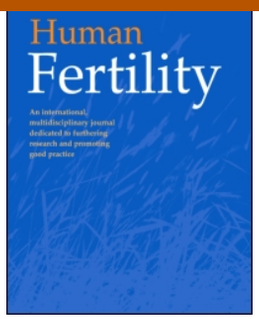

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ORIGINAL ARTICLE

Relation between sperm protamine transcripts with global sperm DNA methylation and sperm DNA methyltransferases mRNA in men with severe sperm abnormalities

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ABSTRACT

This study aimed to evaluate the relationship between mRNA expression of DNA methyltransferases (DNMTs) such as DNMT1, DNMT3A and DNMT3B mRNA and sperm global DNA methylation with protamine transcripts in the sperm from men with severe sperm abnormalities. Sperm from each semen sample were isolated using a standard gradient isolation procedure by layering 1 mL of 40% (v/v) density gradient medium over 1 mL of 80% (v/v). A total of 30 oligoasthenoteratozoospermic ejaculates (OAT) and 30 normozoospermic ejaculates as controls were compared using real-time quantitative reverse transcriptase polymerase chain reaction for mRNA expression of DNMT1, 3A, 3B, protamine1 (P1) and protamine2 (P2). The enzyme-linked immunosorbent assay was used to detect global DNA methylation in sperm. A p-value of <0.05 was considered statistically significant. In OAT ejaculates, the increased level of DNMT3A, 3B mRNA, sperm global methylation, P1 plus P2 mRNA and decrease of P1–P2 ratio were significantly different. Also the content of protamine transcript was not correlated with sperm parameters. The increased total protamine transcript levels were associated with increased mRNA methyltransferases. The increase of DNMT1 may lead to an increased level of global methylation.

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Sperm; DNA; transcription; methylation; protamine

Introduction

During spermiogenesis, sperm chromatin undergoes substantial compaction (Montjean et al., 2012). In an elongating spermatid, the protamine protein replaces the transition proteins leading to DNA stability as well as transcriptional quiescence (Talbert & Henikoff, 2010). Various studies have described altered protamine1 (P1) to protamine 2 (P2) ratios in the sperm from infertile men. Some studies reported elevated P1:P2 ratios (Hammadeh, Hamad, Montenarh, & Fischer-Hammadeh, 2010; Nanassy, Liu, Griffin, & Carrell, 2011), and even the complete absence of P2 (Imken et al., 2009). However, the P1 plus P2 mRNA as the total protamine transcript content and the relation to male infertility were not mentioned.

Regarding the important role of protamine for proper sperm chromatin integrity and the relationship to epigenetic reprogramming in sperm, the detection of 5-methylcytosine (5-mC) has also been suggested for evaluating DNA methylation (Consales et al., 2014). DNA methylation is produced by DNA methyltransferases (DNMTs) and due to its catalytic preference for hemimethylated DNA, DNMT1 is considered to act as the maintenance methyltransferase (Fatemi, Hermann, Gowher, & Jeltsch, 2002; Hirasawa et al., 2008). DNMT3A and DNMT3B are essential for de novo methylation, which establish paternal imprints and regulate normal spermatogenesis and sperm maturation (Jones, 2012).

The DNA methylation profile in sperm from normozoospermic men and isolated by discontinuous density gradients have been shown to be decreased compared with unprocessed samples (Yu et al., 2015). Others, however, showed that there was no relationship between global methylation and sperm quality (Olszewska et al., 2017). In this regard, the results of the study by Montjean et al. (2015) showed that the level of global methylation of sperm DNA is significantly lower in oligozoospermia (Montjean et al., 2015). It is noted that global sperm DNA methylation

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was strongly associated with fertilization rate and embryo quality following assisted reproductive technology (Barzideh, Scott, & Aitken, 2013; Houshdaran et al., 2007). However, it is not clear whether the sperm quality in infertile men is associated with hypermethylation (Dhillon, Shahid, & Husain, 2007; Wu et al., 2010) or hypomethylation (Houshdaran et al., 2007; Tunc & Tremellen, 2009).

