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Influence of tobacco cigarette heavy smoking on DNA methylation patterns and transcription levels of *MAPK8IP3*, *GAA*, *ANXA2*, *PRRC2A*, and *PDE11A* genes in human spermatozoa

Mohammed M. Laqqan^{1*}  and Maged M. Yassin²

Abstract

Background: Tobacco smoking is considered as one of the lifestyles factors that influence the sperm DNA methylation and global sperm DNA methylation and that may affect the sperm phenotype. This study was performed to investigate whether tobacco cigarette heavy smoking influences sperm DNA methylation patterns and semen parameters and to determine whether there is an alteration in the transcription level of *MAPK8IP3*, *GAA*, *ANXA2*, *PRRC2A*, and *PDE11A* genes in heavy smokers compared to non-smokers. Thirty samples were subjected to 450K arrays as a screening study to assess the variation in sperm DNA methylation levels between heavy smokers and non-smokers. Five CpG sites have the highest difference in methylation levels (cg07869343, cg05813498, cg09785377, cg06833981, and cg02745784), which are located in the *MAPK8IP3*, *GAA*, *ANXA2*, *PRRC2A*, and *PDE11A* genes, respectively, and were selected for further analysis using deep bisulfite sequencing in 280 independent samples (120 proven non-smokers and 160 heavy smokers) with a mean age of 33.8 ± 8.4 years. The global sperm DNA methylation, sperm DNA fragmentation, and chromatin non-condensation were evaluated also.

Results: A significant increase was found in the methylation level at seven, three, and seventeen CpGs within the *GAA*, *ANXA2*, and *MAPK8IP3* genes amplicon, respectively ($P < 0.01$) in heavy smokers compared to non-smokers. Additionally, a significant increase was found in the methylation levels at all CpGs within *PRRC2A* and *PDE11A* gene amplicon ($P < 0.01$). A significant increase was found in the level of sperm chromatin non-condensation, DNA fragmentation, and global DNA methylation ($P < 0.001$) in heavy smokers compared to non-smokers.

Conclusion: These results indicate that tobacco cigarette smoking can alter the DNA methylation level at several CpGs, the status of global DNA methylation, and transcription level of the following genes "*MAPK8IP3*, *GAA*, *ANXA2*, *PRRC2A*, and *PDE11A*" in human spermatozoa. These findings may affect negatively semen parameters and men's fertility.

Keywords: DNA methylation, Global methylation, Heavy smokers, Tobacco, Transcription

Background

Infertility is defined as the inability of a sexually active couple to achieve a successful pregnancy after 1 year of unprotected intercourse [1]. Approximately 15% of couples worldwide suffering from infertility problems

*Correspondence: m.laqqan@gmail.com

¹ Faculty of Health Sciences, Department of Medical Laboratory Sciences, Islamic University, Gaza, Palestinian Territories
Full list of author information is available at the end of the article

[2] and about 40% of infertility cases are identified as male factors [3, 4]. There are several factors responsible for the status of male infertility, including lifestyle (food habits, smoking, and alcohol consumption), environmental factors (exposure to relatively more temperature, pollution), and genetic factors [5].

Tobacco cigarette smoking is considered one of the lifestyle factors that contribute to the increased prevalence of male infertility and the reduction in semen parameters and sperm quality [6]. Cigarettes are made up of *Nicotiana tabacum* along with vaporized drugs and harmful substances, where cigarette smoke consists of carbon monoxide, tar, formaldehyde, nicotine, lead, cadmium, and many other substances [7, 8]. Previous studies reported that cigarette smoking impact negatively on spermatogenesis process and functions of spermatozoa [9, 10]. Additionally, several studies reported that the oxygen deficiency produced by cigarette smoking influences testicular function and impairs spermatogenesis, which ultimately leads to impaired sperm morphology, a decline in the progressive sperm, and increased sperm death [11–13]. In addition, several studies have shown that tobacco cigarette smoking-induced genetic alterations have an influence on men's fertility through chromosomal alterations [14], mutations [15], polymorphisms [16], DNA damage [17], and epigenetic alteration [18, 19]. Furthermore, other studies reported that tobacco smoking is considered as one of the lifestyles factors that influence the sperm DNA methylation patterns and global sperm DNA methylation and that may affect the sperm phenotype and males fecundity [20–22].

DNA methylation is an epigenetic mechanism that occurs at the 5th carbon atom of a cytosine that is followed by guanine residues (CpG) to form 5-methylcytosine (5mC) [23], and this process occurs by DNA methyltransferases (DNMTs) enzyme [24]. In humans, CpG dinucleotides can be found in clusters that have been termed CpG islands [25]; these CpG islands are found mostly in the promoter region and remain unmethylated, while the majority of all other CpG islands are methylated [26]. When CpG islands in promoter regions are methylated, the transcription of the corresponding gene is usually suppressed [27]. The methylation status of CpGs located in the promoter and intragenic regions plays a significant role in gene repression and activation, respectively [28]. Actually, DNA methylation is very necessary to facilitate the correct compaction of chromatin in the sperm head and to permanently silence the promoters of genes involved in genetic imprinting [29]. In humans, correct sperm DNA methylation is suggested to be essential for both fertilization and early fetus viability [30, 31].

Several studies have been shown a strong association between tobacco cigarette smoking and the alteration in the spermatozoa DNA methylation patterns [32–34]. Nevertheless, the influence of tobacco smoking on sperm DNA methylation and gene transcription level in sperm remains debatable. Therefore, this study was performed in order to (I) investigate whether tobacco cigarette heavy smoking impacts sperm DNA methylation patterns and semen parameters; (II) determine whether there is an alteration in the transcription level of *MAPK8IP3*, *GAA*, *ANXA2*, *PRRC2A*, and *PDE11A* genes in heavy smokers compared to non-smokers; and (III) investigate the association between the variation in the sperm DNA methylation patterns and the basic semen parameters.

Methods

Study population and participation criteria

The study population included two hundred and eighty adult males with a mean age of 33.8 ± 8.4 years old (120 non-smokers with proven fertile and 160 heavy smokers, who consume at least 25 cigarettes per day for 12 years, sub-fertile and they did not achieve pregnancy in the past 4 years). The exclusion criteria for participation in the study were as follows: consumption of alcohol, the diabetes mellitus, presence of anti-sperm antibodies, varicocele, males underwent a surgical operation in the reproductive system, Y chromosome microdeletions, abnormal hormonal parameters, abnormal body mass index, and males suffering from known infertility problems. On the other hand, the inclusion criteria were age between 20 and 48 years old, males have the same nationality, ethnicity, and food supplementation.

Samples collection and spermatozoa purification

Semen samples were collected by masturbation after 3 days of sexual abstinence. The samples were allowed to liquefy at 37 °C for 30 min, then the sperm count was assessed using the Meckler counting chamber (Sefi-Medica, Israel). The semen parameters were analyzed according to World Health Organization guidelines [35]. Before DNA extraction from human spermatozoa, somatic cells have been removed from all the semen samples through the use of somatic cell lysis buffer (SCLB) which is used widely for sperm cell purification. Briefly, the liquefied semen samples were loaded onto 45% over 90% discontinuous Puresperm gradient (Nidacon International AB, Sweden) and then centrifuged at 500g for 25 min at 25°C. After that, the pure spermatozoa were incubated with SCLB on ice for 30 min and washed three times with phosphate-buffered saline (PBS), and then centrifuged at 500g for 10 min [36]. Finally, the absence of somatic cell contamination was confirmed by microscopic examination.

Evaluation of sperm chromatin non-condensation

The chromomycin A3 (CMA3) staining was used to evaluate the chromatin non-condensation at human spermatozoa. Briefly, four semen smears were prepared from each sample and all smears were fixed by using a fixative solution (methanol-glacial acetic acid, 3:1 respectively) at 4°C for 20 min. The semen smears were air-dried at room temperature. After that, each smear was covered by 50 µl of staining solution (Sigma-Aldrich, USA) and then incubated in a dark place at room temperature for 20 min. The phosphate-buffered saline (PBS) was used to wash all the slides, then the slides were mounted with 1:1 (v/v) glycerol/PBS incubated overnight at 4°C. To evaluate the results of CMA3 staining, the fluorescence microscope (Zeiss Photomicroscope III, Germany) was used to analyze 300 spermatozoa on each smear. Finally, the CMA3 staining was evaluated by differentiating the spermatozoa that stained with bright yellow (positive, bad spermatozoa) from spermatozoa that stained with a dull yellow (negative, good spermatozoa) [37].

