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Impact of heavy smoking on the expression levels of *PGAM5*, *PTPRN2* and *TYRO3* and its correlation to male infertility

Houda Amor¹, Yaser AlKhaled¹, Riffat Bibi², Mohamad Eid Hammadeh¹ and Peter Michael Janowski¹

¹ Department of Obstetrics, Gynecology and Reproductive Medicine, Saarland University clinic, Germany

² Department of Animal Sciences, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, Islamabad, Pakistan.

* Correspondence: houda.amor@uks.eu

Abstract: Smoking have been linked to male infertility by affecting the sperm epigenome and genome. In this study, we aimed to determine possible changes in the transcript levels of the *PTPRN2*, *PGAM5*, and *TYRO3* genes in heavy smokers compared with non-smokers; and to investigate their association with fundamental sperm parameters. One hundred eighteen sperm samples (63 heavy-smokers (G1) and 55 non-smokers (G2) were included in this study. Semen analysis was performed according to WHO guidelines. After total RNA extraction, RT-PCR was used to quantify the transcript levels of studied genes. In G1 a significant decrease in standard semen parameters in comparison to non-smokers has been shown ($p < 0.05$). Moreover, *PGAM5* and *PTPRN2* were differentially expressed ($P \leq 0.03$ and $P \leq 0.01$, respectively), and downregulated in spermatozoa of G1 compared to G2. In contrast, no difference was observed for *TYRO3* ($p \leq 0.3$). In G1, mRNA expression level of studied genes correlated negatively with motility, sperm count, normal form, vitality, and sperm membrane integrity ($p < 0.05$). Therefore, smoking may affect gene expression and male fertility by altering DNA methylation patterns in genes associated with fertility and sperm quality, including *PGAM5*, *PTPRN2*, and *TYRO3*.

Keywords: Male infertility; heavy smoking; transcript level; *PGAM5*; *PTPRN2*; *TYRO3*

1. Introduction

Nearly 15% of infertile couples worldwide are attributable to the male factor [1]. Several factors contribute to male infertility. Reproductive tract infections, genetic and anatomical disorders, and immunological and endocrine disorders are among these factors [2–4].

Lifestyle and environmental factors such as diet, smoking, exercise, and alcohol consumption are as important to our health as our genes. These factors cause changes that affect gene expression.

Gene expression is regulated in several ways in mammals. However, DNA methylation is the most common epigenetic signaling device cells use to lock or unlock genes. DNA methylation works by adding a methyl group at position 5 of cytosine, which is found in cytosine phosphate guanine dinucleotides "CpGs" [5]. Typically, the group is added to specific sites in the DNA where it blocks proteins that bind to the DNA to "read" the gene. This group can be removed through a process called demethylation. Typically, methylation turns genes "off" and demethylation turns genes "on" [5]. In recent decades, DNA methylation has received considerable research attention due to its importance in various cellular processes. DNA methylation plays a crucial role in regulating gene expression by regulating the transcription of DNA to RNA [6,7], and is involved in protecting the genome by inhibiting the movement of DNA transposable elements Integrity [8,9]. DNA methylation processes can also be altered by many other factors, such as genetics [10], and environmental factors, smoking being a typical example [11].

Many studies have investigated the relationship between male infertility and lifestyle or habits such as alcohol consumption, smoking, diet, environmental factors, age, and stress.

Several other factors, including age, stress, and lifestyle, such as obesity, smoking, and alcohol use, have been linked to male infertility [12–16].

Smoking increases harmful oxidants such as reactive oxygen species (ROS) in seminal plasma. This leads to an imbalance between antioxidants and oxidants known as oxidative stress. Oxidative stress can lead to damage to sperm DNA [14,17–19].

Studies have shown that sperm DNA damage may also be caused by the formation of DNA adducts associated with cigarette content [20,21].

Smoking affects the sperm epigenome and genome [22] and possibly the developing embryo [23,24].

In a previous study, after applying bisulfite sequencing, we found 15 CpG sites in the amplicon associated with the PGAM5 (phosphoglycerate mutase family member 5) gene and heavy smokers DNA methylation levels were significantly increased between non-smokers and non-smokers, and there was an amplicon linked to the PTPRN2 gene (protein tyrosine phosphatase, N2-type receptor) at nine CpG sites. On the contrary, the results showed that the DNA methylation levels of three CpG sites of the TYRO3 gene-associated amplicon (tyrosine protein kinase receptor) were significantly reduced in the case group compared with the control group [25].