Aoki, Emery, and Carrell (2006) showed that there is no significant relationship between sperm protamine ratio and DNA global methylation (Aoki, Emery, & Carrell, 2006). Similarly, Bahreinian et al. (2015) also found no relationship between sperm protamine deficiency and global methylation. Hence, as mentioned earlier, the reports of global DNA methylation related to sperm quality and protamine are contradictory. We also found an absence of evidence for the relationship between DNMT1, 3A and 3B mRNA with sperm protamine transcripts. Therefore, this study aimed to evaluate the relationship between protamine transcripts content in terms of protamine transcripts ratio and total protamine transcripts with sperm global DNA methylation, DNMT1, 3A and 3B mRNA expression in a population of infertile men with severe sperm disorders.

Materials and methods

Study participants

Study participants were those seeking treatment for infertility and referred to the Research and Clinical Center for Infertility. The participants gave informed consent in accordance with the Helsinki Declaration of 1975 on human experimentation and all procedures were approved by the Ethics Committee for Clinical Research for Infertility in Yazd (Ref: IRCT201507285261N2). Inclusion criteria were the following: (i) age 25–40 years; (ii) sperm concentration 7–14 million/mL; (iii) total motility <40%; and (iv) normal morphology <4%. Semen samples from 30 individuals with oligoasthenoteratozoospermic (OAT) ejaculates as well as normoazoospermic ejaculates from 30 other men were used as a control.

Semen collection and determination of sperm parameters

Semen samples from patients were collected into sterile vessels by masturbation after 2–7 days of sexual abstinence. Samples were liquefied for at least 30 min at room temperature. Semen analysis was performed according to strict criteria according to WHO (2010). For the assessment of sperm morphology, a 10-mL prepared sperm aliquot smear was dried at room temperature and stained with toluidine blue (Zini & Agarwal, 2011). A total of 200 spermatozoa were counted at 100× magnification. Sperm viability in fresh and thawed samples was determined using the eosin-nigrosin staining technique. Five microlitre of prepared sperm was mixed with 5 μL of eosin–nigrosin stain on a glass slide and assayed using a light microscope to determine the percentage of live sperm. At least 200 spermatozoa were assessed for each sample. The white or unstained sperm were classified as live and pink or red sperm were considered dead.

Sperm preparation by discontinuous density gradients

Density gradients were used in order to eliminate non-sperm cells and other debris from samples (Houshdaran et al., 2007; Rogenhofer et al., 2013). Briefly, sperm from each sample were isolated and purified using a standard gradient isolation procedure according to WHO (2010). The density gradient medium (In Vitro, Fredensborg, Denmark) was prepared in a 15-mL test tube by layering 1 mL of 40% (v/v) over 1 mL of 80% (v/v). About 1 mL of each well-mixed semen sample was placed on top of the density gradient media and then centrifuged at 1200*g* for 15 min. Most of the supernatant was removed from the sperm pellet, which was then resuspended in 5 mL of Hams f10 medium by gentle pipetting. The separated sperm pellet was centrifuged twice at 1200*g* and the sperm pellet was then resuspended in 0.5 mL of PBS. To avoid somatic cell contamination, the processed sperm were examined under light microscopy. Purification using a standard gradient isolation procedure was repeated if there was a somatic cell in the specimen. The specimen was then stored at −80 °C. RNA and sperm DNA were isolated from the same sample aliquot.

Global DNA methylation analysis

Global DNA methylation analysis was conducted using a 5-mC DNA ELISA Kit Catalog Nos. D5326 (ZYMO RESEARCH CORP., USA). Briefly, 100 ng of isolated denatured (single-stranded) sperm DNA from selected samples was dissolved with 5-mC Coating Buffer and incubated at 37 °C for 1 h. After discarding and washing each well three times with 200 μl of 5-mC ELISA Buffer, the samples were incubated at 37 °C for 30 min. Anti-5-methylcytosine and secondary antibody

Table 1. Primer sequence, sequence amplified and product size of study genes.