Evaluation of DNA fragmentation in human spermatozoa

The spermatozoa DNA fragmentation (apoptosis) was evaluated using the terminal deoxyribonucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assay. The TUNEL assay was performed by using the in situ cell death detection kit following the guidelines of the manufacturer company (Roche Diagnostics GmbH, Germany). Briefly, smears were prepared using 10 µl of sperm suspension on microscope slides and allowed to air-dry and then fixed with 4% paraformaldehyde phosphate-buffered saline, pH 7.4 for 2 h at room temperature, then rinsed with PBS. Smears were then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate, pH 6.0 for 15 min at room temperature; 50 µl of the TdT-labeled nucleotide mixture (50 µl of enzyme solution and 450 µl of label solution) was added to each slide and incubated in a humidified chamber at 37°C overnight in the dark. Negative controls without TdT enzyme were run in each replicate. Then, slides were rinsed two times in PBS and left to dry in the air followed by adding 25 µl of 5 µg/ml DAPI stain solution to each slide as a counterstain and then covered by coverslips. For evaluation, a total of 200 spermatozoa were analyzed on each slide, by distinguishing spermatozoa stained bright green (TUNEL positive, fragmented DNA) from those stained dull green (TUNEL negative, with intact DNA). A Zeiss Photomicroscope III was used for the fluorochrome evaluation (Zeiss Photomicroscope III, Germany) [38].

Extraction of nucleic acid from human spermatozoa

The nucleic acids (DNA and RNA) were isolated from the human spermatozoa by using Isolate II DNA/RNA/Protein Kit and all steps were performed according to the guidelines of the manufacturer company (Bioline, UK). The Nanodrop spectrophotometer-2000c (Thermo Scientific, USA) was used to evaluate the concentration and purity of extracted nucleic acids in order to ensure that the isolated nucleic acids are sufficient and suitable for global DNA methylation, deep bisulfite sequencing, and qPCR. To confirm the effectiveness of the protocol used in removing somatic cells from the semen samples that were entered in this study, the publicly available data (GEO # GSE41169) were used to define sample purity and based on a known significantly differentially methylated region (DMR) between somatic cells (White blood cell) and spermatozoa. We assessed DNA from whole blood, DNA from round cell-contaminated sperm samples, and known pure sperm DNA and compared these with sperm DNA of the study population. The data showed that the samples evaluated in this study were free from potential contamination resulting from the presence of immature sperm, white blood cells, or other somatic cells.

Evaluation of global DNA methylation in human spermatozoa

The MethylFlash™ Methylated DNA Quantification ELISA Kit was used to evaluate the level of global DNA methylation (5-methylcytosine) in the human spermatozoa; all the steps were performed according to the manufacturer's guidelines (Epigentek Group Inc, USA). Briefly, 100 ng of extracted DNA was incubated with the DNA binding buffer solution at 37°C for 90 min (blank, a positive, and negative control have been used in triplicate during this assay). After washing the microwell three times, the methylated DNA capture solution was added to each well and incubated for 60 min at room temperature. After that, the detection antibodies were added to each well and incubated at room temperature for 30 min. After three-time washing, the developing solution was added to each well and incubated at room temperature in the dark place for 10 min, and at the end of the 10 min, the stop solution was added. The microplate ELISA reader was used to assess the absorbance at 450 nm. The global DNA methylation status (ng) was calculated using the equation: 5-mC(ng) = [(sample OD - blank OD)/100].

Sodium bisulfite treatment

Four hundred nanograms of isolated spermatozoa DNA was treated with sodium bisulfite using the Epitect

bisulfite conversion kit (Qiagen, Germany) that converts unmethylated cytosines to uracil, while 5-methylcytosine (5MeC) remains unaltered, as described previously [39].

Screening study (infinium 450 K BeadChip array)

Thirty semen samples with a mean age of 34.0 years old (15 proven non-smokers and 15 heavy smokers) were used. After the bisulfite treatment process, the DNA was subjected to the Infinium 450K BeadChip array (Illumina, USA) following the manufacturer's recommendations [40], and the arrays were scanned using the Illumina iScan. Beta-values were then generated by analyzing the intensity of methylation or the absence of methylation at each CpG tile on the array using the calculation: Beta-value = methylated / (methylated + unmethylated). The Beta-value ranges from 0 to 1 and indicates the methylation level for each CpG. A value of 1 represents a completely methylated CpG and a score of 0 means a completely unmethylated CpG. Raw intensity values obtained from Illumina were used to generate Beta-values; the bioinformatic processing and evaluation were performed with the RNBEADS program package [41]. The methylation level at each CpG was considered as being differentially methylated CpG (DMC) when the absolute difference in the means of the average beta values between the two groups was $\geq 20\%$ with a Benjamini–Hochberg-corrected *t*-test FDR (false discovery rate) of 0.05. All CpGs that covered a common SNP site in the dbSNP137 database were excluded, and the findings were considered significant when $p \leq 0.01$. In the current study, and according to the results of the Infinium 450K BeadChip array, five CpG sites have the highest difference in methylation levels (cg07869343, cg05813498, cg09785377, cg06833981, and cg02745784), which are located in the *MAPK8IP3*, *GAA*, *ANXA2*, *PRRC2A*, and

PDE11A genes, respectively, were selected for further analysis using deep bisulfite sequencing according to the manufacturer's instructions [42].

Deep bisulfite sequencing

In this part of the study, 280 independent samples (120 proven non-smokers and 160 heavy smokers) with a mean age of 33.8 ± 8.4 years were used. Briefly, 400 ng of genomic DNA was bisulfite-treated using the Epiect bisulfite conversion kit (Qiagen, Germany). PCRs encompassing the differentially methylated CpG (DMC) identified by the 450K BeadChip array were performed in a 50- μ L total volume reaction using "MyTaqTM HS Red Mix" 2x concentrated (Bioline, UK) according to the manufacturer's protocol. For the amplification, fusion primers were used that consisted of a specific 30 portion and a universal 50 portion containing the necessary nucleotide sequences for Illumina sequencing (listed in Table 1). Primers were designed using the BiSearch primer design tool (<http://bisearch.enzim.hu/?m=search>). In this assay, 5 μ L of each PCR reaction was loaded onto a 2% agarose gel stained with ethidium bromide (New England Biolabs, USA). The PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter, USA) and measured using Quant-iTTM DNA Assay Kit (Fisher Scientific, USA) according to the manufacturer's recommendations, then diluted and pooled. Deep sequencing was performed on the Illumina MiSeq according to the manufacturer's protocols aiming at 10,000 reads per amplicon. All data obtained from the deep sequencing step were processed, filtered, and aligned using BiQ Analyzer HT software [43], excluding all reads containing $\geq 10\%$ of missing CpG sites (maximal fraction of unrecognized sites ≥ 0.1). The obtained alignment sequences showed an absence of alterations at CpG positions (no SNPs were detected).

Table 1 Primer sequences, number of differentially methylated CpGs (DMCs), and PCR annealing temperatures used to amplify regions including the target CpGs analyzed by deep bisulfite sequencing

cgID	Chr.	nt (hg19)	AT (°C)	Product size (bp)	DMC	Primer sequence (5'–3')	
cg07869343	chr16	1797050	52	334pb	22	F	GAGGTAAGTGTAAGTATT
						R	CTAAATAACCTATACTTCCA
cg05813498	chr17	78093353	55	268pb	10	F	AGTTGGGTATTAATTATTTTAA
						R	AACTCAAATACATACCCAC
cg09785377	chr15	60644157	52	210pb	4	F	TGAGGAAAAATAATAAGAGT
						R	CTAAACAATACCATTCAAAC
cg06833981	chr6	31597708	54	206pb	3	F	TGATGATTTTTTTTATGTGGATTT
						R	ACCCTCCCTCATCACTATAA
cg02745784	chr2	178970365	52	398pb	1	F	AAAATGATGTAAAAATAAAA
						R	TTTATATCAAATCCATCCC

Chr. Chromosome, At Annealing temperature, bp Base pair, F Forward, R Reverse

Reverse transcription and qPCR

The isolated RNA from spermatozoa was converted to complementary DNA (cDNA) in a 30 µl reaction volume by using the miScript reverse transcription kit and all procured following the manufacturer’s guidelines (Qiagen, Germany). The transcription level of *MAPK8IP3*, *GAA*, *ANXA2*, *PRRC2A*, and *PDE11A* and the house-keeping gene as a reference gene (*GAPDH*) were evaluated by using the qPCR instrument (7500 Fast applied Bio-systems, USA). The cDNA was used as a template, and all the primers included in this study (QuantiTect Primer) were used according to the guidelines of the manufacturer company (Qiagen, Germany). The reverse transcriptase control (NRT) and template control (NTC) were not involved in runs. All samples were analyzed in triplicate, and all the values of C_t were normalized to *GAPDH*.

