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Impact of cigarette smoking on DNA methylation levels and its influence on human sperm parameters

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Contents

Abstract

Cigarette-smoking is still one of the most common habits worldwide. According to WHO, almost one-third of global populations above 15-years-old are smokers. Several of the previous studies have found a negative association between cigarette-smoking and standard semen parameters, sperm penetration, and fertilization capacity. Moreover, cigarette-smoking also produces changes in the epigenome, such as DNA methylation. DNA methylation plays a vital role in genome stability, imprinting genes, X-chromosome inactivation, and the regulation of gene transcription. The main aims of this present thesis can be summarized as the following: (I) to assess the variation in sperm DNA methylation levels between smokers as cases and non-smoker males as controls, as well as to study the association between the change in sperm DNA methylation patterns and sperm parameters in male smokers (II) to assess whether there is an alteration in the sperm DNA methylation levels between subfertile and proven fertile males; and furthermore, (III) to evaluate the correlation between changes in sperm DNA methylation levels and sperm parameters in subfertile males.

Infinium 450K BeadChip arrays were used to recognize the alteration in the sperm DNA methylation levels between the various study groups (cases and controls). Deep bisulfite sequencing was therefore used to validate the results obtained from Infinium 450K BeadChip arrays. Following CpGs, cg00648582, cg0932376, cg19169023, cg23841288, cg27391564, cg19455396, and cg07869343) were subjected to bisulfite sequencing. The cg00648582, cg19169023, cg23841288, cg19455396, cg07869343 are located in the gene body and are related to *PGAM5, TYRO3, PTPRN2, TAP2* and *MAPK8IP3* genes respectively whereas the cg0932376, and cg2739156 are located in the intergenic regions. Only six CpGs showed a significant difference between the case and control groups. Moreover, when local deep bisulfite sequencing was applied, different CpG sites were obtained besides the target CpG* that resulted from the Infinium 450k beadchip array. The following CpG sites showed significant differences in the case group compared to control one. These sites are related to specific genes and were shown to be as follows: fifteen out of twenty-five CpG sites in a *PGAM5* gene-related amplicon, three out of four CpG sites in a gene *TYRO3*-related amplicon, nine out of twelve CpG sites in a gene *PTPRN2*-related amplicon and six out of twenty-two CpG sites in gene *MAPK8IP3*-related amplicons. A significant difference was also observed in four out of five CpG sites adjacent to cg09432376, and seven out of fifteen

CpG sites bordering the cg27391564-related amplicon. Moreover, a significant correlation was found between the variation in sperm DNA methylation levels and standard sperm parameters in the fertile male smokers, namely the case group.

On the other hand, the variations between sperm DNA methylation levels among the proven fertile and subfertile were assessed as follows: the CpG sites (cg19779893, cg19406113, cg23081194, cg04807108, cg25750688, cg07227024, cg16338278, cg05799088, and cg08408433) were validated by deep sequencing where cg19779893, cg23081194, cg07227024, cg16338278, cg05799088, and cg0840843 were found to be located in the gene body of *ADAMTS14, UBE2G2, ALS2CR12, ALDH3B2, PRICKLE2, and PTGIR* genes respectively, and the three remaining cg19406113, cg04807108, and cg25750688 were located in the intergenic regions. When a local deep bisulfite sequencing was applied, different CpGs were detected close to the target CpGs* that resulted from the screening study. The following CpG sites showed significant differences in the case group compared to the control one. These sites are related to specific genes and were demonstrated as follows: three CpGs related to the *ADAMTS14 gene,* six out of eleven CpG sites related to cg19406113, three CpGs in the *UBE2G2* gene, two CpG sites related to the cg25750688, and eight out of fifteen related to the cg04807108. In addition, two CpGs related to the *PRICKLE2* gene, two CpGs related to the *ALS2CR12* gene, seven CpGs related to the *ALDH3B2* gene, and nine CpGs related to the *PTGIR* gene also demonstrated significant differences in the case groups compared to the control ones. Moreover, significant differences were also observed between the alterations in sperm DNA methylation levels in some CpGs adjacent to those afore-mentioned CpG sites and standard sperm parameters.

In conclusion, this present study revealed biological differences in the DNA methylation levels of CpGs located in the gene body (cg00648582, cg19169023, cg23841288, cg19455396, cg07869343) and those located in the intergenic regions (cg0932376, and cg27391564). These alterations could potentially be related to the effects caused by smoking on the developmental stages of spermatozoa. In addition, a variation in sperm DNA methylation patterns between proven fertile and subfertile males was detected. Furthermore, an association between the changes in sperm DNA methylation and semen parameters was found in the case groups.

Zusammenfassung

Zigarettenrauchen ist immer noch eines der häufigsten Gewohnheiten weltweit. Laut WHO sind fast ein Drittel der über 15-jährigen Raucher. Mehrere Studien fanden bereits eine negative Assoziation zwischen Zigarettenrauchen und Standard-Spermienparametern, Spermienpenetration und Befruchtungskapazität. Darüber hinaus führt Zigarettenrauchen auch zu Veränderungen im Epigenom und beeinträchtigt auch die DNA-Methylierung. Die DNA-Methylierung spielt eine entscheidende Rolle bei der Genomstabilität, der Prägung von Genen, der Inaktivierung von X-Chromosomen und der Regulation der Gentranskription. Das Ziel der vorliegenden Arbeit war (I), die Variation der Spermien-DNA-Methylierungslevels zwischen Rauchern und Nichtrauchern (als Kontrollgruppe) zu untersuchen. Außerdem sollte der Zusammenhang zwischen Veränderungen der Spermien-DNA-Methylierungslevel und Spermienparameter bei männlichen Rauchern untersucht werden (II). Es sollte beurteilt werden, ob es eine Veränderung der Spermien-DNA-Methylierungslevel bei subfertilen und fertilen Männern gibt. Weiterhin sollte die Korrelation zwischen Veränderungen der Spermien-DNA-Methylierung und Spermienparametern bei subfertilen Männern untersucht werden.

Infinium 450K BeadChip-Arrays wurden verwendet, um die Veränderung der Spermien-DNA-Methylierungslevel zwischen den verschiedenen Studiengruppen (Rauchern als Fallgruppe und Nichtrauchern als Kontrollgruppe) zu erkennen.