Gene	Primer sequence (5′–3′)	Sequence amplified	Product size (bp)
P1	F-AGAGCCGGAGCAGATATTACC R-TCTACATCGCGGTCTGTACCT	NM_002761.2	119
P2	F-ATCCACAGGCGGCAGCATCG R-TTCCAGCTGGGGGTGAGGGG	NM_002762.3	144
DNMT1	F-TGGACGACCCTGACCTCAAAT R-GCTTACAGTACACACTGAAGCA	NM_001318731.1	168
DNMT3A	F-TATTGATGAGCGCACAAGAGAG R-GGGTGTTCCAGGGTAACATTGAG	NM_001320893.1	111
DNMT3B	F-GGCAAGTTCTCCGAG GTCTCTG R-TGGTACATGGCTTTTCGAAGGA	NM_001207056.1	113
B2M	F-AGATGAGTATGCCTGCCGTG R-TGCGGCATCTTCAAACCTC	NM_004048.2	106

Key: F: forward; R: reverse; B2M: beta-2-microglobulin.

in 5-mC ELISA Buffer was added and incubated at 37 °C for 1 h. After washing the wells, HRP (Horseradish Peroxidase) Developer was added to each well and the colour allowed to develop for 20 min at room temperature. The absorbance was measured at 450 nm using an ELISA plate reader (star fax-2100, Awareness Technology, Palm City, FL). A standard curve was generated using negative and positive internal controls on the same plate using different dilutions. The negative control was unmethylated DNA containing no 5-methylcytosine, whereas the positive control was methylated DNA containing 5% of 5-methylcytosine. The global methylation of each sample was conducted in duplicate and the final value was the average.

Human sperm RNA extraction

A suspension of 1×10^6 sperm in a PBS buffer was used. The total RNA was isolated from the sperm pellet of each subject using the RNeasy Plus Universal Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Scientific, 2000c, Waltham, MA). Regarding the extraction of RNA, the expected concentration of a sperm is 50 fg. Therefore, in a volume of 30 μL, each sample will be 1.5–2 ng. Our results also corresponded to the same amount. RNA integrity number (RIN) was measured using the 28S to 18S rRNA ratio. The RIN of more than 50% of gradient separated sperm RNA was 1. After RNA normalization for each sample, the complementary DNA (cDNA) was prepared.

Quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR)

Approximately 100 ng RNA was reverse-transcribed into cDNA using RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Loughborough, UK). The quantitative polymerase chain reaction (PCR) was performed using Fast SYBR®Green Master Mix, Applied Biosystems (Foster City, CA). Briefly, 100 ng cDNA per sample was used for amplification of target genes with the primers of human genes; P1, P2, DNMT1, DNMT3A and DNMT3B (Table 1). Using a thermal cycler (step one, Applied Biosystems, Foster City, CA), the thermal cycling programme included an initial incubation at 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 58 °C for 30 s. A final 58–95 °C step was used to form the melt curve. Values recorded for quantification were the fractional cycle numbers for which the background-corrected amplification curves crossed a threshold value. The threshold value was set within the log-linear phase of the amplification curves. Two replicates of each reaction were performed, and the CT values were averaged. The 2_ΔCT was calculated to represent the levels of gene expression after normalization to that of B2M (beta-2-microglobulin), where $\Delta CT = (CTgenes - CT\ B2M)$. The specificity of amplified products was confirmed by gel electrophoresis as well as the melting curve. Each sample was conducted in duplicate.

Statistical analysis

Non-parametric Mann–Whitney test was used to analyze differences in sperm parameters, mRNA expression and global DNA methylation between two study groups. Spearman's test was used to calculate correlations. All analyses were performed using PASW Statistics 18 (SPSS, Chicago, IL). A p value <0.05 was considered statistically significant.