Data analysis

The data obtained from this study were analyzed using SPSS version 24.0 (SPSS Inc., USA). Data included in this study were non-normally distributed (nonparametric) according to the values of the skewness test, Kurtosis test, Z-value, and Shapiro test. The independent-sample U-test (Mann–Whitney test) was used to compare the quantitative variables between the study groups. Spearman’s rank correlation coefficient was applied to assess the correlation between the variation in sperm DNA methylation patterns and other investigated parameters. The comparative ΔC_t method was used separately to calculate the relative RNA quantity in all samples. The ΔC_t was calculated by subtracting the C_t values of *GAPDH* from the C_t values of the target RNA, where $\Delta C_t = [C_t \text{ NA of target RNA}] - [C_t \text{ RNA of GAPDH}]$. Then, the $\Delta\Delta C_t$ was calculated by subtracting the mean ΔC_t of the non-smokers from the ΔC_t of the heavy smoker ($\Delta\Delta C_t =$

ΔC_t of heavy smokers – ΔC_t of non-smokers). The fold change of transcription level was calculated by using the following equation: $2^{-\Delta\Delta C_t}$ equation [44]. All the results of the abovementioned tests were considered statistically significant when $P < 0.05$.

Results

Descriptive characteristics of heavy smokers compared to non-smokers

Table 2 illustrates the locations of the most differentially methylated CpGs (DMC), based on the DNA methylation difference of > 20% between the heavy smokers and non-smokers groups. Five CpG positions following the applied criteria (cg07869343, cg05813498, cg09785377, cg06833981, and cg02745784) were selected to validate the observed methylation difference in 280 samples. All of these CpGs located in gene bodies and CpG islands and these genes were found to be related to male fertility, sperm concentration, and sperm function.

Table 3 summarizes the descriptive characteristics of the study population. The mean age of males included in this study was 33.8 ± 8.4 years old. The study population was divided into two groups: a heavy smokers group was composed of 160 males with an average age of 33.1 ± 8.4 years old, and the non-smoker’s group was composed of 120 males with an average age of 34.7 ± 8.4 years old. A significant reduction has been found in sperm concentration, percentage of total sperm motility, and sperm progressive motility in heavy smokers compared to non-smokers ($P < 0.001$). In contrast, a significant increase has been observed in the percentage of sperm non-progressive motility, non-motile sperm, and sperm abnormal form ($P < 0.001$). A significant increase has been found in the level of sperm chromatin non-condensation, sperm

Table 2 Location of CpG dinucleotides that differ in their spermatozoa DNA methylation levels in heavy smokers compared to non-smokers (n= 30)

CgID	Chromosome	Start	Strand	Heavy smokers	Non-smokers	Mean difference	Difference methylation P-value	Annotation
cg07869343	Chr16	1797050	+	0.9297	0.3092	0.6205	3.00E–06	MAPK8IP3
cg05813498	Chr17	78093353	+	0.9061	0.3351	0.571	3.04E–06	GAA
cg09785377	Chr15	60644157	+	0.8854	0.2956	0.5898	2.99E–06	ANXA2
cg06833981	Chr6	31597708	+	0.9362	0.2943	0.6419	3.04E–06	PRRC2A
cg02745784	Chr2	178970365	–	0.9137	0.3245	0.5892	3.02E–06	PDE11A
cg09737095	chr11	128787688	–	0.5024	0.1092	0.3932	7.83862E–07	KCNJ5
cg14791502	chr7	4243368	+	0.9339	0.5624	0.3715	1.56812E–06	SDK1
cg26303777	chr1	230311676	–	0.9107	0.5488	0.3619	1.47551E–06	GALNT2
cg14230280	chr9	132502800	–	0.8887	0.5144	0.3743	1.97437E–06	PTGES
cg00545199	chr4	2305515	–	0.8851	0.5807	0.3044	3.40579E–06	ZFYVE28

Table 3 Descriptive characteristics of heavy smokers compared to non-smokers (n = 280)

Variables	Heavy smoker (n = 160)	Non-smoker (n = 120)	P-value
	Mean ± SD	Mean ± SD	
Male age (years)	33.1 ± 8.4	34.7 ± 8.4	0.115
Semen volume (ml)	3.58 ± 1.31	4.17 ± 2.17	0.178
Sperm concentration (Mill/ml)	17.29 ± 14.97	87.67 ± 70.68	< 0.001
Total sperm motility (%)	35.74 ± 20.16	54.27 ± 17.76	< 0.001
Sperm progressive motility (%)	14.60 ± 11.36	39.99 ± 18.68	< 0.001
Sperm non-progressive motility (%)	21.78 ± 13.99	13.33 ± 8.06	< 0.001
Non-motile sperm (%)	63.72 ± 20.60	46.68 ± 19.37	< 0.001
Sperm abnormal form (%)	93.52 ± 5.47	61.67 ± 25.62	< 0.001

All values are expressed as mean ± SD; SD standard deviation; P ≥ 0.05, not significant; P < 0.05, significant

DNA fragmentation (Fig. 1), and sperm global DNA methylation (Fig. 2) in heavy smokers compared to non-smokers (P < 0.001).

Correlation between semen parameters and other parameters in the heavy smokers

As represented in Table 4, a significant negative association was found between the sperm concentration and the age of heavy smokers (r = -0.232, P = 0.003). Conversely, a positive correlation was found between the sperm concentration and the level of sperm chromatin non-condensation (r = 0.183, P = 0.021). However, no significant correlations were found between sperm DNA fragmentation, global DNA methylation status, and the basic semen parameters in heavy smokers have been found.

Deep bisulfite sequencing results

The results revealed that not only the target CpGs obtained from the 450K bead array experiments showed a difference in the methylation level, but also the neighboring CpGs. A significant increase in the methylation level was found in heavy smokers compared to non-smokers at seventeen of twenty-two tested CpGs tested within the *MAPK8Ip3* gene-related amplicon CpG1, CpG2, CpG4, CpG5, CpG6, CpG7, CpG8, CpG10, CpG12, CpG13, CpG14, CpG15, CpG16, CpG18, CpG20, CpG21, and CpG22 (P < 0.001, P = 0.004, P = 0.001, P < 0.001, P = 0.025, P = 0.002, P < 0.001, P = 0.001, P < 0.001, P = 0.002, P = 0.001, P = 0.001, P = 0.002, P = 0.031, P < 0.001, P < 0.001, and P = 0.001, respectively) (Fig. 3A–E). Similarly, a significant increase was found at seven of ten tested CpGs within the *GAA*