Folglich wurde eine tiefe Bisulfitsequenzierung verwendet, um die Ergebnisse zu bestätigen, die von Infinium 450K BeadChip-Arrays erhalten wurden. Folgende CpGs wurden der Bisulfitsequenzierung unterzogen: cg00648582, cg0932376, cg19169023, cg23841288, cg27391564, cg19455396 und cg07869343). cg00648582, cg19169023, cg23841288, cg19455396, cg07869343, sind im Genkörper lokalisiert und mit *PGAM5*-, *TYRO3*-, *PTPRN2*-, *TAP2*- *und MAPK8IP3*-Genen assoziiert, während die cg0932376- und cg2739156-CpG stellen in den Bereichen zwischen den Genen lokalisiert sind. Nur sechs CpGs zeigten einen signifikanten Unterschied zwischen Fall- und Kontrollgruppe. Wenn lokale tiefe Bisulfit-Sequenzierung angewendet wurden, wurden außerdem verschiedene CpG-Stellen neben dem Ziel-CpG * erhalten, welches aus dem Infinium-450k-Beadchip-Array resultierte. Die folgenden CpG-Sites zeigten signifikante Unterschiede im Vergleich zu den Kontrollfällen. Sie sind mit spezifischen Genen verwandt und wurden wie folgt

nachgewiesen: Fünfzehn von fünfundzwanzig CpG-Stellen in einem *PGAM5*-Gen-, drei von vier CpG-Stellen im *TYRO3*-, waren mit neun von zwölf CpG-Stellen in Gen-*PTPRN2* und sechs von zweiundzwanzig CpG-Stellen in Gen-*MAPK8IP3*.

Darüber hinaus wurde ein signifikanter Unterschied in vier von fünf CpG-Stellen von benachbart zu cg09432376 und sieben von fünfzehn CpG-Stellen, die dem cg27391564-verwandten Amplikon benachbart sind, beobachtet. Es wurde eine signifikante Korrelation zwischen der Variation des Spermien-DNA-Methylierungslevels und den Standard-Spermienparametern bei männlichen Rauchern (Fallgruppe) gefunden. Zusätzlich wurde die Variation der Spermien-DNA-Methylierungslevel zwischen den nachgewiesenen fertilen und subfertilen Proben beurteilt. Folgende CpG-Stellen (cg19779893, cg19406113, cg23081194, cg04807108, cg25750688, cg07227024, cg16338278, cg05799088 und cg08408433) wurden durch Tiefensequenzierung validiert. Es zeigte sich, dass cg19779893, cg23081194, cg07227024, cg16338278, cg05799088 und cg0840843 im Genkörper von *ADAMTS14*-, *UBE2G2*-, *ALS2CR12*-, *ALDH3B2*-, *PRICKLE2*- und *PTGIR*-Genen lokalisiert waren, und die drei verbleibenden cg19406113, cg04807108 und cg25750688 waren in Bereichen zwischen den Genen lokalisiert. Wenn eine lokale Tiefenbisulfitsequenzierung angewendet wurde, wurden verschiedene CpGs in der Nähe der Ziel-CpGs * nachgewiesen. Die folgenden CpG-Sites zeigten signifikante Unterschiede im Vergleich zur Kontrollgruppe. Diese Stellen sind mit spezifischen Genen verwandt und wurden wie folgt nachgewiesen: drei mit ADAMTS14-Gen verwandte CpGs, sechs von elf mit *cg19406113* in Beziehung stehender CpG-Stelle, drei CpGs im *UBE2G2*-Gen, zwei mit cg25750688 verwandte CpG-Stellen und acht von fünfzehn verwandten cg04807108. Darüber hinaus zeigten zwei mit dem *PRICKLE2*-Gen verwandte CpGs, zwei mit *ALS2CR12* verwandte CpGs, sieben mit dem *ALDH3B2*-Gen verwandte CpGs und neun mit dem PTGIR-Gen verwandte CpGs signifikante Unterschiede im Vergleich zur Kontrollgruppe. Darüber hinaus wurden signifikante Unterschiede zwischen Veränderungen der Spermien-DNA-Methylierungslevels in einigen CpG-Adjacents und den zuvor erwähnten CpG-Stellen, sowie den Standard-Spermienparametern beobachtet.

Zusammenfassend wurden in der vorliegenden Arbeit biologische Unterschiede im DNA-Methylierungsgrad von CpGs im Genkörper (cg00648582, cg19169023, cg23841288, cg19455396, cg07869343) und in Bereichen zwischen den Genen (cg0932376 und cg27391564) gefunden. Diese Veränderungen könnten möglicherweise mit Auswirkungen des Rauchens auf die Entwicklungsstadien der Spermatozoen zusammenhängen. Zusätzlich

wurde eine Variation in den Spermien-DNA-Methylierungsmustern zwischen fertilen und subfertilen Männern festgestellt. Darüber hinaus wurde in den Fallgruppen ein Zusammenhang zwischen den Veränderungen der Spermien-DNA-Methylierung und den Spermienparametern gefunden.

Papers included in this thesis

This thesis contains six published articles; these are:

1. **Alkhaled, Y.,** Laqqan, M., Tierling, S., Lo Porto, C., Amor, H. & Hammadeh, M. E. (2018). Impact of cigarette-smoking on sperm DNA methylation and its effect on sperm parameters. Andrologia.

2. **AlKhaled, Y.,** Tierling, S., Laqqan, M., Lo Porto, C. & Hammadeh, M. E. (2018). Cigarette-smoking induces only marginal changes in sperm DNA methylation levels of patients undergoing intracytoplasmic sperm injection treatment. Andrologia, 50(1).

3. **Alkhaled, Y.,** Laqqan, M., Tierling, S., Porto, C. L. & Hammadeh, M. E. (2018). DNA methylation level of spermatozoa from subfertile and proven fertile and its relation to standard sperm parameters. Andrologia.

4. 4) Laqqan, M., Tierling, S., **Alkhaled, Y.,** Porto, C. L., Solomayer, E. F. & Hammadeh, M. E. (2017). Aberrant DNA methylation patterns of human spermatozoa in current smoker males. Reproductive Toxicology.

5. Laqqan, M., Tierling, S., **Alkhaled, Y.,** LoPorto, C., & Hammadeh, M. E. (2017). Alterations in sperm DNA methylation patterns of oligospermic males. Reproductive Biology.

6. Laqqan, M., Tierling, S., **Alkhaled, Y.,** Lo Porto, C., Solomayer, E. F. & Hammadeh, M. (2017). Spermatozoa from males with reduced fecundity exhibit differential DNA methylation patterns. Andrology.