Results

Values for sperm concentration, progressive motility, non-progressive motility, immotility, normal morphology and viability were significantly different ($p < 0.001$) between OAT and normozoospermic (control) ejaculates (Table 2). The mRNA expression of P1, P2 and P1 plus P2 as the total protamine transcripts was significantly higher in OAT. However, the P1 to P2 mRNA as the protamine ratio was significantly lower ($p < 0.04$) in OAT (0.27) compared with the control group (0.51). The median of global DNA methylation was 3.1% in OAT compared with 2.0% in the control

Table 2. Comparing different variables in control and OAT ejaculates.

	Control group median (minimum, maximum)	OAT group median (minimum, maximum)	*p* Value
Sperm concentration ($\times 10^6$ per ml)	138.0 (50.0, 150.0)	10.0 (9.9, 12.0)	<0.001
Progressive motility (%)	46.0 (42.0, 51.9)	25.0 (15.0, 30.0)	<0.001
Non-progressive motility (%)	17.0 (14.0, 22.0)	11.5 (6.5, 16.0)	<0.001
Immotile (%)	35.0 (28.6, 39.0)	83.0 (66.0, 90.0)	<0.001
Normal morphology (%)	15.0 (12.0, 17.7)	1.0 (0.6, 1.4)	<0.001
Viability (%)	70.0 (64.0, 74.0)	50.0 (47.0, 57.0)	<0.001
Global DNA methylation%	2.0 (1.7, 2.55)	3.1 (2.9, 3.8)	<0.001
P1mRNA	0.0321 (−0.299, 0.31)	6.4 (2.2, 9.5)	<0.001
P2mRNA	0.12 (−0.57, 2.24)	12.9 (3.9, 39)	<0.001
P1plusP2 mRNA	0.156 (−0.8, 6.5)	20.7 (7.3, 47.7)	<0.001
P1toP2 mRNA	0.51 (0.43, 0.74)	0.27 (0.19, 0.52)	0.04
DNMT1 mRNA	0.046 (0.16, 0.49)	0.058 (0.04, 0.09)	0.92
DNMT3A mRNA	0.004 (0.005, 0.039)	0.06 (0.04, 0.12)	0.04
DNMT3B mRNA	0.016 (0.01, 0.03)	0.12 (0.09, 0.17)	0.03

Key: The Mann–Whitney *U* test was done. OAT: oligoasthenoteratozoospermia. A p value < 0.05 was considered statistically significant.

group ($p < 0.001$). The mRNA expression of DNMT1 was not statistically different between the two groups. However, the mRNA expressions of DNMT3A and DNMT3B were significantly higher in OAT compared to the control ($p = 0.04$, $p = 0.03$, respectively) (Table 2). In OAT, neither the total protamine content nor the ratio was related to sperm parameters. However, in the control group, P1 plus P2 mRNA was significantly negatively correlated to sperm progressive motility (Table 3). In the two study groups, the total protamine transcripts showed high correlation with DNMT3A and 3B mRNA. Moreover, the DNMT1 transcript was associated with total protamine transcripts in OAT (Table 4). In OAT, DNMT1 mRNA was related to DNMT3A, 3B transcripts and global DNA methylation (Table 5).

Discussion

This study evaluated the protamine transcript content in relation to sperm global DNA methylation, DNMTs expression in sperm from OAT and normozoospermic ejaculates. We detected that P1 and P2 transcripts were significantly higher in OAT which is in agreement with Aoki, Liu, and Carrell (2005) who noted the retention of these transcripts in spermatogenesis impairment. However, we showed P1 to P2 mRNA was significantly lower in sperm from OAT ejaculates compared to those from normozoospermic ones. In OAT sperm, it seemed that P1 was less expressed than P2. Some findings showed that abnormally increased P1 and P2 mRNA retention appears to be associated with diminished P1 protein concentration. A significant negative relationship between P1 mRNA levels and the aberrant P1 to P2 protein ratio was identified (Aoki, Liu, & Carrell, 2006; Ni et al., 2014). In a study in patients with an abnormally low ratio of P1 to P2 protein, there was a significant increase in P1 and P2 transcript retention (Aoki, Liu, et al., 2006).