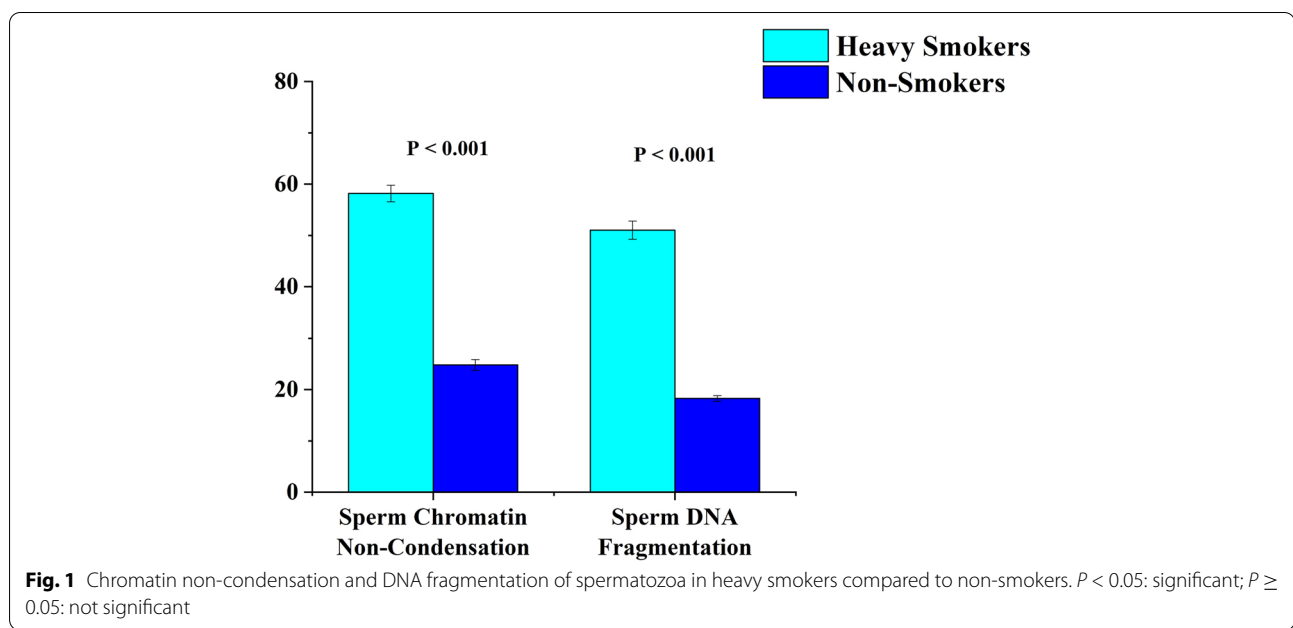


Fig. 1 Chromatin non-condensation and DNA fragmentation of spermatozoa in heavy smokers compared to non-smokers. P < 0.05: significant; P ≥ 0.05: not significant

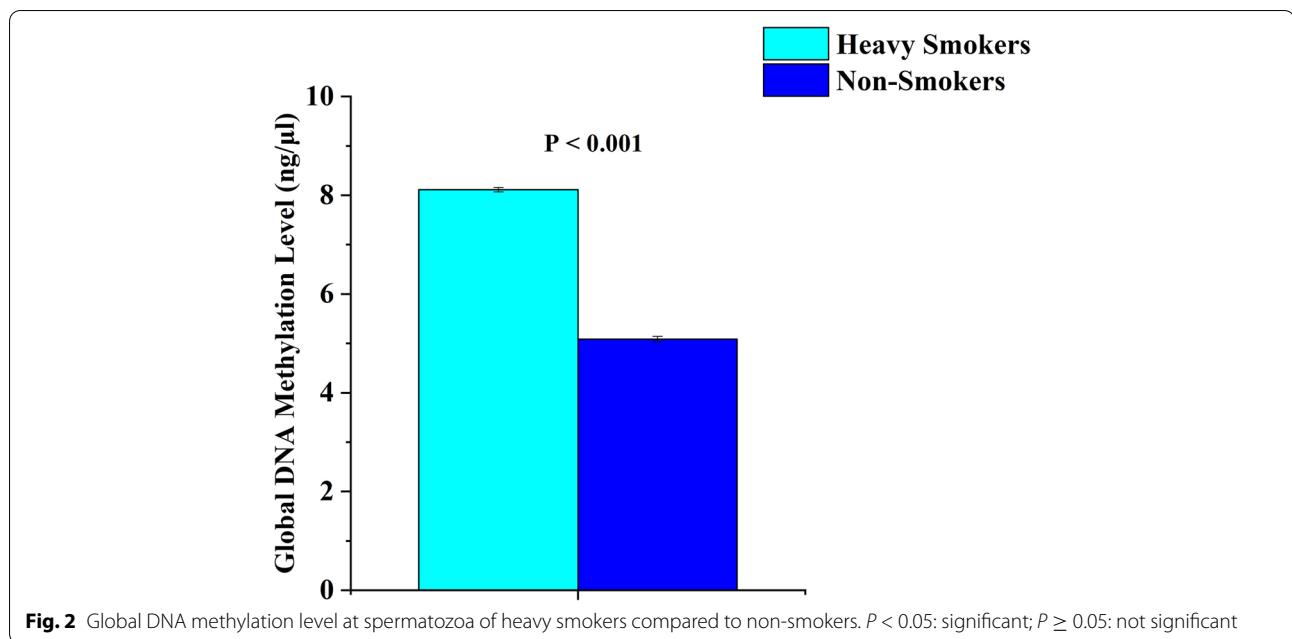


Fig. 2 Global DNA methylation level at spermatozoa of heavy smokers compared to non-smokers. $P < 0.05$: significant; $P \geq 0.05$: not significant

Table 4 Association between the semen parameters and other investigated parameters in the heavy smokers ($n=160$)

Variables	Male age (years)		Sperm chromatin non-condensation		Sperm DNA fragmentation		Global DNA methylation level	
	<i>R</i>	<i>P</i> -value	<i>r</i>	<i>P</i> -value	<i>r</i>	<i>P</i> -value	<i>r</i>	<i>P</i> -value
Semen volume (ml)	-0.114	0.151	-0.082	0.304	-0.060	0.453	0.082	0.300
Sperm concentration (Mill/ml)	-0.232	0.003	0.183	0.021	0.145	0.068	-0.066	0.405
Total sperm motility (%)	0.004	0.957	0.118	0.139	0.031	0.697	-0.098	0.216
Sperm progressive motility (%)	0.099	0.214	0.077	0.333	-0.071	0.376	-0.046	0.567
Sperm non-progressive motility (%)	-0.077	0.335	0.083	0.299	0.078	0.329	-0.066	0.404
Non-motile sperm (%)	0.004	0.960	-0.094	0.236	-0.013	0.874	0.085	0.285
Sperm abnormal form (%)	0.024	0.767	0.121	0.126	-0.103	0.194	0.028	0.723

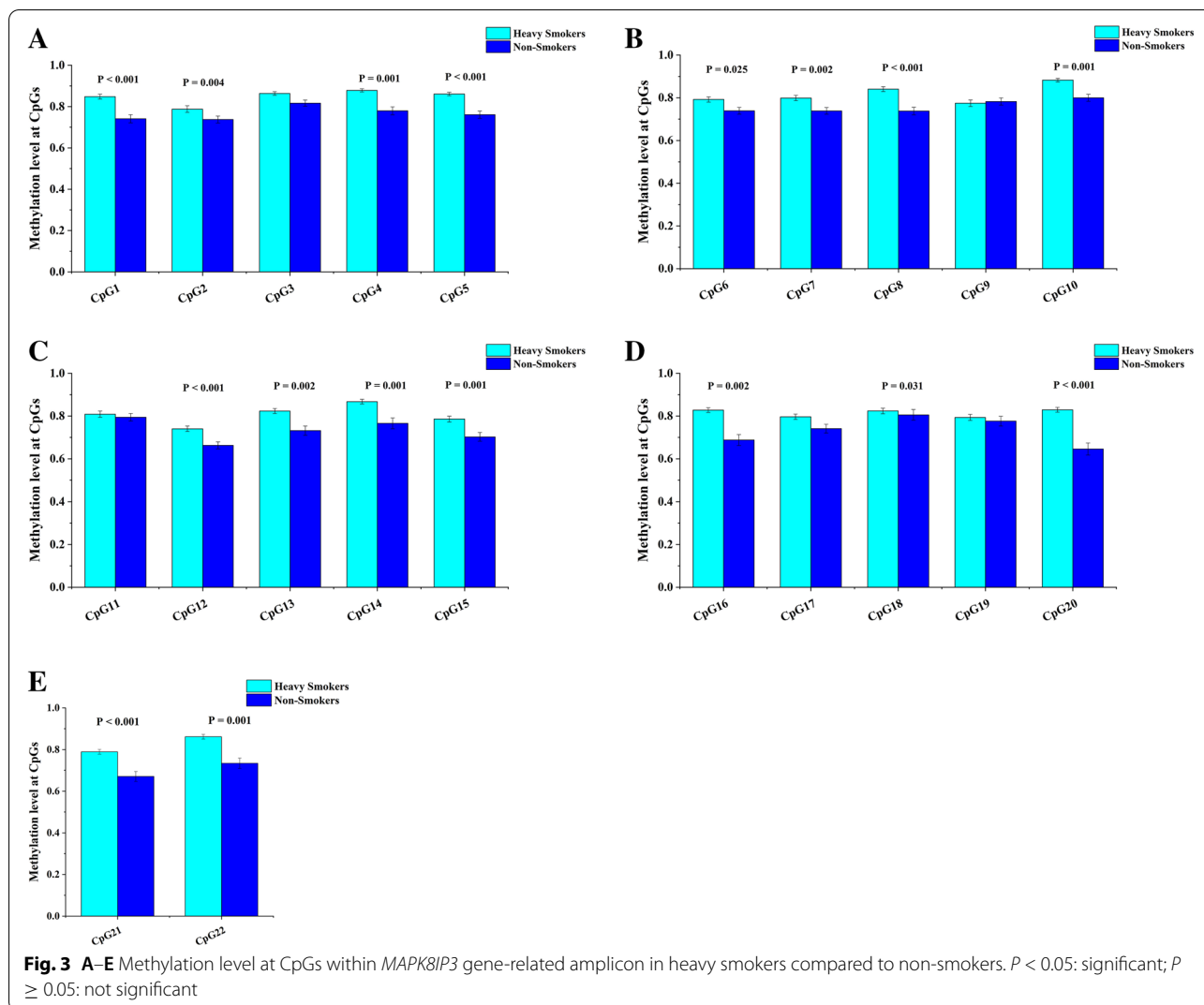
Spearman's test; *r*, correlation coefficient; $P \geq 0.05$, not significant; $P < 0.05$, significant