Abbreviations

CpG	Cytosine-Phosphate-Guanine
DNA	Deoxyribonucleic Acid
DMCs	Different Methylation CpGs
DNMTs	DNA Methyl Transferase
HOS	Hypo-Osmotic Solution
IGF2	Insulin Growth Factor 2
ICR	Imprinting Control Region
ICSI	Intracytoplasmic Sperm Injection
miRNA	microRNA
P1	Protamine 1
P2	Protamine 2
ROS	Reactive Oxygen Species
RNAi	RNA interference
SAH	S - Adenosyl Homocysteine
SAM	S - Adenosyl Methionine
si RNA	Small Interference RNA
TP	Transition Protamine
TET	Ten Eleven Translocation
WHO	World Health Organization

List of Figures and Tables

1. Introduction

1.2 Spermatogenesis

Defined as the process for the production of haploid cells called spermatozoa from diploid spermatogonial stem cells, the spermatozoa cells are genetically very specialized male cells that can fertilize the oocytes and transfer the genetic material to the offspring. The spermatogenesis process is initiated by the effect of the hypothalamus controlling, the secretion of the gonadotrophin-releasing hormone (GnRH).This then stimulates the interior pituitary gland to release two important hormones, namely, the luteinizing hormone (LH) and the follicle-stimulating hormone (FSH). These two hormones have several functions as they travel through the bloodstream to the interstitial tissue space between the seminiferous tubules in the testes. The follicle-stimulating hormone (FSH) stimulates the Sertoli cells to release an androgen-binding protein, which binds with the testosterone hormone to induce the spermatogonium (male germ cells) to synthesise the spermatozoa (**Griswold., 1995**) while, the luteinizing hormone will enter the interstitial tissue space and stimulate the leydig cells to secrete the testosterone hormone (**Ewing *et al.*, 1993**). The spermatogenesis process comprises a sequence of developmental stages whereby the spermatogonial stem cells produce spermatozoa. The spermatogonial stem cells are divided into spermatogonium types A and B: the spermatogonium type A divides into a different type of spermatogonium A to maintain the stem cell pool, whereas the spermatogonium type B divides mitotically to produce primary spermatocytes, which are duplicates of genetic material (4N). Primary spermatocytes undergo a meiosis I division to produce secondary spermatocytes which contain haploid genetic material (2N); this is then followed by meiosis II to produce four haploid spermatids with either (22, Y) or (22, X) chromosome (**De Kretser *et al.*, 1998**) (Fiqure1).The produced spermatids interact with the Sertoli cells to be differentiated into sperm xcells which are then released into the lumen cavity of the seminiferous tubules and are finally transferred to the epididymis where they mature into spermatozoa and arestored before they are released into the outside environment.

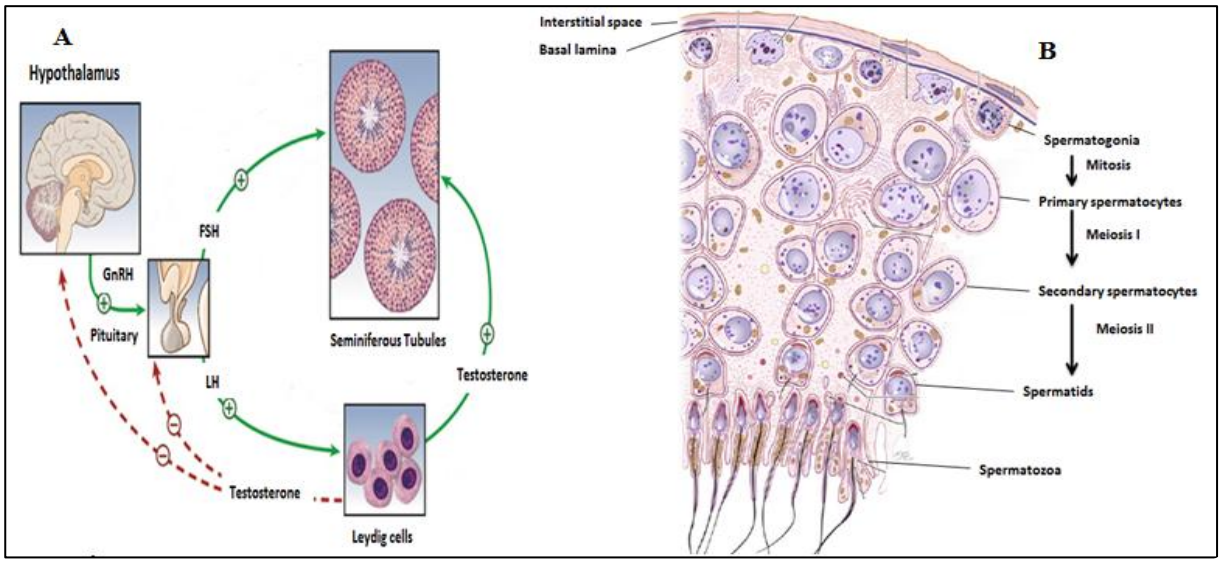

Figure 1: A schematic explanation of the different steps involved in spermatogenesis, including the hormonal and morphological changes. (A) represents the role of hormones and their feedback system while (B) represents a section in the seminiferous tubules where the spermatogonia divide into spermatogonium, then split mitotically into primary spermatocytes, which subsequently meiosis I and II and eventually produce four haploid spermatids. [Adapted from **Sharma & Agarwal (2011**).

1.3 Etiology concepts of male infertility

Male infertility is considered to be one of the major concerns of today's health-care community. According to the American Society for Reproductive Medicine (ASRM) and the World Health Organization (WHO), infertility is defined as the failure to achieve conception despite a regular unprotected intimate relationship between couples for at least one year of intercourse **(Rowe &Comhaire, 2000; Bayer *et al*., 2007)**. It is estimated that about 7% of men suffer from fertility problems which can be the result of congenital or acquired irregularity and have been classified into the following two groups: obstructive and non-obstructive causes (Figure 2) (**Forti & Krausz, 1998; Raheem & Raphel, 2011**). However, about 60-70% of male infertility cases are idiopathic (**Filipponi & Feil, 2009)**. Genetic anomalies constitute ahigh percentage of the idiopathic causes of male infertility which impact negatively on male fecundity and also lessen the chance of successful artificial conception; idiopathic male infertility may even be transferred to the offspring **(Vogt, 2004)**. Genetic abnormalities such as a change in the chromosome count or in the chromosome structure lead to failure in the meiotic division during spermatogenesis **(Alves *et al*., 2002)**. A

change in the chromosome count is associated with azoospermia, and the Klinefelter syndrome (47, XXY) is the most commonly observed manifestation of this abnormality, whereas an aberration in the chromosome structure can include translocation, deletion, inversion and insertion. Robertsoniation translocation, which is a type of non-exchanged translocation that takes place between two acrocentric chromosomes, is in fact a common case and is related to oligozoospermia (**Kumar *et al.*, 2008 and 2009**). The Y chromosome includes various genes which have an important role in the different stages of spermatogenesis. In addition, the long arm of the Y chromosome contains the azoospermia factor (AZF) genes (R); this chromosome has a higher susceptibility to the mutation, in comparison with autosomes, due to the absence of the meiotic recombination and the presence of only a single copy of the Y chromosome. Y chromosome microdeletion is a factor causing male infertility, due to the deletion of several important genes. Several studies have found that microdeletion is related to men who have azoospermia more than it is to men with oligospermia (**Schlege, 2004; Foresta *et al.*, 2001; Dohle *et al.*, 2002**). Despite numerous genes being related to the process of spermatogenesis, most of these genes have not yet been completely identified. Table (1) illustrates the function of some of the genes that contribute to the different processes of spermatogenesis.