Furthermore, this study showed a significant correlation between changes in sperm DNA methylation levels and standard sperm parameters in the case cohort [25].

Therefore, in this study, we aimed to determine possible changes in the transcript levels of the PTPRN2, PGAM5, and TYRO3 genes in heavy smokers compared with non-smokers; and to investigate their association with fundamental sperm parameters.

2. Materials and Methods

2.1. Semen sample collection

Before the beginning of this study, an institutional review board approval (No. PHRC/HC/13/14) was achieved by the Ethics Committee of Helsinki. Moreover, approval consent was taken from each participant enrolled in this study. The study was conducted in the laboratory of biochemistry and molecular biology of reproductive medicine, Department of Obstetrics and Gynecology at the University Hospital-Homburg /Saar, Saarland Germany.

All participant males were in the reproductive age group (25- 45 years old) and the following parameters were excluded: cases suffering from varicocele, anti-sperm antibodies, Y chromosome microdeletions, males subjected to surgical operation in the reproductive system, and heavy body mass index.

Patients were classified into two groups. The control group includes 55 proven fertile candidates who show no previous history of smoking, and the case group includes 63 fertile heavy smokers who smoked more than 20 cigarettes per day (in the last 3 years until their enrolment in this study). Semen samples were collected by masturbation after 3 to 5 days of sexual abstinence in clean, dry, sterile and leak-proof plastic containers in a collection room attached to the laboratory. Following liquefaction of semen samples at 37°C for 30 min to 1hour, samples were analyzed according to World Health Organization guidelines [26].

2.3. Spermatozoa purification and analysis

The sample was kept on the heating stage for 30 minutes for liquefaction at 37°C. After that, the sperm samples were evaluated according to the WHO laboratory manual to determine Sperm count, motility, morphology, vitality (eosin test) and, sperm membrane integrity (Hos test) [26].

Gradient centrifugation was used to purify samples. Briefly, each sample was treated by a discontinuous Puresperm gradients (40%–80%) (Nidacon International, Sweden). Then, to guaranty the elimination of somatic cells, the samples were washed with lysis buffer (0.1% SDS, 0.5% Triton X-100 in double-distilled water).

2.3.1. Assessment of Sperm Morphology

Sperm morphology was evaluated according to strict criteria as following:

Smears were prepared by spreading 20 μ l of ejaculate on a glass slide. After fixation, the slides were stained using Papanicolaou method. A total of 200 spermatozoa from each slide were evaluated under oil immersion at a magnification of 1,000 x using bright field illumination. At least 10 high power fields from different areas of the slide were estimated.

2.3.2. Assessment of Sperm Vitality (Eosin Test)

On a glass slide, 10 μ l of ejaculate was mixed with 10 μ l of 0.5% aqueous yellowish Eosin Y solution. The mixture covered with a cover slide, then evaluated after 3-5 min by distinguishing between the dead spermatozoa (Red stained) and the live spermatozoa (not stained). 200 spermatozoa from each slide were evaluated.

2.3.4. Assessment of the Sperm Membrane Integrity (Hypo-Osmotic Test (HOS))

The HOS test is a sperm vitality test that predicts sperm membrane integrity. For this test, 100 μ l of fresh ejaculate mixed with 1.0 ml of the hypo osmotic solution. Then, the mixture was incubated at 37°C for 30-60 minutes. Influx of the fluid due to hypo-osmotic stress causes the sperm tail to swell which indicates the presence of sperm having a functional and intact plasma membrane. A minimum of 200 swollen and/or not swollen spermatozoa were examined per slide under phase contrast microscope.

2.4. RNA Extraction and synthesis of the cDNA (Reverse transcription)

Total RNA was isolated from the purified semen samples according to a modified protocol of the Isolate II RNA/DNA/Protein Kit (Phenol-free) (Bioline, UK). The Nanodrop spectrophotometer ND-2000c (Thermo Scientific, USA) was used to measure the concentration and the purity of isolated RNA. The integrity of the isolated RNA was checked on an RNA Nano 6000 chip via an Agilent Bioanalyzer (Agilent technologies, Santa Clara, California, United States). The extracted RNA was stored at -80°C until the time of usage.