gene-related amplicon CpG1, CpG2, CpG5, CpG6, CpG7, CpG8, and CpG10 ($P < 0.001$, $P < 0.001$, $P < 0.001$, $P = 0.002$, $P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively) in heavy smokers compared to non-smokers (Fig. 4A, B). The DNA methylation levels at three out of four CpGs related to the ANXA2 gene amplicon (CpG1, CpG2, and CpG4) showed a significant difference in the heavy smokers compared to the non-smokers ($P = 0.002$, $P = 0.002$, and $P = 0.005$, respectively) (Fig. 5). Additionally, the results showed that all CpGs related to the PRRC2A gene amplicon (CpG1, CpG2, and CpG3) had a significant difference in the methylation level (0.001, 0.004, and 0.001 respectively) in heavy smokers compared to non-smokers (Fig. 6). Besides that, all CpGs related

to the PDE11A gene amplicon (CpG1, $P = 0.002$) showed a significant difference in the heavy smokers compared to non-smokers (Fig. 7).

Transcription level of studied genes

This study showed a significant reduction in the transcription levels of MAPK8IP3, GAA, ANXA2, and PRRC2A genes ($P = 0.002$, $P < 0.001$, $P = 0.005$, and $P < 0.001$, respectively) in heavy smokers compared to non-smokers (Fig. 8). Additionally, the results found upregulation in the MAPK8IP3, ANXA2, and PRRC2A transcription levels with fold change 1.43, 4.58, and 2.52, respectively in heavy smokers compared to non-smokers. Conversely, downregulation has been found in the GAA gene transcription level with a fold change



of 0.10 in heavy smokers compared to non-smokers (Table 5). No significant difference between heavy smokers and nonsmokers male in the transcription level of PDE11A ($P = 0.099$).

Correlation between methylation levels at different CpGs and basic semen parameters in the heavy smokers

As illustrated in Table 6, a significant negative correlation was found between the methylation levels at CpG3, CpG4, CpG5, CpG9, CpG11, CpG12, CpG13, CpG16, CpG21, and CpG22 within the *MAPK8IP3* gene-related amplicon and sperm concentration ($P = 0.006$, $P = 0.001$, $P = 0.002$, $P = 0.012$, $P = 0.005$, $P = 0.034$, $P = 0.005$, $P = 0.005$, $P = 0.002$, and $P = 0.005$, respectively). In addition, a significant negative correlation was found between percentage of total sperm motility, sperm non-progressive motility, and the methylation levels at CpG3 in the *GAA* gene-related amplicon (P

$= 0.039$ and $P = 0.017$, respectively). Likewise, a significant negative correlation was found between the methylation in the (CpG9 within *GAA* gene-related amplicon, CpG3 within *ANXA2* gene-related amplicon, CpG1 within *PRRC2A* gene-related amplicon) and percentage of sperm progressive motility ($P = 0.017$, $P = 0.008$, and $P = 0.025$, respectively). In contrast, a significant positive correlation was found between the methylation level at the CpG3 within *ANXA2* gene-related amplicon, CpG10 within *GAA* gene-related amplicon and sperm concentration ($r = 0.163$, $P = 0.040$; $r = 0.208$, $P = 0.008$, respectively). Moreover, a significant positive correlation has been found between the methylation level at CpG1 ($r = 0.184$; $P = 0.020$), CpG19 ($r = 0.172$; $P = 0.029$), and CpG21 ($r = 0.157$, $P = 0.048$) within *MAPK8IP3* gene-related amplicon, and percentage of sperm abnormal form.

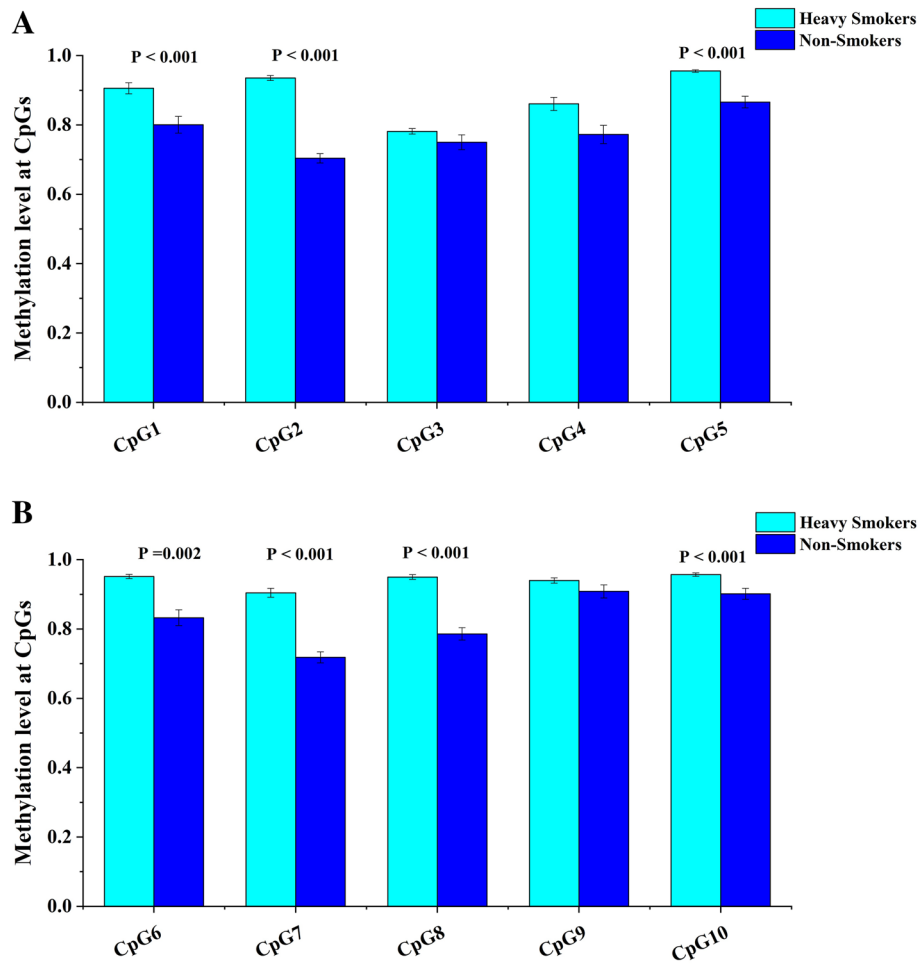


Fig. 4 A, B Methylation level at CpGs within *GAA* gene-related amplicon in heavy smokers compared to non-smokers. $P < 0.05$: significant; $P \geq 0.05$: not significant

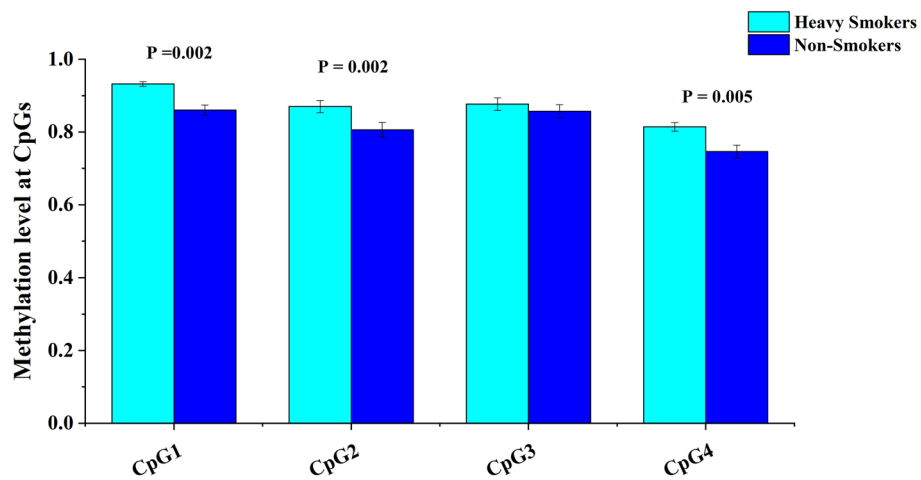


Fig. 5 Methylation level at CpGs within *ANXA2* gene-related amplicon in heavy smokers compared to non-smokers. $P < 0.05$: significant; $P \geq 0.05$: not significant