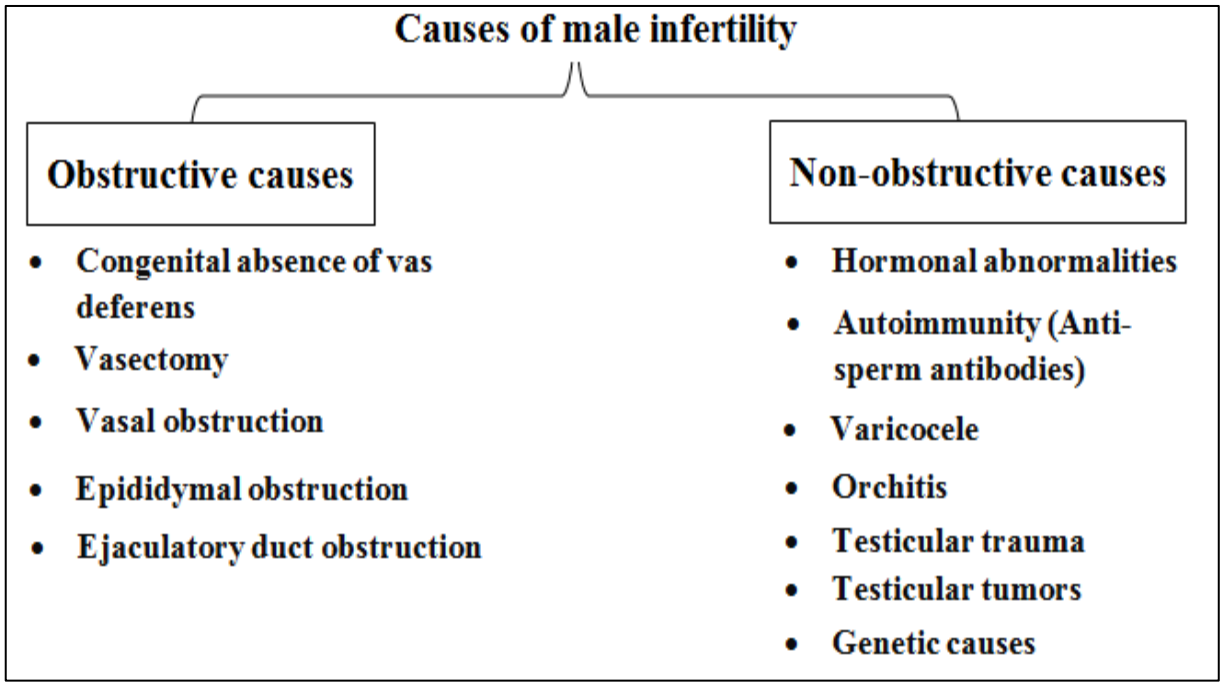

Figure 2: Schematic elucidation of the causes of male infertility, which are either due to obstructive causes or to non- obstructive ones.

Table 1: Genes related to male fertility and sperm function

Genes	Encoded protein	Function	Disruption of the gene
TEKT	Tektins 1-5	Expressed during sperm tail formation and plays an important role in the normal tail structure and function.	Sperm with a bent tail and its progressive motility is thereby reduced. (***Tanaka et al., 2004; Royet al., 2009***).
VDACs	Voltage dependent anion channel	Has a crucial role in the function of mitochondria required for the stability of the axoneme in the tail.	Reduces the sperm motility due to the defective structure of the axenome. (Hinsch et al., 2004).
SEPP1	Selenoprotein plasma	Extracellular glycoproteins on the flagella of the spermatid supply selenium to the testes and play a vital role in the flagella functions.	Reduces motility due to a defect in the axoneme microtubules and in the outer dense fibres from the principal piece of the sperm tail (**Olson *et al*., 2005**).
AKAP4	A-kinase anchor proteins	Signal transduction in the fibrous sheath of the developing spermatids.	Normal sperm count but reduced motility due to the lack of a fibrous sheath, which results in a shortened flagella (**Miki *et al*., 2002**).
SPAG6	Sperm associated antigen6	Associated with the microtubules for the flagella function of the sperm (**zhang *et al*., 2005**).	Low sperm motility because of the disorganized flagellar structure (**Sapiro *et al*., 2002**).
ADCY10 (sAC)	Adenylyl cyclase	The signalling molecule required for sperm motility and considered a major source of the cAMP in sperm which is required for the capacitation of the sperm to acquire the ability to fertilize the oocyte (**Lefievre *et al*., 2002**).	No sperm motility despite a normal count and morphology (**Esposito *et al*., 2004**).
CATSPER	Cation channel of human sperm	Acts as ion channel-mediated signalling and if located in the mid-piece of the sperm. It is required for sperm hyperactivated motility (**Jin *et al*., 2007; Qi *et al*., 2007**)	Sperm fails to develop hyperactive motility, resulting in male infertility (**Nikpoor *et al*., 2004**).
TNPs 1 & 2	Transition Protein 1 & 2	A sufficiently high concentration of proteins to support normal nuclear condensation.	Low condensed sperm nuclei, moreover, with a high level of DNA fragmentation form defects such as heads bent back on midpiece (**Yu *et al*., 2000; Zhao *et al*., 2004**)
PRMs 1 & 2	Protamine 1&2	The DNA packed in the sperm nucleus. This is essential for sperm DNA condensation and head formation (**O'Brien & Zini., 2005**).	Male infertility due to the defect of nuclear condensation (**Cho *et al*., 2003**).
GOPC	Golgi associated PDZ and coiled-coil motif containing	Plays an important role in acrosome biogenesis.	Male infertility because of the disruption to the mitochondrial sheath. In addition, there is the production of round sperm heads without the acrosome (**Yao *et al*., 2002**).
SPEM1	spermatid maturation 1	Nuclear elongation and cytoplasm removal during the spermatogenesis (**Zheng *et al*., 2007**).	Sperms with bent-back heads.
GBA2	Beta glycosidase 2	The encoded protein ß glycosidase2, expressed in the Sertoli cells in the testes, plays a crucial role in the endoplasmic reticulum.	Accumulation of the lipid in the endoplasmic reticulum of the Sertoli cells and resulting in round sperm lacking acrosome (**Roy *et al*., 2006**)
CSNK2A2	Protein Casein Kinas 2	It is expressed in the spermatids and plays an important role in the acrosome biogenesis.	Male infertility due to the absence of the acrosome in the sperm.
PIWIL	P-elements induced wimpy testes	Contributes to post-translation modification of late spermatid gene expression, and it is important for the meiotic phase of male germ cell developments.	Meiotic arrest in spermatogenesis stops the gene expression of spermatid (**Carmell et al., 2007**).