The reverse transcription procedure performed using miScript II RT Kit according to the standard protocol provided by the manufacturer (Cat No. 218161, Qiagen, Germany). Briefly, 300 ng extracted RNA were added to 4 μ l of 5x MiScriptHiLflex buffer, 2 μ l of nucleotide mix, and 2 μ l of reverse transcriptase mix which contains all the components required for the synthesis of the complementary DNA (cDNA), then RNA free water was added to complete the required volume. After that, the reaction buffer incubated at 37°C for 60 min, then at 95°C for 5 min to suppress the activity of the reverse transcriptase mix.

2.5. Real-Time quantitative PCR (qPCR)

After the synthesis of cDNA, real time qPCR performed to amplify and quantify the transcript level of each of the studied genes PGAM5, TYRO3, PTPRN2, and GAPDH (as endogenous control).

Briefly, the produced cDNA used as the template for the qPCR reaction mixture. This reaction was prepared using 2x quantiTect SYBR Green PCR Mix (Cat No. 204143, Qiagen, Germany) and QuantiTect primers assay for PGAM5, TYRO3, PTPRN2, and GAPDH (as reference gene) (Cat No. QT00079247, Qiagen, Germany) according to the recommendations of the manufacturer.

At the end, the prepared mixture distributed in triplicate for each sample into 96 well plate plate. The plate placed in StepOnePlus™ System (7500 Fast Applied Biosystems, USA) and the appropriate program applied according to the instructions provided by the manufacturer.

In addition, a no template control (NTC) and a no reverse transcriptase control (NRT) were included in each run. All qPCR experiments performed in triplicate and the resulting CT values normalized to GAPDH.

2.6. Statistical analysis

The quantification of gene expression was determined through CT value (threshold cycle) which, provided from Real-Time PCR instrument by the software when the PCR reaction reaches the start of its exponential stage and imported into a spreadsheet program such as Microsoft Excel. The average of Ct for each triple sample was calculated, the change of target gene expression Firs the Ct average of each triples sample was determined, then the expression of target and control genes were normalized to the endogenous gene by ΔC_T (C_T target gene – C_T GAPDH) and (C_T control – C_T GAPDH). $\Delta\Delta C_T$ calculated through (ΔC_T target – ΔC_T control) and the fold change of gene expression was calculated through the equation fold change = $2^{-\Delta\Delta C_T}$ [27].

For data analysis, IBM SPSS for Windows software package version 23.0 (SPSS Inc., USA) was used. Samples included in this study were non-normal distributed (non-parametric) according to the value of skewness test, Kurtosis test, Z-value and Shapiro test. The independent-sample t-test (Mann-Whitney test) used to compare means of quantitative variables.

In addition, Spearman’s test used to assess the correlation coefficient between expression levels in fertile heavy smokers and sperm parameters. To be qualified as statistically significant, the results should show a p-value was less than 5 % ($p \leq 0.05$).

3. Results

One hundred and eighteen sperm samples were divided into two groups. The first group include 55 proven fertile candidates who show no previous history for smoking as control group with a mean age (36.33 ± 6.18). The second group include 63 fertile smokers who smoked more than 20 cigarettes per day with a mean age (37.42 ± 5.24) as case group.

3.1. Characteristics of the study population

Sperm parameters were compared between heavy smokers and non-smokers as shown in table 1. Total sperm count, total motility, progressive motility, normal form, vitality, and sperm membrane integrity were significantly higher in non-smokers group in comparison to heavy smokers group: 52.38 ± 34.52 vs 64.42 ± 39.18 Mill/ml, $P \leq 0.01$; 44.61 ± 22.47 vs $53.67 \pm 20.51\%$, $P \leq 0.003$; 33.17 ± 21.87 vs $40.96 \pm 20.78\%$, $P \leq 0.01$; 17.22 ± 8.26 vs $22.98 \pm 12.62\%$, $P \leq 0.05$; 59.95 ± 13.08 vs $64.87 \pm 15.18\%$, $P \leq 0.001$, and 72.19 ± 10.03 vs $78.73 \pm 11.21\%$, $P \leq 0.02$ respectively. Exception was for immotile sperms that was significantly higher in heavy smokers group (51.13 ± 24.23 vs $42.21 \pm 18.24\%$; $P \leq 0.002$).

