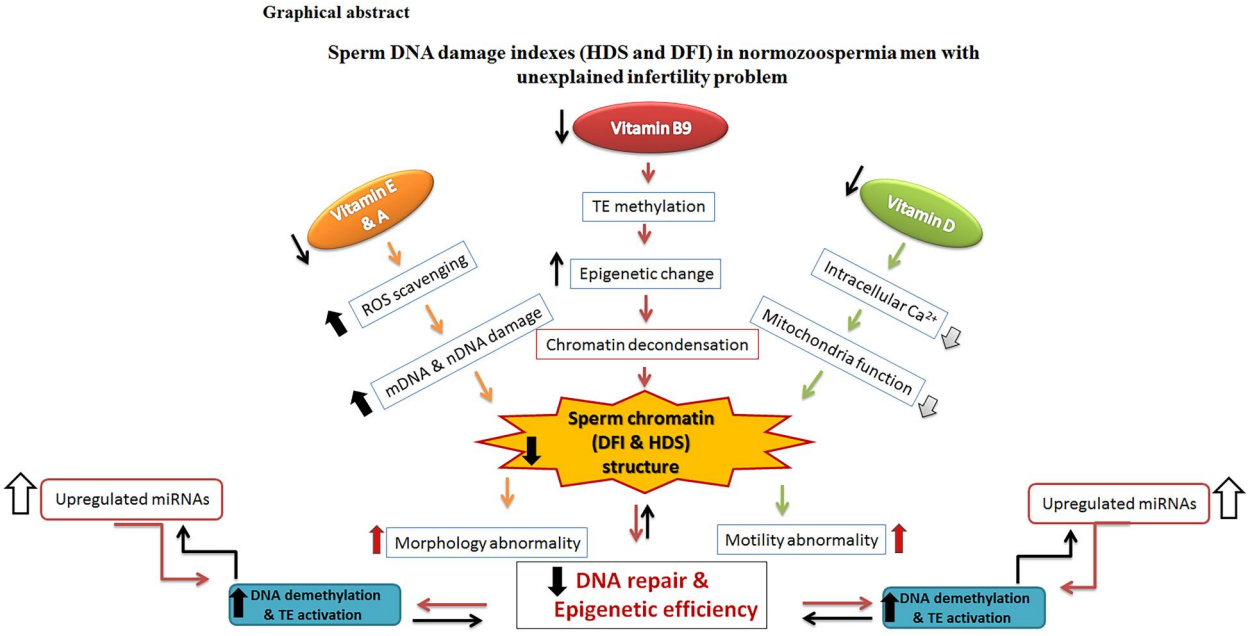


**Figure 4.** Overview of the origins of sperm chromatin structure disruption (DFI and CMA3) and its association with sperm abnormal morphology and motility. The main factors can be deficiencies in DNA repair and epigenetic systems. DFI and CMA3 may result from underlying mechanisms, such as defects in intracellular Ca homeostasis, mitochondria function, TE and repetitive DNA methylation, and ROS scavenging. Moreover, clinical (age, infection, cancer, hormonal imbalances, obesity, and diabetes) and environmental (heat exposure, environmental toxins, radiation, smoking, drug abuse, and diet) risk factors can help in these abnormalities. TE: transposable elements; ROS: reactive oxygen species; mDMA: mitochondrial DNA; nDNA: nuclear DNA; Ca: calcium ion.

SDF post-sperm selection and non-viable embryos was detected, independent of the age of the partners, the sperm selection procedures used, and the AMH levels of the female partner. Similar to the literature data, a positive correlation between SDF post-sperm selection and the percentage of low-quality embryos and a negative correlation with viable embryo formation was found in this study. In particular, an SDF $>$ 2.9% increased the risk of obtaining a non-viable embryo by almost 4-fold. Our data confirm that sperm DFI evaluation could be informative during the infertile couple's workup to improve counseling for couples undergoing ART.

Vitamin D also has regulatory roles in the reproductive system and sperm fertility by considerable expression of its receptors (VDR) and enzymes in testicular tissues and spermatozoa. Vitamin D directly or indirectly affects sperm quality and regulates calcium homeostasis to support the proper maturation of human spermatozoa in the epididymis. There is a high calcium concentration in the epididymis and prostate fluid of fertile men that supports sperm quality and fertility rates [8–10]. Seminal vitamin D increases intracellular calcium ions that enhancing the activity of mitochondrial dehydrogenases in the OXPHOS system and promoting ATP synthesis and spermatozoa motility. Intracellular calcium induces the cAMP/PKA-depended pathway and mitochondria function [10,28].

Also, inappropriate epigenetic modifications and defects in histone-to-protamine conversion are other reasons that can lead to abnormalities in chromatin structure and sperm quality [3,29].

Folic acid deficiency has been associated with aberrant DNA methylation and chromatin remodeling. Seminal low levels of folic acid thus increase sperm DFI and $\gamma$-H2AX, a significant marker for DNA damage. Abnormalities in DNA methylation of sperm have been identified in men with poor seminal fluid parameters (such as low sperm motility). Reduced levels of sperm DNA methylation have been associated with increased fractions of DFI and CMA3 in the nucleus of sperm [29,30].

Epigenetics is now recognized as a biological process that controls semen characteristics and contributes to fertilization. In this regard, sperm-born miRNAs are influenced by SDF and involved in the stages of spermatogenesis and post-fertilization events [27].