1.4 Structure of human sperm chromatin

It is essential to understand how DNA is packaged in human sperm for it has crucial implications for male infertility. The paternal genome in human spermatozoa is condensed in a unique style, not only to protect the DNA during the transit from the male to the female reproductive tract until it reaches the oocyte but also to ensure that the DNA is delivered in such a physical and chemical form that it allows the embryo to gets intact genetic information (**Fuentes *et al*., 2000; D'Occhio *et al*., 2007).** Unlike the somatic cells, the human sperm chromatin differs in both composition and structure. During the stages of the spermatogenesis process, major events occur such as the acquisition of the nuclear form and the removal of the histones, which are replaced by transition proteins that are about 90% formed during this stage. The transition proteins are then replaced by sperm-specific nuclear basic proteins (protamines), which are a synthesis in the last spermatid stage and play a vital role in the condensation and stabilization of sperm chromatin (**Kierszenbaum, 2001; Zhao *et al*., 2001; Balhorn, 2007; Carrell *et al*., 2007**). Moreover, the chromatin of human sperm is tightly compact, being at least six times more than the chromatin of the somatic cell, due to its unique supercoiled structure (toroids) (**Conwell *et al*., 2003; Hammoud *et al*., 2009**). Protamines account for 85% of the nuclear proteins in mature spermatozoa; protamine1 (PRM1) are produced as mature proteins and protamine 2 (PRM2) are produced as precursor proteins. Both PRM1 and PRM2 are expressed in the same quantities with a mean P1/ P2 ratio of about 1 in fertile males (**Bench *et al*., 1996; Carrell & Liu, 2001; Torregrosa *et al*., 2006**; **Oliva, 2006**). On the other hand, the retained 15% of histones remain in the mature spermatozoa to reduce the compaction of the chromatin structure, which allows for the decondensation process after fertilization, fusing the parents' genomic materials. Histones thus play a vital role in sperm functions and early embryonic development (**Wykes & Krawetz, 2003; Rajender *et al*., 2011**). Any change in the ratio P1/P2 or between histone and protamines in the human sperm will be associated with the low compaction of human chromatin, which results in DNA fragmentation, lower fertilization rates, and reduced pregnancy rates (**Oliva, 2006; García-Peiró *et al*., 2011; Simon *et al*., 2011**).

1.5 Epigenetic

The prefix Epi in the expression 'epigenetic' comes from the Greek and means 'over above or around.' In 1942 Conrad Hal Waddington was the first to establish the concept of epigenetic to clarify the then incomplete understanding of the whole process of embryo development and the actual mechanism of gene expression. According to Waddington, the interaction between the environmental stimuli and the genotype leads to a change in the phenotype and this is known as the canalization of developments **(Waddington, 1942, 2011; Felsenfeld, 2014).** Recently the epigenetic has been defined as the study of the biochemical alteration of the DNA and the histone structure without changing the genome sequence itself, which can be transferred through mitotic or meiotic division **(Carrell, 2012).** The epigenetic includes different processes such as DNA methylation, histone modification, and chromatin remodelling **(Richardson, 2002; Anway *et al*, 2005; Vaissière *et al*, 2008)**. Genetic information is very stable and needs several generations to obtain mutations and evolve, whereas the epigenetic alterations are reversible and able to quickly respond to exogenous and endogenous signals. Epigenetic modifications therefore play a crucial role in adapting to the environment and in regulating cell differentiation during development **(Reik, 2007; Baccarelli & Bollati, 2009**; **Zaidi *et al*., 2010; Margueron & Reinberg, 2010)**. Moreover, epigenetic modification plays an important role in embryonic development through its inactivation of particular genes in X chromosomes, and of imprinted genes. In the case of imprinting genes, the expression in the progeny depends upon which alleles are imprinted in the parents for instance, if the maternal alleles are imprinted, it will not be expressed in the offspring but instead the paternal alleles will be expressed and vice-versa (**Wolffe & Matzke, 1999; Reik & Walter, 2001**). Furthermore, epigenetic modifications influence the transcription process through either silencing or activating the genes. For instance, of the genes found in all the cells of an organism only some will be activated in any specific cell at a specific time. These processes determine which protein will be produced or not. The gene which is responsible for coding the protein troponin found almost in all cells is activated only in the skeletal muscle cells but silenced in the other types of cells. On the other hand, the housekeeping genes are turned on in all types of cells, and the majority of epigenetic mechanisms that control the gene expression process are DNA methylation and histone modification (acetylation and deacetylation) **(Nan *et al*., 1998; Bird, 2002; Kondo & Issa, 2003; Bell *et al*., 2011).**