Table 1. Descriptive characteristics of the study population (N=118): comparison between heavy smokers (N=63) and non-smokers (N=55).

Variables	Heavy Smokers (Mean ± SD)	Non-smokers (Mean ± SD)	P-value
Age	37.42 ± 5.24	36.33 ± 6.18	≤ 0.4*
Count [mill/ml]	52.38 ± 34.52	64.42 ± 39.18	≤ 0.01*
Total motility (%)	44.61 ± 22.47	53.67 ± 20.51	≤ 0.003*
Progressive motility (%)	33.17 ± 21.87	40.96 ± 20.78	≤ 0.01*
Immotile (%)	51.13 ± 24.23	42.21 ± 18.24	≤0.002*
Normal form (%)	17.22 ± 8 .26	22.98 ± 12.62	≤ 0.05*
Vitality (%)	59.95 ± 13.08	64.87 ± 15.18	≤ 0.001*
Membrane integrity (HOS) (%)	72.19 ± 10.03	78.73± 11.21	≤ 0.02*

SD, stander deviation. *Mann-Whitney test. $P > 0.05$: not significant. $P \leq 0.05$: significant. ≤ 0.01 highly significant

3.2. Quantification of mRNA

The quantification Real-time PCR was used to quantify the expression level of the selected genes (PGAM5, TYRO3, and PTPRN2). The CT represents the threshold cycle and provides information about the cycle of the fluorescent signal that increases exponentially to cross the threshold.

Relative amounts of PGAM5, TYRO3, and PTPRN2 mRNA delta Ct (Δ CT) were differentially expressed among the compared groups (Table 2).

This difference between the group of non-smokers and the group of heavy smokers was significant for PGAM5 ($P \leq 0.03$) and PTPRN2 ($p \leq 0.01$) but not significant for TYRO3 ($p \leq 0.3$).

For PGAM5 gene, a down regulation by 0.57-fold change was determined (Figure 1). Similarly, for PTPRN2 gene, it was a down regulation by a fold change equal to 0.66 (Figure 2). In contrast, an upregulation by 1.21-fold change for TYRO3 gene was determined (Figure 3).

Table 2. Expression levels of PGAM5, TYRO3 and PTPRN2 genes from spermatozoa in Heavy smokers compared to non-smokers controls groups (n = 118).

Target	Δ CT		$\Delta\Delta$ CT	Fold Change		P-value
	Heavy smokers	Non-smokers		2- $\Delta\Delta$ CT	Regulation	
PGAM5	3.7 $\pm$ 1.6	2.9 $\pm$ 1.1	0.804	0.573	Down	≤ 0.03 *
TYRO3	2.3 $\pm$ 1.1	2.4 $\pm$ 1.0	-0.275	1.209	Up	≤ 0.3 *
PTPRN2	4.5 $\pm$ 1.0	3.9 $\pm$ 0.9	0.587	0.666	Down	≤ 0.01 *

*Mann-Whitney test. $P > 0.05$: not significant. $P \leq 0.05$: significant. ≤ 0.01 highly significant.

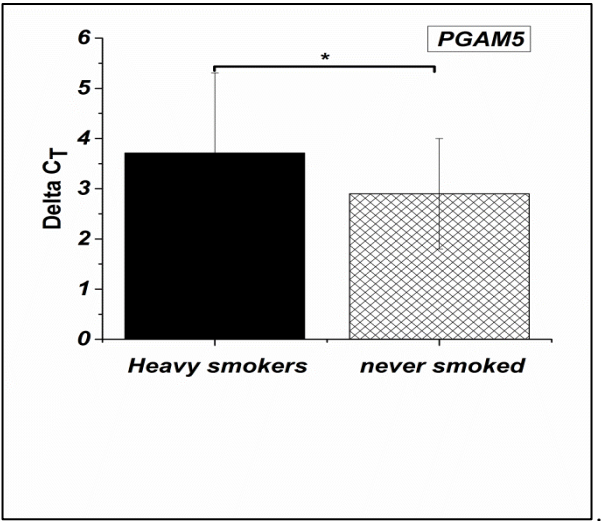


Figure 1. Expression levels of the PGAM5 gene in Heavy smokers compared to non-smokers males (P-value ≤ 0.03). * $P \leq 0.05$: significant.