In sperm from OAT, we observed that protamine mRNA content was not associated with sperm parameters. Data from Mengual, Ballescá, Ascaso, and Oliva (2003) showed no significant relationship between progressive sperm motility and protamine ratio in a group of oligoasthenospermic men (Mengual, Ballescá, Ascaso, & Oliva, 2003). In contrast, Rogenhofer et al. (2013) showed a significant relationship between the ratio of protamine mRNA with motility and morphology of sperm (Rogenhofer et al., 2013). Data from Depa-Martynow, Kempisty, Jagodziński, Pawelczyk, and Jedrzejczak (2012) also showed a significant correlation between protamine mRNA and sperm progressive motility in 92 infertile couples (Depa-Martynow et al., 2012).

We showed that global DNA methylation was higher in sperm from OAT ejaculates compared to those from normozoospermic ones. Other studies showed that global hypermethylation status of the DNA was characterized by poor-quality spermatozoa. Hypermethylation was visualized by an anti-5-methylcytosine antibody randomly distributed throughout the chromatin (Barzideh et al., 2013). Moreover, a significant hypermethylation was detected in poor sperm population which accords with a large number of single-gene studies in specific genomic loci in cases of male infertility (Hammoud, Purwar, Pflueger, Cairns, & Carrell, 2010; Ouko et al., 2009; Urdinguio et al., 2015).

The DNMT3A and 3B transcripts were high in OAT sperm, which suggests a failure of the impaired sperm to obtain the proper methylation level. In a comparable study, an increased level of DNMT3A enzyme was detected in impaired testicular samples (Adiga et al., 2011). DNA methylation analyses suggested that there were alterations in specific location patterns in the human genome (Yu et al., 2015). These changes in the methylation pattern largely occurred in transcriptional

Table 3. The correlation of P1 to P2 mRNA ratio and P1plusP2 mRNA with different sperm parameters in control and OAT groups.

		Sperm concentration ($\times 10^6$ per ml)	Progressive motility (%)	Non-progressive motility (%)	Immotility (%)	Normal morphology (%)	Viability (%)
Control group	*P1*plus*P2*mRNA	0.134	−0.385	0.267	0.252	0.177	−0.354
		0.480	0.036	0.154	0.179	0.350	0.055
	P1toP2 mRNA	−0.230	−0.132	−0.291	0.290	−0.519	−0.245
		0.239	0.504	0.133	0.134	0.005	0.209
OAT group	*P1*plus*P2*	−0.208	−0.147	−0.053	0.097	−0.125	0.137
	mRNA	0.289	0.456	0.789	0.625	0.525	0.487
	P1toP2 mRNA	0.186	−0.015	−0.023	0.137	−0.100	0.199
		0.325	0.936	0.906	0.470	0.600	0.292

Key: In each cell the upper data is the *r* value and the lower data the *p* value. A *p* value of < 0.05 was considered statistically significant.

Table 4. The correlation of P1 to P2 mRNA ratio and P1plusP2 mRNA with different sperm parameters in control and OAT ejaculates.

		% global DNA methylation	*P1*mRNA	*P2*mRNA	*DNMT1* mRNA	*DNMT3A* mRNA	*DNMT3B* mRNA
Control group	*P1toP2* mRNA	−0.177	0.181	−0.265	0.139	−0.278	−0.050
		0.368	0.357	0.174	0.480	0.161	0.805
	*P1*plus*P2* mRNA	−0.263	0.884	0.968	0.004	0.711	0.576
		0.161	<0.001	<0.001	0.982	<0.001	0.001
OAT group	*P1toP2* mRNA	−0.075	0.108	−0.184	−0.236	−0.158	−0.214
		0.692	0.571	0.331	0.209	0.405	0.255
	*P1*plus*P2* mRNA	−0.137	0.931	0.994	0.502	0.481	0.647
		0.487	<0.001	<0.001	0.006	0.010	<0.001

Key: In each cell the upper data is the r value and the lower data the *p* value. A *p* value of < 0.05 was considered statistically significant.