1.5.1 Epigenetic mechanism

The epigenetic mechanisms play a vital role in the chromatin structure and modify the accessibility to the transcription regulatory elements. These mechanisms involve chromatin remodelling, histone modification, DNA methylation, and RNA interference (**Reik, 2007; Vaissière *et al*., 2008**). Different enzymes include histone modifying and DNA methyltransferase working in conjunction with mi RNA mechanisms to form a specific chromatin structure that prevents gene transcription. A histone modification is known as a co-valent post-translational modifications of histone proteins. The nucleosome is the main unit of the chromatin structure, which itself contains 147 bp of genomic DNA, wrapped around a core histone octamer (H2A, H2B, H3, and H4). Accessibility to the transcription elements is coordinated by amendments to the N-terminal of the histone tail and DNA, including methylation, acetylation, phosphorylation and ubiquitilation (**Linggi *et al*., 2005; Kouzarides, 2007**). Euchromatin is characterized by the relaxation of the bonds between DNA and histone, thereby allowing the transcription factor to bind to the transcription site; this process is correlated to histone acetylation and demethylation. In contrast, the heterochromatin is highly packed and associated with de-acetylation and methylation, and the transcription is therefore inactive (**Kurdistani & Grunstein, 2003; Nightingale *et al*., 2006; Shukla *et al*., 2008**). RNA interference (RNAi) is a biological process inside the cell, acting as post-translation gene silencing. RNAi mechanisms include micro-RNA (miRNA) and small interference RNA (siRNA) that both play an important role in the regulation of the genes (**Saumet & Lecellier, 2006; Li *et al*., 2006**). Both miRNA and siRNA have the same function but differ in their biogenesis where miRNA is endogenously expressed from an organism genome as a stem-loop with an incomplete double-strand RNA whereas the siRNA is exogenous and processed from a long complementary double-strand RNA (**Hamilton & Baulcombe,1999**; **Tomari & Zamore, 2005**). RNAi mechanisms start by cleaving the premature long double-strand RNA (dsRNA) into short mature (dsRNA) with approximately 20-24 base pairs with nucleotide overhang in the 3^- end through the specific endonuclease enzyme ribonuclease III (Diser). These short double strands interact with a catalytic RNA-induced silencing complex (RISC) and unwind into a passenger and a guide strand, followed by a release-out and degradation; the passenger strand and the guide strand remain binding to the RISC complex, which specifically attaches to the complementary mRNA molecules and cleave them, resulting in a complete loss of translation (**Pare & Hobman, 2007; Wilson &**

Doudna, 2013). This work is focused on DNA methylation since DNA methylation is a good marker for epigenetic modification.

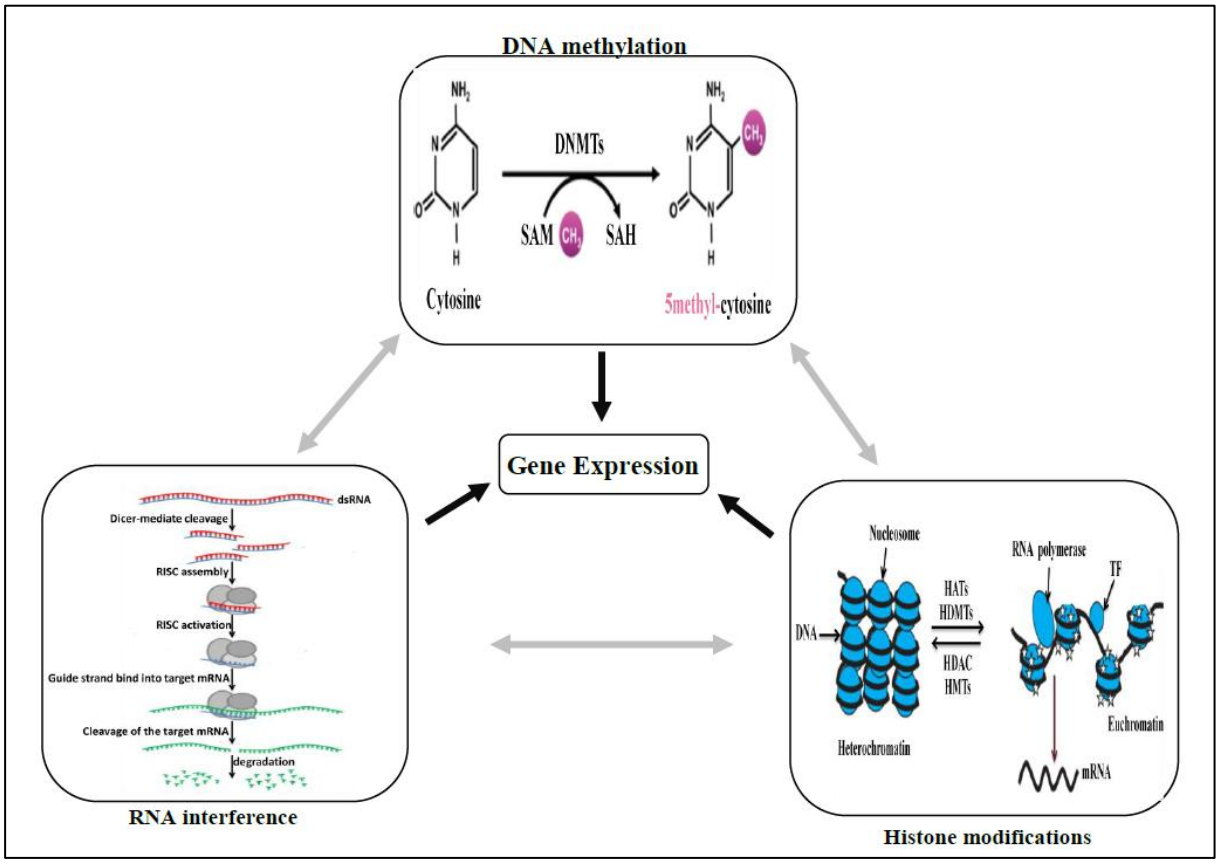

Figure 3: The mechanisms of epigenetic regulations. (A) DNA methylation is a co-valent binding of a methyl group to the C5 atom of cytosine pyrimidine in the promoter, which results in the prevention of gene transcription, (B) Histone modification or post-translation modifications include histone acetylation, de-acetylation, methylation, de-methylation, and phosphorylation.(C) RNA interference or post-translation gene silencing. These epigenetic mechanisms are controlled by each other and form a complex regulatory machinery. Modified from **(Barnes et al.,2005; Petrova *et al.*, 2013; Zakhari., 2013).**

1.5.2 DNA methylation

DNA methylation is defined as a biochemical process that alters the function of the gene without changing its own structure. This process id catalyzed by the enzyme DNA methyltransferase (DNMTs), which transfers a methyl group from S - adenosylmethionine (SAM) to C^5 atom in cytosine pyrimidine ring, resulting in the SAM being transformed into S- adenosylhomocysteine (SAH) and the cytosine is converted to 5- methylcytosine (5mC) (Figure) (4) (**Bird., 2002**; **Ulrey *et al.*, 2005**).