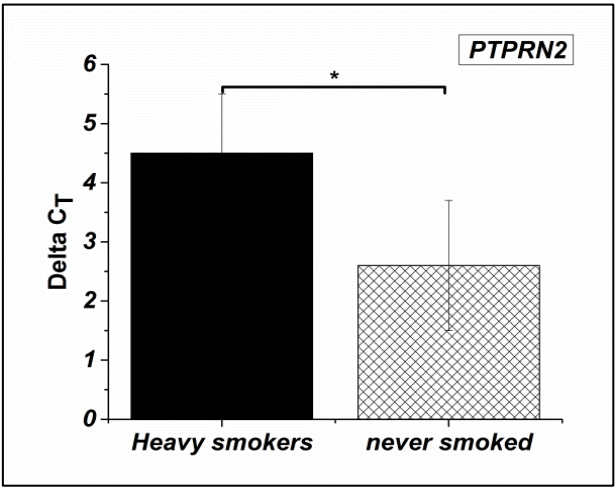


Figure 2. Expression levels of the PTPRN2 gene in Heavy smokers compared to non-smokers males (P-value ≤ 0.01). * $P \leq 0.05$: significant.

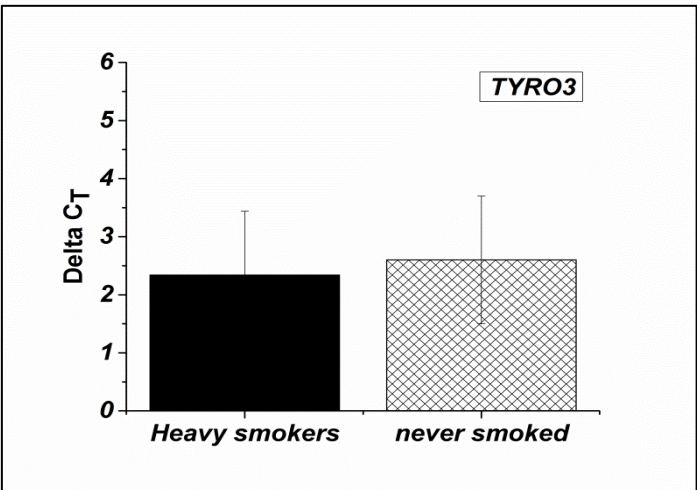


Figure 3. Expression levels of the TYRO3 gene in Heavy smokers compared to non-smokers males (P-value ≤ 0.3).

3.3. Correlation between PGAM5, TYRO3, and PTPRN2 mRNA expression and sperm parameters

The association between expression level of PGAM5, TYRO3 and PTPRN2 and sperm parameters was estimated.

The data included in the present study were not-normally distributed (non-parametric), therefore the spearman correlation coefficient (Spearman Roh) was used. Table 3 demonstrated that PGAM5 mRNA expression level was correlated negatively with motility ($r = -0.336$, $P \leq 0.005$) progressive motility ($r = -0.274$, $P \leq 0.01$) and positively with vitality ($r = 0.253$, $P \leq 0.02$).

TYRO3 mRNA expression level showed a significant negative correlation with sperm count ($r = -0.331$, $P \leq 0.009$), and vitality ($r = -0.230$, $P \leq 0.04$), and a negative correlation with motility ($r = 0.339$, $P \leq 0.008$), progressive motility ($r = 0.308$, $P \leq 0.01$), normal form ($r = 0.259$, $P \leq 0.04$), , and sperm membrane integrity ($r = 0.269$, $P \leq 0.01$) (Table 3).

Furthermore, PTPRN2 mRNA expression level correlated significantly negative with total sperm count ($r = -0.273$, $P \leq 0.01$), motility ($r = -0.276$, $P \leq 0.01$), progressive motility ($r = -0.293$, $P \leq 0.009$), normal form ($r = -0.228$, $P \leq 0.04$), and vitality ($r = -0.344$, $P \leq 0.02$) (Table 3).