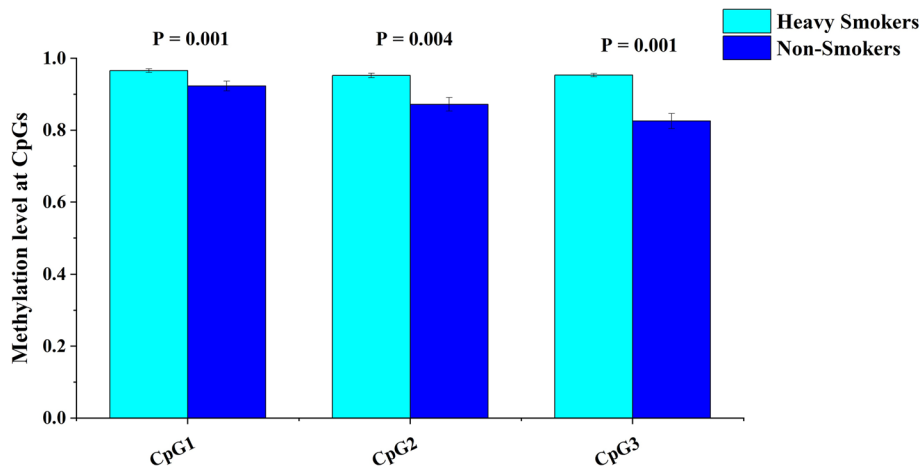


Fig. 6 Methylation level at CpGs within *PRRC2A* gene-related amplicon in heavy smokers compared to non-smokers. $P < 0.05$: significant; $P \geq 0.05$: not significant

Discussion

Tobacco cigarette consumption is prevalent in more than a third of the world population [45]. Tobacco smoke contains around 4700 chemical compounds and several hazardous substances which are inhaled by smokers, which have harmful impacts on human germ cells [46]. Previous studies noted an association between a quality deterioration of semen including (sperm motility, sperm

concentration, and sperm morphology), and smoking cigarettes [13, 47].

This study evaluated the methylation levels at differentially methylated CpGs (DMC) within the *MAPK8IP3*, *GAA*, *ANXA2*, *PRRC2A*, and *PDE11A*, gene-related amplicon in human spermatozoa obtained from heavy smokers compared to a proven fertile nonsmoker. In spermatozoa, DNA methylation plays a necessary role

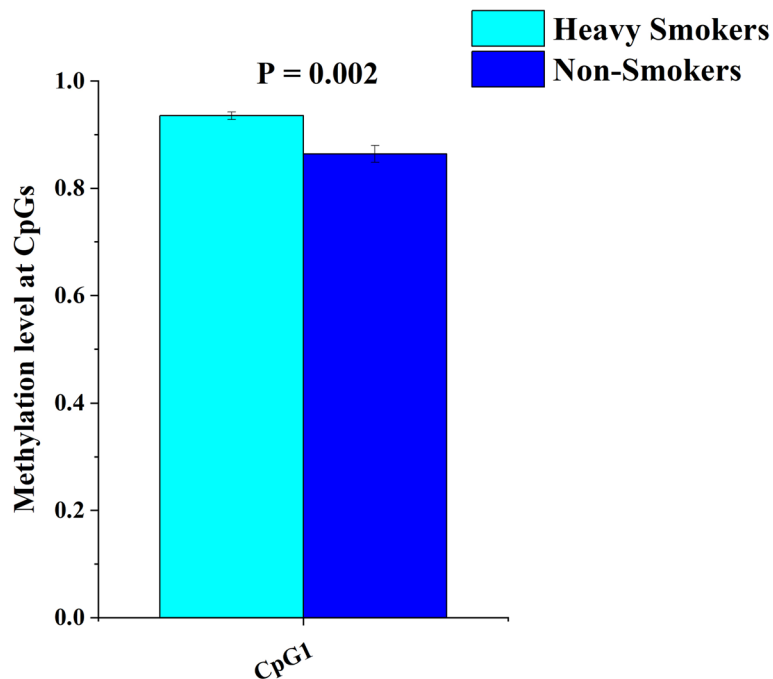


Fig. 7 Methylation level at CpGs within *PDE11A* gene-related amplicon in heavy smokers compared to non-smokers. $P < 0.05$: significant; $P \geq 0.05$: not significant

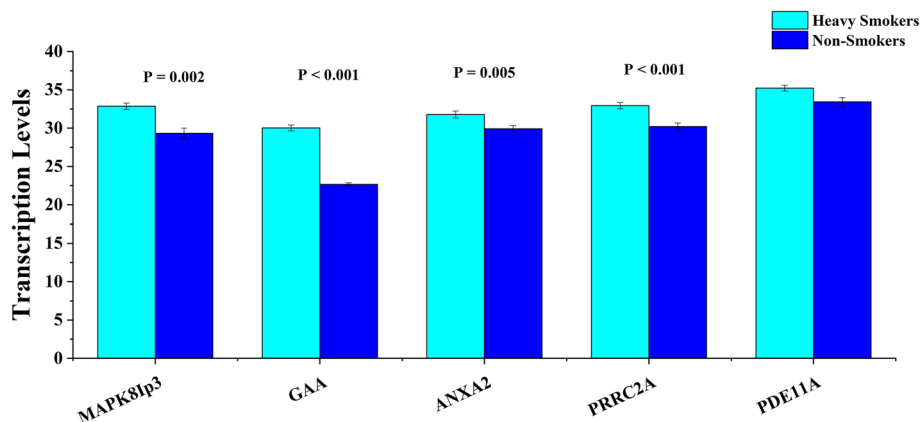


Fig. 8 Transcription level of *MAPK8IP3*, *GAA*, *ANXA2*, *PRRC2A*, and *PDE11A* genes in spermatozoa of heavy smokers compared to non-smokers. $P < 0.05$: significant; $P \geq 0.05$: not significant. C_t levels are inversely proportional to the amount of target nucleic acid in the sample

in the proper spermatogenesis process [48], and several studies have observed that the decline in the reproductive potential of men and the defects in spermatogenesis could be caused by abnormal methylation in genes expressed in the testes [49, 50]. The results obtained from the validation study showed a significant increase in the methylation levels at seventeen of twenty-two tested CpGs within the *MAPK8IP3* gene-related amplicon (CpG1, CpG2, CpG4, CpG5, CpG6, CpG7, CpG8, CpG10, CpG12, CpG13, CpG14, CpG15, CpG16, CpG18, CpG20, CpG21, and CpG22) and at seven of ten tested CpGs within the *GAA* gene-related amplicon (CpG1, CpG2, CpG5, CpG6, CpG7, CpG8, and CpG10). Besides there is a significant increase at three out of four CpGs related to the *ANXA2* gene amplicon and all CpGs related to the *PRRC2A* and *PDE11A* genes amplicon. These findings are in agreement with previous studies that showed that the methylation levels in sperm DNA tend to be altered in heavy smokers compared to non-smokers males [51, 52]. In addition, several studies have shown a significant association between tobacco cigarette smoking and the alteration in DNA methylation level at multiple CpGs in human spermatozoa [32, 53]. Another study observes a significant difference in methylation level of 11 CpGs

in spermatozoa of current smokers compared to never smokers [54]. It is worth mentioning that the glucosidase alpha acid, encoded by the glucosidase alpha acid (*GAA*) gene, plays an essential role in the degradation of glycogen to glucose in lysosomes and that any defects in this gene lead to glycogen storage disease II “Pompe’s disease” [55], whereas the lysosomes found in the spermatozoa acrosome contain protease and hyaluronidase along with acid phosphatase, which plays a critical role in the maturation of spermatozoa and fertilization process (provided by RefSeq, Jul 2008, NCBI website). Besides, it should also be noted that members of the PDE protein superfamily, that is, phosphodiesterase 11A (*PDE11A*), play important roles in the control of cyclic nucleotide signaling [56]. Other studies reported that the expression of this gene was prominent in the prostate and testes [57, 58]. Another study founded that strong immune signals were obtained for *PDE11A* in the acrosomal cap and flagella of spermatozoa [59]. Additionally, the Annexin A2 (*ANXA2*) gene encodes a member of the Annexin family, a family of Ca^{2+} -regulated phospholipid-binding and membrane-binding proteins that are found in the acrosome and flagellum of the spermatozoa and could be implicated in different events that are known to be