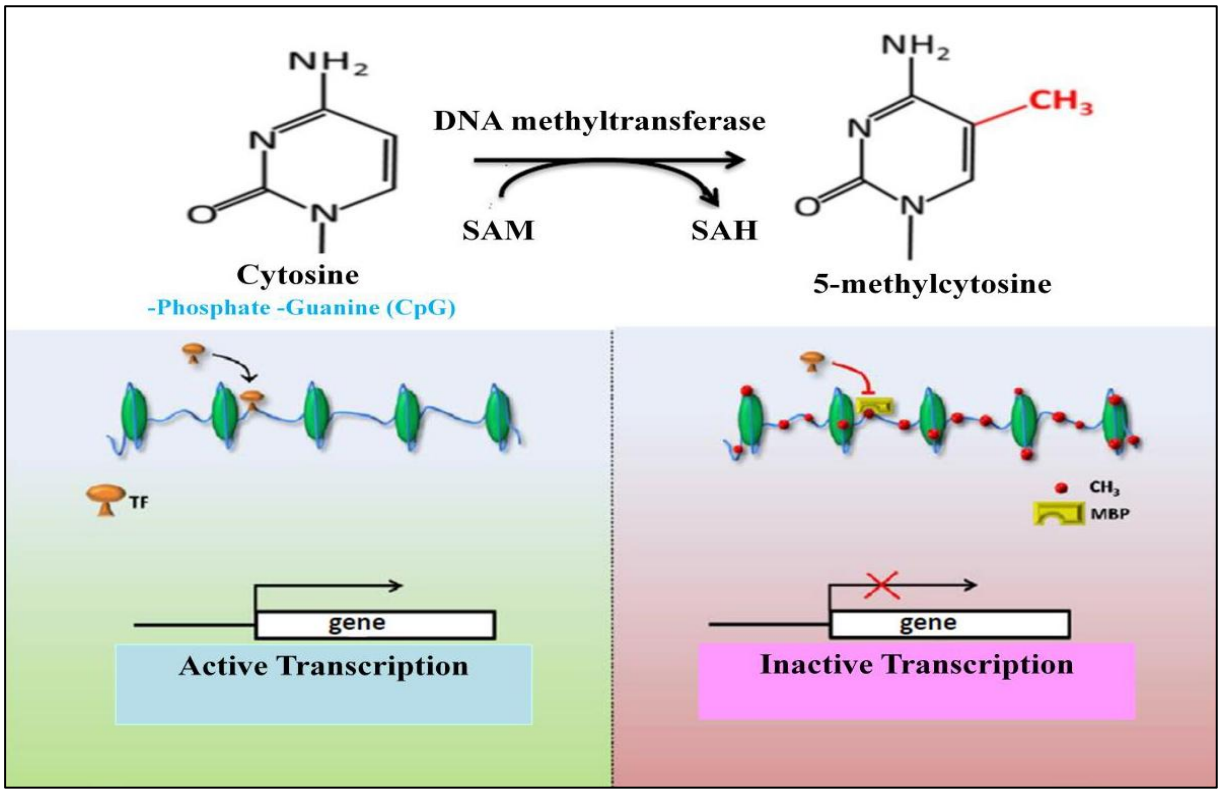

Figure 4: The mechanism of DNA methylation. DNA methylation usually happens through the transfer of the methyl group from S-adenosyl methionine (SAM) by methyltransferase into the 5th carbon atom in cytosine, followed by guanine base in a set known as CpGs Island. This is associated either with gene activation or gene silencing according to the position of the methylated CpGs in the genome. Modified from **Zhong *et al*. (2016).**

DNA methylation occurs solely in a region of the genome with a high abundance of CG, known as CpG islands (CGIs), where the cytosine is followed by a guanine base. CpG (cytosine phosphodiester bond-guanine) islands are almost 1k bp long. Most (abiut 50%) of the CpGs exist in the promoter and transcription start site (TSS) and the residual percent dole out intragenetic (gene body) and intergenetic (between genes) (**Takai & Jones, 2002; Illingworth *et al*., 2010**). Approximately 70–80 % of human genome promoter contains CpGs, and methylation in the promoter leads to the suppression of the transcription process either by the addition of methyl group into the major groove of DNA or through providing a binding site for the methyl-CpG-binding domain (MBD)-containing proteins such as MeCP2, which recruits repressor complexes, resulting in the chromatin structure (heterochromatin) being condensed. Both methods act as a barrier to prevent the binding of a transcription factor (RNA polymerase II) to the promoter, which results in the deactivation of the transcription process (**Jones *et al*., 1998; Feng & Zhang, 2001; Fuks *et al*., 2003)**. DNA methylation in

the gene body is described as beig associated with an activation of the transcription process. This correlation is thought to be dependent on the cell type; for instance, the embryonic stem cells did not indicate activation correlated with gene body methylation, whereas another study demonstrated that methylation in the gene body is associated positively with the efficiency of transcription elongation (**Lorincz *et al.*, 2004; Ball *et al.*, 2009**; **Lister *et al.*, 2009**). The intergenic region is a base sequence of nucleotides found between a set of genes that occasionally play a functional role in the regulation of transcription of the adjacent genes. The best understanding of the functional role of the intergenic is that it is concerned with the regulation of two imprinting genes, namely *H19* and *IGF2*, which are regulated by the same intergenic region known as the imprinting control region (ICR). Methylation of ICR prevents the binding of CTCF protein, which results in an interaction of the distal enhancer with the promoter of insulin growth factor 2 gene (*IGF2)* and, as a result, inhibits the expression of gene *H19*. The CTCF protein, on the other hand, will bind into unmethylated ICR, and this will block the interplay of the enhancer with *IGF2* and activate the transcription of the gene *H19*. Figure 5 (**Szabó *et al.*, 2004; Moarefi & Chedin, 2011; Bartolomei & Ferguson-Smith, 2011**).