Table 5. The correlation of different DNA methytransferase transcripts with global DNA methylation in sperm from OAT and normozoospermic ejaculates.

		DNMT1mRNA	DNMT3AmRNA	DNMT3BmRNA	% global DNA methylation
control group	*DNMT1*		0.114	−0.303	0.159
	mRNA	–	0.555	0.111	0.401
	DNMT3A			0.620	−0.111
	mRNA	–	–	<0.001	0.568
	DNMT3B				−0.089
	mRNA	–	–	–	0.645
OAT	*DNMT1*		0.689	0.736	0.381
	mRNA	–	<0.001	<0.001	0.038
	DNMT3A			0.724	0.338
	mRNA	–	–	<0.001	0.06
	DNMT3B				0.210
	mRNA	–	–	–	0.266

Key: In each cell the upper data is the *r* value and the lower data the *p* value. A *p* value of <0.05 was considered statistically significant.

factor to inhibit proper gene expression during spermatogenesis (Pedersen & Helin, 2010).

Abnormal replacement of protamine and the overall decrease in the sperm quality may be due to transcriptional and translation incompatibility system by aberrant methylation (Carrell, Emery, & Hammoud, 2008). The aberrant methylation level in chromatin may affect the transcription of other genes involved in protamine translation. In OAT sperm, the content of protamine transcript was not correlated with sperm parameters. However, by increasing the total protamine transcript levels, the methyltransferases mRNA also increased. Also, by increasing DNMT1, the high level of global methylation occurred.

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Disclosure statement

All investigators disclose no conflict of interest in this study.

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References

Adiga, S.K., Ehmcke, J., Schlatt, S., Kliesch, S., Westernstroer, B., Luetjens, C.M., … Gromoll, J. (2011). Reduced expression of DNMT3B in the germ cells of patients with bilateral spermatogenic arrest does not lead to changes in the global methylation status. *Molecular Human Reproduction*, *17*, 545–549. doi: 10.1093/molehr/gar023.

Aoki, V.W., Emery, B.R., & Carrell, D.T. (2006). Global sperm deoxyribonucleic acid methylation is unaffected in protamine-deficient infertile males. *Fertility and Sterility*, *86*, 1541–1543. doi: 10.1016/j.fertnstert.200.

Aoki, V.W., Liu, L., & Carrell, D.T. (2005). Identification and evaluation of a novel sperm protamine abnormality in a population of infertile males. *Human Reproduction*, *20*, 1298–1306. doi: 10.1093/humrep/deh798.

Aoki, V.W., Liu, L., & Carrell, D.T. (2006). A novel mechanism of protamine expression deregulation highlighted by abnormal protamine transcript retention in infertile human males with sperm protamine deficiency. *MHR: Basic Science of Reproductive Medicine*, *12*, 41–50. doi: 10.1093/molehr/gah258.

Bahreinian, M., Tavalaee, M., Abbasi, H., Kiani-Esfahani, A., Shiravi, A.H., & Nasr-Esfahani, M.H. (2015). DNA hypomethylation predisposes sperm to DNA damage in individuals with varicocele. *Systems Biology in Reproductive Medicine*, *61*, 179–186. doi: 10.3109/19396368.2015.1020116.

Barzideh, J., Scott, R., & Aitken, R. (2013). Analysis of the global methylation status of human spermatozoa and its association with the tendency of these cells to enter apoptosis. *Andrologia*, *45*, 424–429. doi: 10.1111/and.12033.

Carrell, D.T., Emery, B.R., & Hammoud, S. (2008). The aetiology of sperm protamine abnormalities and their potential impact on the sperm epigenome. *International Journal of Andrology*, *31*, 537–545. doi: 10.1111/j.1365-2605.2008.00872.x.