Table 5 Transcription level of *MAPK8IP3*, *GAA*, *ANXA2*, and *PRRC2A* genes and fold change in spermatozoa of heavy smokers compared to non-smokers ($n = 280$)

Genes	Heavy smokers (ΔC_t)	Non-smokers (ΔC_t)	$\Delta \Delta C_t$	Fold change	Regulation
<i>MAPK8IP3</i>	3.47	3.988	-0.518	1.43	Up
<i>GAA</i>	0.639	-2.678	3.317	0.10	Down
<i>ANXA2</i>	2.397	4.592	-2.195	4.58	Up
<i>PRRC2A</i>	3.551	4.887	-1.336	2.52	Up
<i>PDE11A</i>	5.836	8.097	-2.261	4.79	Up

C_t , Cycle threshold

Table 6 Association between the methylation levels at different CpGs and basic semen parameters in the heavy smokers (n = 160)

DMC		Semen volume (ml)	Sperm concentration (Mill/ml)	Total sperm motility (%)	Sperm progressive motility (%)	Sperm non-progressive motility (%)	Non-motile sperm (%)	Sperm abnormal form (%)
Methylation level at CpGs in MAPK8ip3 gene-related amplicon								
CpG1	<i>r</i>	-0.108	0.040	0.111	0.081	0.080	-0.104	0.184
	<i>P</i> -value	0.175	0.617	0.162	0.307	0.316	0.192	0.020
CpG2	<i>r</i>	-0.113	-0.131	0.032	0.109	-0.019	-0.054	0.094
	<i>P</i> -value	0.156	0.099	0.686	0.170	0.815	0.500	0.239
CpG3	<i>r</i>	-0.197	-0.216	0.059	0.186	-0.034	-0.075	0.014
	<i>P</i> -value	0.013	0.006	0.462	0.018	0.669	0.343	0.856
CpG4	<i>r</i>	-0.144	-0.254	0.023	0.147	-0.060	-0.017	-0.029
	<i>P</i> -value	0.069	0.001	0.769	0.064	0.449	0.832	0.713
CpG5	<i>r</i>	-0.011	-0.240	-0.041	0.095	-0.134	0.038	-0.143
	<i>P</i> -value	0.887	0.002	0.607	0.232	0.091	0.633	0.072
CpG6	<i>r</i>	0.042	-0.098	0.010	0.059	-0.020	-0.028	0.070
	<i>P</i> -value	0.600	0.216	0.897	0.460	0.800	0.728	0.378
CpG7	<i>r</i>	-0.046	-0.093	0.048	0.088	-0.006	-0.064	-0.042
	<i>P</i> -value	0.561	0.241	0.549	0.268	0.937	0.423	0.597
CpG8	<i>r</i>	-0.005	-0.050	0.020	0.016	0.004	-0.021	0.075
	<i>P</i> -value	0.949	0.530	0.803	0.837	0.958	0.790	0.343
CpG9	<i>r</i>	-0.097	-0.198	0.050	0.118	0.005	-0.065	0.062
	<i>P</i> -value	0.225	0.012	0.533	0.136	0.952	0.414	0.438
CpG10	<i>r</i>	-0.079	-0.095	0.035	0.129	-0.060	-0.035	0.103
	<i>P</i> -value	0.320	0.233	0.658	0.103	0.449	0.659	0.194
CpG11	<i>r</i>	-0.142	-0.220	0.032	0.155	-0.046	-0.054	0.050
	<i>P</i> -value	0.072	0.005	0.691	0.051	0.562	0.494	0.531
CpG12	<i>r</i>	-0.125	-0.167	0.000	0.116	-0.077	-0.011	0.039
	<i>P</i> -value	0.116	0.034	1.000	0.145	0.335	0.894	0.622
CpG13	<i>r</i>	-0.056	-0.222	-0.009	0.069	-0.056	-0.017	0.083
	<i>P</i> -value	0.480	0.005	0.912	0.389	0.484	0.829	0.299
CpG14	<i>r</i>	-0.115	-0.068	0.048	0.074	0.001	-0.032	-0.094
	<i>P</i> -value	0.146	0.395	0.549	0.354	0.992	0.686	0.235
CpG15	<i>r</i>	-0.095	-0.103	0.070	0.140	0.006	-0.078	0.144
	<i>P</i> -value	0.233	0.194	0.379	0.077	0.938	0.329	0.069
CpG16	<i>r</i>	-0.010	-0.219	-0.105	0.038	-0.158	0.082	-0.031
	<i>P</i> -value	0.904	0.005	0.185	0.632	0.046	0.300	0.696
CpG17	<i>r</i>	-0.043	-0.105	0.097	0.188	-0.022	-0.100	0.102
	<i>P</i> -value	0.593	0.186	0.222	0.017	0.784	0.207	0.199
CpG18	<i>r</i>	-0.148	-0.100	-0.022	0.015	-0.048	0.015	0.137
	<i>P</i> -value	0.062	0.209	0.778	0.851	0.546	0.852	0.085
CpG19	<i>r</i>	-0.016	-0.139	0.094	0.163	0.004	-0.087	0.172
	<i>P</i> -value	0.837	0.079	0.239	0.039	0.957	0.274	0.029
CpG20	<i>r</i>	-0.015	-0.134	-0.046	0.052	-0.082	0.052	0.132
	<i>P</i> -value	0.853	0.091	0.565	0.510	0.301	0.518	0.097
CpG21	<i>r</i>	-0.081	-0.245	0.026	0.080	-0.012	-0.048	0.157
	<i>P</i> -value	0.307	0.002	0.742	0.315	0.877	0.545	0.048
CpG22	<i>r</i>	-0.137	-0.219	-0.173	-0.008	-0.199	0.153	-0.047
	<i>P</i> -value	0.085	0.005	0.028	0.922	0.011	0.054	0.557
Methylation level at CpGs in GAA gene-related amplicon								
CpG1	<i>r</i>	-0.010	-0.111	0.115	0.097	0.067	-0.098	0.075
	<i>P</i> -value	0.904	0.164	0.147	0.221	0.396	0.220	0.343

Table 6 (continued)