Table 3. Correlation coefficient between the gene expression level and semen parameters for heavy smokers cases group (n = 63).

		Total sperm count (mill/ml)	Motility (%)	Progressive (%)	Immo- tile (%)	Normal form (%)	Vitality (%)	Membrane integrity (HOS) (%)
PGAM5	R	0.078	-0.336**	-0.274*	-0.001	-0.017	0.253*	0.020
	P	0.668	0.005	0.015	0.994	0.925	0.026	0.913
TYRO3	R	-0.331**	0.239**	0.308*	-0.218	0.259*	-0.230*	0.269*
	P	0.009	0.008	0.016	0.057	0.045	0.044	0.018
PTPRN5	R	-0.273*	-0.276*	-0.293**	0.268*	-0.228*	-0.344*	-0.194
	P	0.016	0.015	0.009	0.018	0.046	0.021	0.202

Spearman’s test, r: Correlation coefficient. *P ≤0.05: significant. **<0.01 highly significant.

4. Discussion

Infertility or subfertility is the result of many pathological factors. In general, 50% of infertility cases are attributed to idiopathic subfertility. About 15 percent of these cases are due to genetic defects, with the remainder due to environmental and lifestyle factors such as diet, alcohol consumption, physical activity, and smoking.

These factors have been found to have effects on the sperm epigenome and genome [22], which may have implications for the developing embryo [23,24].

Several studies have shown an association between decreased sperm quality (decreased sperm concentration, motility, normal morphology) and heavy smoking [14,28–30].

In comparison to the control group, we have identified a significant decrease in standard semen parameters (count, total motility, progressive motility, normal form, vitality, and membrane integrity) in heavy smokers group ($P \leq 0.05$).

These results are consistent with other studies showing the negative effects of smoking on sperm quality and its DNA structure [28–31]. A meta-analysis showed that that smoking negatively affect sperm parameters [32]. Conversely, other researchers have found that tobacco has no impact on standard semen parameters [33–35].

Thus, more research are needed to understand the molecular mechanism of how tobacco smoking influences male fecundity.

The genotoxic components of tobacco cause DNA adduct crosslinks, single- or double-strand breaks, chromosomal abnormalities and aneuploidy, and other genetic changes in spermatozoa [23].

We focused in this study on the relative quantification of PGAM5, TYRO3, and PTPRN2 genes expression and investigated the influence of tobacco smoke on the transcript level of these genes.

The relative amounts of PGAM5, TYRO3, and PTPRN2 mRNA delta Ct (Δ CT) were differentially expressed among the compared groups (Table 2).

This difference between the group of non-smokers and the group of heavy smokers was significant for PGAM5 ($P \leq 0.03$) and PTPRN2 ($P \leq 0.01$) but not significant for TYRO3 ($P \leq 0.3$).

PGAM5 gene were down regulated in the spermatozoa of heavy smokers compared to that of nonsmokers by 0.57 fold change (Figure 1). Similarly, for PTPRN2 gene, it was a down regulation by a fold change equal to 0.66 (Figure 2). In contrast, an upregulation by 1.21 fold change for TYRO3 gene was determined (Figure 3).

These results are in accordance to a study that demonstrated a downregulation in the transcription levels of H2BFWT, TNP1, TNP2, PRM1 and PRM2 genes in the spermatozoa of heavy smokers compared to that of nonsmokers [28]. Other studies demonstrated a significant decline in the transcription level of PRM1 and PRM2 in smokers spermatozoa compared to non-smokers [36], and that reactive oxygen species (ROS) resulting from smoking might be responsible for the decrease of the transcription level of genes like I κ B α [37]. Another study showed a downregulation in the transcription levels of MAPK8IP3, GAA, ANXA2, PRRC2A, and PDE11A genes in heavy smokers compared to non-smokers and an upregulation in the MAPK8IP3, ANXA2, PRRC2A, and PDE11A transcription levels in heavy smokers compared to non-smokers [38].