Consales, C., Leter, G., Bonde, J.P.E., Toft, G., Eleuteri, P., Moccia, T., … Spano, M. (2014). Indices of methylation in sperm DNA from fertile men differ between distinct geographical regions. *Human Reproduction*, *29*, 2065–2072. doi: 10.1093/humrep/deu176.

Depa-Martynow, M., Kempisty, B., Jagodziński, P.P., Pawelczyk, L., & Jedrzejczak, P. (2012). Impact of protamine transcripts and their proteins on the quality and fertilization ability of sperm and the development of preimplantation embryos. *Reproductive Biology*, *12*, 57–72. doi: 10.1016/S1642-431X(12)60077-1.

Dhillon, V.S., Shahid, M., & Husain, S.A. (2007). Associations of MTHFR DNMT3b 4977 bp deletion in mtDNA and GSTM1 deletion, and aberrant CpG island hypermethylation of GSTM1 in non-obstructive infertility in Indian men. *MHR: Basic Science of Reproductive Medicine*, *13*, 213–222. doi: 10.1093/molehr/gal118.

Fatemi, M., Hermann, A., Gowher, H., & Jeltsch, A. (2002). Dnmt3a and Dnmt1 functionally cooperate during de novo methylation of DNA. *European Journal of Biochemistry*, *269*, 4981–4984. doi: 10.1046/j.1432-1033.2002.03198.x.

Hammadeh, M., Hamad, M., Montenarh, M., & Fischer-Hammadeh, C. (2010). Protamine contents and P1/P2 ratio in human spermatozoa from smokers and non-smokers. *Human Reproduction*, *25*, 2708–2720. doi: 10.1093/humrep/deq226.

Hammoud, S.S., Purwar, J., Pflueger, C., Cairns, B.R., & Carrell, D.T. (2010). Alterations in sperm DNA methylation patterns at imprinted loci in two classes of infertility. *Fertility and Sterility*, *94*, 1728–1733. doi: 10.1016/j.fertnstert.2009.09.010.

Hirasawa, R., Chiba, H., Kaneda, M., Tajima, S., Li, E., Jaenisch, R., & Sasaki, H. (2008). Maternal and zygotic Dnmt1 are necessary and sufficient for the maintenance of DNA methylation imprints during preimplantation development. *Genes & Development*, *22*, 1607–1616. doi: 10.1101/gad.1667008.

Houshdaran, S., Cortessis, V.K., Siegmund, K., Yang, A., Laird, P.W., & Sokol, R.Z. (2007). Widespread epigenetic abnormalities suggest a broad DNA methylation erasure defect in abnormal human sperm. *PLoS One*, *2*, e1289. doi: 10.1371/journal.pone.0001289.

Imken, L., Rouba, H., El Houate, B., Louanjli, N., Barakat, A., Chafik, A., & McElreavey, K. (2009). Mutations in the protamine locus: Association with spermatogenic failure? *Molecular Human Reproduction*, *15*, 733–738. doi: 10.1093/molehr/gap056.

Jones, P.A. (2012). Functions of DNA methylation: Islands, start sites, gene bodies and beyond. *Nature Reviews Genetics*, *13*, 484–492. doi: 10.1038/nrg3230.

Mengual, L., Ballescá, J.L., Ascaso, C., & Oliva, R. (2003). Marked differences in protamine content and P1/P2 ratios in sperm cells from percoll fractions between patients and controls. *Journal of Andrology*, *24*, 438–447. doi: 10.1002/j.1939-4640.2003.tb02692.x.

Montjean, D., De La Grange, P., Gentien, D., Rapinat, A., Belloc, S., Cohen-Bacrie, P., … Benkhalifa, M. (2012). Sperm transcriptome profiling in oligozoospermia. *Journal of Assisted Reproduction and Genetics*, *29*, 3–10. doi: 10.1007/s10815-011-9644-3.