DMC		Semen volume (ml)	Sperm concentration (Mill/ml)	Total sperm motility (%)	Sperm progressive motility (%)	Sperm non-progressive motility (%)	Non-motile sperm (%)	Sperm abnormal form (%)
CpG2	<i>r</i>	−0.001	0.042	−0.042	−0.112	−0.028	0.068	−0.046
	<i>P</i> -value	0.985	0.598	0.601	0.160	0.728	0.392	0.563
CpG3	<i>r</i>	−0.009	−0.097	−0.163	−0.121	−0.189	0.181	0.005
	<i>P</i> -value	0.912	0.221	0.039	0.129	0.017	0.022	0.946
CpG4	<i>r</i>	−0.199	0.008	0.024	0.041	0.019	−0.032	0.086
	<i>P</i> -value	0.012	0.923	0.759	0.605	0.807	0.685	0.282
CpG5	<i>r</i>	0.021	0.121	0.039	−0.143	0.134	−0.018	−0.026
	<i>P</i> -value	0.788	0.127	0.622	0.070	0.092	0.826	0.744
CpG6	<i>r</i>	0.109	0.075	0.006	−0.093	0.098	−0.017	−0.067
	<i>P</i> -value	0.170	0.346	0.939	0.243	0.218	0.834	0.400
CpG7	<i>r</i>	0.167	0.119	0.020	−0.148	0.117	0.004	−0.054
	<i>P</i> -value	0.035	0.133	0.800	0.062	0.142	0.959	0.500
CpG8	<i>r</i>	0.065	0.169	0.005	−0.080	0.024	0.025	−0.131
	<i>P</i> -value	0.413	0.032	0.952	0.316	0.766	0.756	0.100
CpG9	<i>r</i>	−0.074	0.116	−0.083	−0.188	0.045	0.080	0.037
	<i>P</i> -value	0.349	0.143	0.299	0.017	0.574	0.316	0.641
CpG10	<i>r</i>	0.047	0.163	−0.041	−0.109	−0.009	0.067	−0.114
	<i>P</i> -value	0.557	0.040	0.610	0.169	0.914	0.398	0.149
Methylation level at CpGs in ANXA2 gene-related amplicon								
CpG1	<i>r</i>	0.051	0.062	0.114	0.089	0.159	−0.134	0.045
	<i>P</i> -value	0.519	0.435	0.152	0.265	0.044	0.092	0.575
CpG2	<i>r</i>	0.003	0.016	0.029	−0.081	0.104	−0.004	0.054
	<i>P</i> -value	0.966	0.843	0.712	0.311	0.192	0.963	0.496
CpG3	<i>r</i>	0.084	0.208	−0.112	−0.208	0.025	0.104	−0.019
	<i>P</i> -value	0.293	0.008	0.157	0.008	0.751	0.193	0.810
CpG4	<i>r</i>	−0.110	−0.058	−0.017	0.044	−0.043	−0.003	0.106
	<i>P</i> -value	0.167	0.466	0.831	0.585	0.586	0.970	0.183
Methylation level at CpGs in PRRC2A gene-related amplicon								
CpG1	<i>r</i>	−0.043	0.122	−0.116	−0.178	−0.093	0.139	−0.093
	<i>P</i> -value	0.587	0.123	0.144	0.025	0.241	0.079	0.240
CpG2	<i>r</i>	0.058	−0.029	−0.032	−0.085	0.010	0.033	−0.096
	<i>P</i> -value	0.465	0.715	0.691	0.287	0.898	0.681	0.229
CpG3	<i>r</i>	0.047	−0.036	−0.069	−0.128	−0.025	0.084	−0.094
	<i>P</i> -value	0.552	0.647	0.383	0.106	0.751	0.293	0.235
Methylation level at CpGs in PDE11A gene-related amplicon								
CpG1	<i>r</i>	−0.015	−0.143	−0.004	0.022	−0.012	0.005	−0.008
	<i>P</i> -value	0.846	0.072	0.958	0.778	0.878	0.950	0.922

Spearman's test; *r* Correlation coefficient, DMC Differentially methylated CpGs; *P* ≥ 0.05, not significant; *P* < 0.05, significant

calcium-dependent, such as flagella motility, acrosome reaction and fertilization [60]. Also, the protein encoded by the mitogen-activated protein kinase 8 interacting protein 3 (*MAPK8IP3*) gene is one of the *MAPK* family, which is found in the spermatozoa and was observed to be associated with a direct or indirect function in human sperm capacitation [61].

Several studies investigated the relationship between DNA methylation and cigarette smoking [62, 63], and these studies proposed that tobacco cigarette smoking could alter the sperm DNA methylation patterns through two mechanisms: the first mechanism is the recruitment of DNA methyltransferases after DNA damage resulting from smoking, and the second mechanism involves the

alterations in the transcription and expression of genes due to the influence of nicotine and hypoxia caused by carbon monoxide, a principal component of cigarette smoke [64]. The results of this study showed a significant increase in the level of global DNA methylation, sperm chromatin non-condensation, and spermatozoa DNA fragmentation in heavy smokers compared to non-smokers males; these findings are matching with a previous study which was performed by Hamad and his colleagues who have found an increase in the level of global sperm DNA methylation and sperm chromatin decondensation in smokers compared to non-smokers [22]. Other studies have found an increase in the rate of spermatozoa DNA fragmentation in smokers than in non-smokers [65, 66]; besides other studies showed a significant increase in the abnormal level of sperm chromatin condensation ($P \leq 0.001$) in smokers compared to nonsmokers [66, 67].

Regarding the semen parameters, the results found a significant reduction in sperm concentration, percentage of total sperm motility, sperm progressive motility, and sperm normal form in heavy smokers compared to non-smokers males. Conversely, a significant increase has been noted in the percentage of non-motile sperm in heavy smokers compared to non-smokers males, and these results are consistent with other studies reported the same variation in semen parameters in smoker compared to non-smokers [66, 68]. Nevertheless, these results are not consistent with previous studies that showed no significant differences between smokers and non-smokers males in the sperm count and percentage of total sperm motility [69, 70].

This study showed a significant reduction in the transcription levels of the following genes (*MAPK8IP3*, *GAA*, *ANXA2*, *PRRC2A*, and *PDE11A*) in heavy smokers compared to non-smokers. Furthermore, the results were found upregulation in the *MAPK8IP3*, *ANXA2*, *PRRC2A*, and *PDE11A* transcription levels in heavy smokers compared to non-smokers. In contrast, a downregulation has been found in the *GAA* gene transcription level in heavy smokers compared to non-smokers. These findings are matching with a previous study that showed an alteration in the gene expression in smokers compared to non-smokers; besides another study showed a significant decline in the transcription level of *PRM 1* and *PRM 2* in smokers' spermatozoa compared to non-smokers [71]. Another study reported that the smoking-related ROS might cause decreased in the transcription level of genes like *IκBα* and might be correlated with defective spermatogenesis [72]. All of these findings are in agreement with a hypothesis that reported that cigarette smoke may play a critical role in the alteration of

the transcription level of human genes found in spermatozoa by its influence on chromatin remodeling, global DNA methylation status, and DNA methylation at different CpG sites. Further, previous studies have reported that cigarette smoking leads to increased sperm DNA damage [73].

Finally, the study investigated the correlation between the alteration in DNA methylation level at tested CpGs and semen parameters. The results showed a negative significant correlation between the methylation levels at ten out of twenty-two within the *MAPK8IP3* gene-related amplicon and sperm concentration. Moreover, a negative significant correlation was found between the percentage of total sperm motility, sperm non-progressive motility, and the methylation levels at CpG3 in the *GAA* gene-related amplicon. Likewise, a negative significant correlation was found between the methylation in one CpGs within the *GAA*, *ANXA2*, *PRRC2A*, and the percentage of sperm progressive motility. These findings are in line with other studies that observed an association between the alterations in the sperm DNA methylation patterns and semen parameters of tobacco cigarette smokers [74, 75]. Another study found a strong association between reduced sperm count, sperm motility, sperm normal form, and cigarette consumption [45, 76]. Nevertheless, the associations observed in this study contradict with other studies that showed no association between sperm global DNA methylation level and sperm parameters of smokers [77].

Conclusion

This study clearly offers evidence that tobacco cigarette smoking can alter the DNA methylation level at several CpGs, the status of global DNA methylation, and transcription level of the following genes "*MAPK8IP3*, *GAA*, *ANXA2*, *PRRC2A*, and *PDE11A*" in human spermatozoa. Besides an association has been found between alterations in the methylation level at these CpGs and basic semen parameters in smokers, and these findings may affect negatively semen parameters and men's fertility.

Abbreviations

DNMTs: DNA methyltransferases; 5mC: 5-Methylcytosine; SCLB: Somatic cell lysis buffer; PBS: Phosphate-buffered saline; CMA3: Chromomycin A3; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; qPCR: Quantitative polymerase chain reaction; ELISA: Enzyme-Linked Immunosorbent Assay; DMC: Differentially methylated CpG; cDNA: Complementary DNA.

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Authors' contributions

M. M. L. collected and processed the samples. Additionally, sample analysis including DNA/RNA extraction, bisulfite treatment, PCR, PCR purification, and qPCR and data analysis were done by M. M. L., and he was a major contributor in writing the manuscript. M. M. Y. performed a review for data analysis, discussion preparation, and the writing of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations**Ethics approval and consent to participate**

This study was approved by the Health Research Council, Palestinian Territories (Reference No. PHRC/HC/13/14), and consent was provided according to the Declaration of Helsinki Committee. Besides, all participants signed an informed approval form to participate in this study. The samples were analyzed according to the guidelines and standard procedures of the Al Basma Fertility Center.

Consent for publication

Not applicable.

Competing interests

There is no potential conflict of interest relevant to this article.

Author details

¹Faculty of Health Sciences, Department of Medical Laboratory Sciences, Islamic University, Gaza, Palestinian Territories. ²Faculty of Medicine, Department of Human Physiology, Islamic University, Gaza, Palestinian Territories.

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