In patients with unexplained infertility, DNA hypomethylation has been observed in specific regions with repetitive sequences named LINE-1, Alu Yb8, NBL2, and D4Z4. These loci are enriched with histones containing the repressive H3K9me3 marks, while in

low-quality sperms they may be replaced with histones containing H3K4me1 marks and CTCF. Alterations in methylation of genes associated with the PIWI pathway (e.g. PIWIL2 and TDRD1) and LINE-1 sequences correlate with sperm severe defects. CTCF binds to hypomethylated DNA with H3K4me1 marks and causes alterations in the chromatin context and architecture where the expression and function of fertile genes are changed [31,32].

Clustering of the repetitive elements (e.g. *Alu* elements and *miRNA* genes) exists on chromosome X that produces piRNAs and miRNAs and has unique compositions expressed in the testis to control spermatogenesis and to reprogram oocyte to zygote after fertilization. The majorities of miRNAs that are expressed in the testis are X-encoded and have the potential to reprogram oocytes while Y is kept poor. The potential to fertilize oocytes and activate early zygotic genes is related to paternally derived miRNAs [22,33,34]. Over-expression of some miRNAs can be negatively associated with spermatogenesis and sperm fertility quality (e.g. miR-19a/b: *FSHR [follicle stimulating hormone receptor]* and *BRCA2 [BRCA2; DNA repair associated* gene*]* [22,35,36]. Epi-miRNAs are the most abundant miRNAs in human spermatozoa (e.g. *hsa-miR-140/21/152/148a)*. A cluster of six distinct miRNAs (*miR-890/888/892a/892b)* is exclusively expressed in primate spermatozoa (human Xq27.3) [33,37,38].

On the other hand, pollution has a marked impact on the expression of small non-coding RNAs and basic spermatozoa nuclear proteins in germ cells, which reflects significant environmental effects on sperms and especially on the male reproductive system. Pollutants induce consequences on sperm morphology, number, and motility, by causing oxidative damage to spermatozoa membrane lipids, particularly PUFAs and its DNA [12].

DNA integrity along with sperm morphology and PR has been reported as epigenetic-dependent parameters [5,16]. Alterations in epigenetics may result from defects in DNA repair or surveillance systems and lead to abnormalities in microfilament structures, genetic material mismatch, unequal cell division during spermatogenesis, and sperm incompetence [3,15].

Furthermore, studies report an association between viral infection and sperm morphological and functional damage. Recent molecular studies and semen analysis have shown that spermatogenesis and erectile function can be irreversibly impaired by viral infection (such as HPV) which causes poor semen quality [13].

It should be mentioned here that this study has not directly investigated molecular factors and their abnormality relationships with sperm parameters. The study has tried to show that DNA quality and chromatin structure are the main molecular parameters controlling the fertility potential of males who despite having normal sperm concentrations are infertile and discuss the possible molecular mechanism involved.

### Limitations

This was a retrospective cross-sectional study that included 1992 recorded data from normozoospermic men with unexplained infertility problem. Selection of the semen samples and normospermia were in accordance with the WHO 2010 criteria.

### Interpretation

Here, a direct relationship was shown between sperm quality and DNA integrity parameters (DFI and CMA3). Normal parameters of sperm gradually decrease with the increase of DNA damage indexes [5,16,21]. A valuable negative association exists between sperm parameters with DFI and CMA3 at the same age, which results from physiological events.

### Generalizability

Here, the CMA3 index indicates the level of histone-DNA complexes in the sperm genome, which makes the DNA susceptible to damaging agents and disrupts the chromatin structure [3,5,23].

Nutritional factors, such as lack of vitamins and environmental factors, such as infections, pollution, and occupational exposures increase the risk of DNA damage in spermatozoa by causing defects in the function of epigenetic factors and converting histone to protamine. These extrinsic factors reduce sperm quality and cover the effects of age and genetics.

Epigenetic factors (such as DNA methylation, histone marks, and small non-coding RNAs) control the expression of repetitive sequences and transposable elements in the spermatozoa genome which in turn determine the expression of fertile genes and sperm quality [31,32].

Therefore, in patients with unexplained infertility, spermatozoa maturation and fertility quality are strongly affected by internal and external factors, which eventually affect sperm chromatin structure and DNA integrity (Figure 4).

Therefore, genetic background and internal factors controlling male fertility are important markers in helping professionals make a better diagnosis, choose the appropriate treatment, and monitor treatment.

## Disclosure statement

The authors declare no conflicts of interest.

## Ethical approval

The Ethics Committee of Shahid Sadouqhi University of Medical Sciences (IR.SSU.SRH.REC.1401.011) approved the study.

## Author contributions

- *Conceptualization*: Fatemeh pourrajab, Mehdi Abedinzadeh,
- *Investigation:* Fateme Sadeghi-Nodoushan, Sara Abedinzadeh,
- *Methodology:* Sara Abedinzadeh, Fatemeh pourrajab,
- *Supervision:* Fateme Sadeghi-Nodoushan, Fatemeh pourrajab,
- *Writing – original draft:* Fateme Sadeghi-Nodoushan, Fatemeh pourrajab,
- *Writing – review & editing*: Mehdi Abedinzadeh, Fatemeh pourrajab.

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## Availability of data and materials

Data or any datasets used in the research can be available on request. Dr. Fatemeh Pourrajab should be contacted if someone wants to request the data from this study.

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