Montjean, D., Zini, A., Ravel, C., Belloc, S., Dalleac, A., Copin, H., … Benkhalifa, M. (2015). Sperm global DNA methylation level: Association with semen parameters and genome integrity. *Andrology*, *3*, 235–240. doi: 10.1111/andr.12001.

Nanassy, L., Liu, L., Griffin, J., & Carrell, D.T. (2011). The clinical utility of the protamine 1/protamine 2 ratio in sperm. *Protein & Peptide Letters*, *18*, 772–777. doi: 10.2174/092986611795713934.

Ni, K., Steger, K., Yang, H., Wang, H., Hu, K., & Chen, B. (2014). Sperm protamine mRNA ratio and DNA fragmentation index represent reliable clinical biomarkers for men with varicocele after microsurgical varicocele ligation. *The Journal of Urology*, *192*, 170–176. doi: 10.1016/j.juro.2014.02.046.

Olszewska, M., Barciszewska, M.Z., Fraczek, M., Huleyuk, N., Chernykh, V.B., Zastavna, D., … Kurpisz, M. (2017). Global methylation status of sperm DNA in carriers of chromosome structural aberrations. *Asian Journal of Andrology*, *19*, 117–124. doi: 10.4103/1008-682X.168684.

Ouko, L.A., Shantikumar, K., Knezovich, J., Haycock, P., Schnugh, D.J., & Ramsay, M. (2009). Effect of alcohol consumption on CpG methylation in the differentially methylated regions of H19 and IG-DMR in male gametes—Implications for fetal alcohol spectrum disorders.

Alcoholism: Clinical and Experimental Research, *33*, 1615–1627. doi: 10.1111/j.1530-0277.2009.00993.x.

Pedersen, M.T., & Helin, K. (2010). Histone demethylases in development and disease. *Trends in Cell Biology*, *20*, 662–671. doi: 10.1016/j.tcb.2010.08.011.

Rogenhofer, N., Dansranjavin, T., Schorsch, M., Spiess, A., Wang, H., von Schonfeldt, V., … Steger, K. (2013). The sperm protamine mRNA ratio as a clinical parameter to estimate the fertilizing potential of men taking part in an ART programme. *Human Reproduction*, *28*, 969–978. doi: 10.1093/humrep/des471.

Talbert, P.B., & Henikoff, S. (2010). Histone variants—Ancient wrap artists of the epigenome. *Nature Reviews Molecular Cell Biology*, *11*, 264–275. doi: 10.1038/nrm2861.

Tunc, O., & Tremellen, K. (2009). Oxidative DNA damage impairs global sperm DNA methylation in infertile men. *Journal of Assisted Reproduction and Genetics*, *26*, 537–544. doi: 10.1007/s10815-009-9346-2.

Urdinguio, R.G., Bayón, G.F., Dmitrijeva, M., Toraño, E.G., Bravo, C., Fraga, M.F., … Fernández, A.F. (2015). Aberrant DNA methylation patterns of spermatozoa in men with unexplained infertility. *Human Reproduction*, *30*, 1014–1028. doi: 10.1093/humrep/dev053.

Wu, W., Shen, O., Qin, Y., Niu, X., Lu, C., Xia, Y., … Wang, X. (2010). Idiopathic male infertility is strongly associated with aberrant promoter methylation of methylenetetrahydrofolate reductase (MTHFR). *PLoS One*, *5*, e13884. doi: 10.1371/journal.pone.0013884.

Yu, B., Zhou, H., Liu, M., Zheng, T., Jiang, L., Zhao, M., … Huang, Z. (2015). Epigenetic alterations in density selected human spermatozoa for assisted reproduction. *PLoS One*, *10*, e0145585. doi: 10.1371/journal.pone.0145585.

Zini, A., & Agarwal, A. (2011). *Sperm chromatin: Biological and clinical applications in male infertility and assisted reproduction*. New York: Springer-Verlag.