Regarding the correlation between the expression level of the studied genes and sperm parameters in the heavy smokers group (Table 3), we found that PGAM5 mRNA expression level correlated negatively with total motility, progressive motility ($P \leq 0.01$) and positively with vitality ($P \leq 0.02$).

TYRO3 mRNA expression correlated negatively with sperm count and vitality ($P \leq 0.01$), but highly positive with total motility, progressive motility, sperm membrane integrity ($P \leq 0.01$), and normal form ($P \leq 0.04$).

PTPRN2 mRNA expression level showed a negative correlation with total sperm count, total motility, progressive motility ($P \leq 0.01$), normal form ($P \leq 0.04$), and vitality ($P \leq 0.02$) (Table 3).

The PGMA5 gene encodes two mitochondrial genes: PGAM5s and PGMA5L (phosphoglycerate mutase family member 5) [39]. Both proteins are expressed in the testis and play an important role in mitophagy, a cellular process that eliminates damaged mitochondria [40,41].

Studies have shown that mutation or downregulation of the PGMA5 gene can lead to mitochondrial dysfunction, depletion or loss of mtDNA [42,43]. mtDNA is important for energy production in cells, including sperm, which can lead to decreased sperm motility and fertility.

PTPRN2 is a gene encoding a protein called tyrosine phosphatase non-receptor type 2, which is involved in the regulation of insulin signaling and glucose metabolism [39]. A previous study found that semen parameters (semen volume, sperm concentration, motility and morphology) were reduced in diabetic patients compared with non-diabetic patients [44].

With regard to male infertility, PTPRN2 has been reported to be located near hypermethylated regions of sperm DNA from patients who failed to conceive [45]. Another study showed that the PTPRN2 gene showed highly differential methylation differences between severe oligospermia and obstructive azoospermia [46].

A case-control study published in 2019 identified candidate genes important for spermatogenesis and fertility [47]. Of the 170 genes highlighted in this study, at least 38 genes (including PTPRN2) were differentially methylated and/or exhibited high testis expression between infertility cases and controls [47].

TYRO3 is a gene encoding a receptor tyrosine kinase protein [39]. This protein is involved in cell signaling and has been shown to play a role in the development and function of the male reproductive system [48–50]. Research suggests that variations in the TYRO3 gene may be associated with decreased sperm motility and fertility [48–50].

Smoking causes the formation of free radicals, which cause oxidative stress in the body. Oxidative stress can damage DNA in sperm and lead to reduced fertility [51]. PGAM5 has been shown to play a role in protecting cells from oxidative stress [43], so downregulation of this gene may make sperm more susceptible to damage from smoking-related oxidative stress. In addition, smoking has been shown to impair mitochondrial function in various tissues, including sperm [52,53]. PGAM5 plays a role in regulating mitochondrial function, so downregulation of this gene may lead to further mitochondrial dysfunction in response to smoking.

Smoking causes inflammation in the body, and chronic inflammation has been linked to decreased fertility. PTPRN2 has been shown to play a role in regulating inflammation. Therefore, the downregulation of this gene may make sperm more vulnerable to the inflammatory damage caused by smoking.

Changes in the transcript levels of these genes confirmed our previous study [25]. Increased DNA methylation levels in PGAM5 and PTPRN2 genes in heavy smokers lead to repression of the expression of these genes, resulting in downregulation of PGAM5 and PTPRN2 genes. Conversely, a significant decrease in DNA methylation (demethylation) levels in TYRO3 in cases compared with controls could lead to upregulation.

This study confirms that cigarette smoke plays a crucial role in altering human gene transcription levels in sperm as it affects chromatin remodeling [15], and global DNA methylation status [54], and DNA methylation at different CpG sites [8].

5. Conclusions

Overall, this study suggests that smoking may affect gene expression and male fertility by altering DNA methylation patterns in genes associated with fertility and sperm quality, including PGAM5, PTPRN2, and TYRO3. However, more research is needed to fully understand the mechanisms involved and the potential effects of smoking on these genes associated with male fertility. Quitting smoking can improve overall health and fertility and may also help improve gene expression and mitochondrial function in sperm.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (No. PHRC/HC/13/14).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to ethical restrictions.

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Conflicts of Interest: The authors declare no conflict of interest.

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