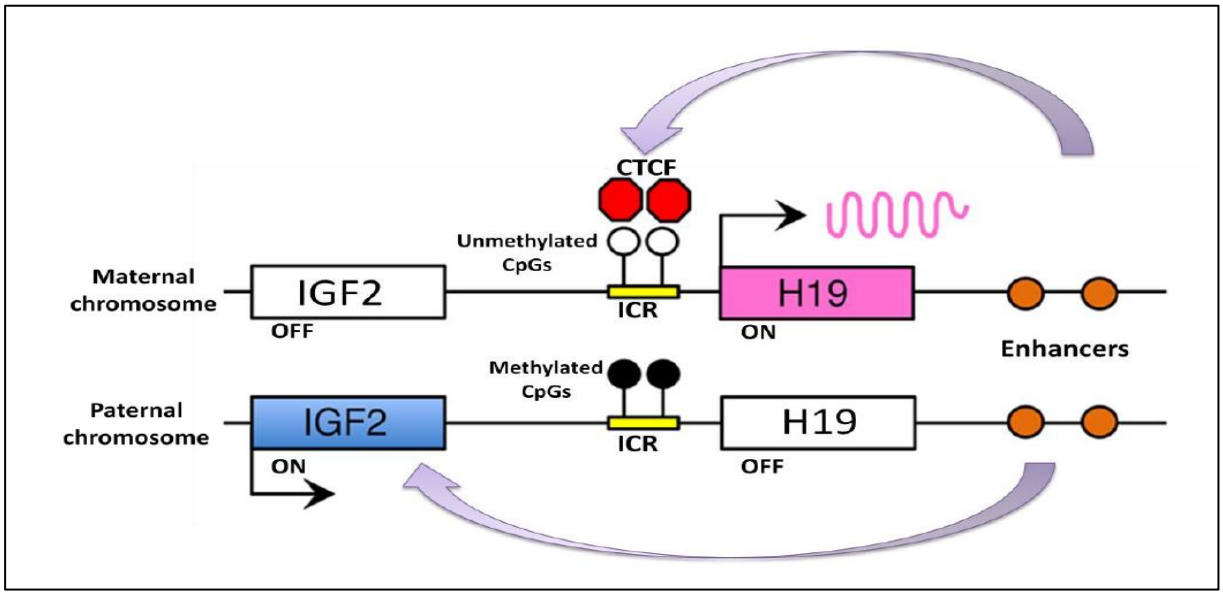

Figure 5: Methylation in the intergenic region CpGs and its role in the regulation of imprinting genes *H19* and *IGF2*. Unmethylated intragenic CpGs of ICR allow CTCF protein, which activates the interaction between the enhancer and the *H19* gene, thus suppressing the expression of the *IGF2* gene; on the other hand, methylated CpGs of ICR induce an interaction of the enhancer with the *IGF2* gene and inhibit the transcription of the *H19* gene. Modified from **Kameswaran & Kaestner (2014**).

The DNA methylation process is catalysed by different DNA methyltransferase (DNMTs) enzymes, such as DNMT1, DNMT3a, and DNMT3b. DNMT1 plays an important role in maintaining DNA methylation during replication through adding methyl groups to the new strand, with DNMT3a and DNMT3b being associated with *de novo* and establishing the DNA methylation pattern during the embryonic development (spermatogenesis) (**Okano *et al*., 1999; Bestor, 2000; Bird, 2002**). DNA methylation is considered to be an important epigenetic process which has a remarkable impact on several biological functions, such as imprinting genes, X chromosome inactivation, the maintenance and stability of the genome through repression of the movement elements (retrotransposons), the regulation of gene expression, early development of spermatozoa (Spermatogenesis), and embryo developments (**Holmes & Soloway, 2006; Chow & Heard, 2009; Xu & Song, 2014**). During the development, the male gremlin undergoes widespread epigenetic reprogramming, which includes DNA methylation and DNA de-methylation. Where DNA de-methylation occurs in the primordial germ cell during the development stage, the re-methylation process subsequently occurs with DNMT3a and DNMT3a (*de novo* methylation) before spermatogenesis, proper methylation in spermatozoa is therefore essential for normal fertilization, early embryo viability, adequate implantation and healthy live births (**Morgan *et al*., 2005; Carrell & Hammoud., 2009; Wu *et al*., 2015; Jenkins *et al*., 2016**).

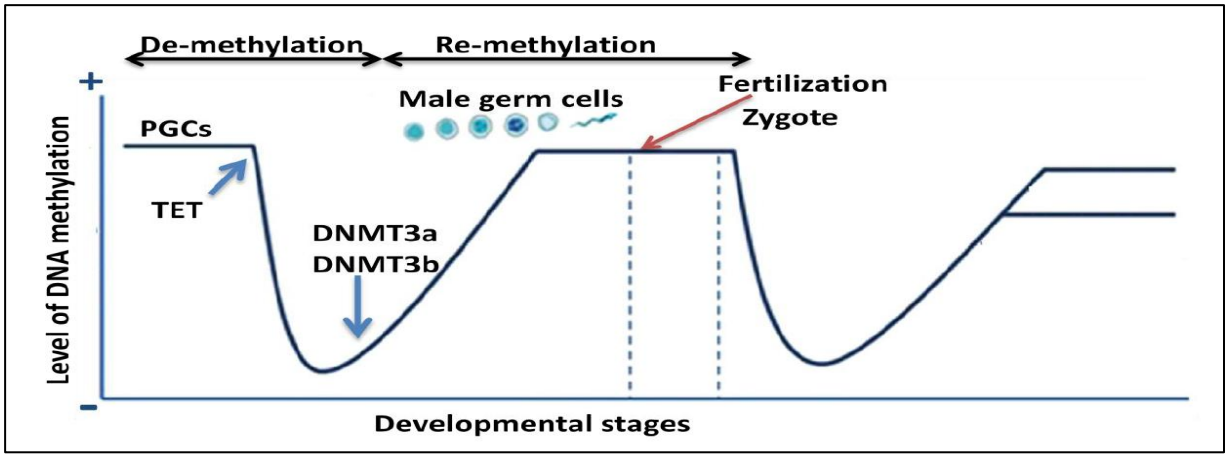

Figure 6: DNA methylation reprogramming in human germ cells. The primordial germ cells undergo de-methylation by the TET enzyme in the early stage of development followed by a *de novo* methylation (re-methylation) process in the presence of DNMTs 3a and DNMTs3b enzymes during the maturation of the germ cells and spermatogenesis. After the fertilization the second stage of DNA methylation reprogramming starts and demethylation is actively achieved through deamination. Modified from **Dean *et al*., 2005; Cui *et al*. (2016**).

1.5.3 Factors affecting DNA methylation

The best understanding of epigenetic alteration is DNA methylation where there are different factors affecting this epigenome (DNA methylation) mark; these factors are environmental or related to lifestyle. The environmental factors comprise toxins, metal, air pollution, UV radiation, and bisphenol A, whereas the lifestyle ones include nutrition, stress, exercise, smoking, and alcohol consumption (Figure 7). Folic acid, methionine, vitamin B6, vitamin B12 and selenium are considered to be vital dietary components and play a crucial role in DNA methylation (**Alegría-Torres *et al*., 2011; Feil & Fraga, 2012**). Folic acid and other supplementary components, such as vitamins B6 and B12, are essential for the activity of enzymes, for that intermediate synthesis of methionine and S- adenosylmethionine (SAM), a common donor for the methyl group, to maintain the DNA methylation patterns. Deficiency in these dietary components therefore leads to change in DNA that is related to genomic instability and genomic hypomethylation. Furthermore, DNA methylation is affected by selenium and polyphenols, which influence the metabolism of folate. The pregnancy period is principally susceptible to epigenetic disorders and nutrition can have a variety of impacts on the embryo; different studies have shown an association between prenatal nutrition and DNA methylation (**Rampersaud *et al*., 2000; Fang *et al*., 2007; Heijmans *et al*., 2008; Tobi *et al*., 2009; Waterland *et al*., 2010**).

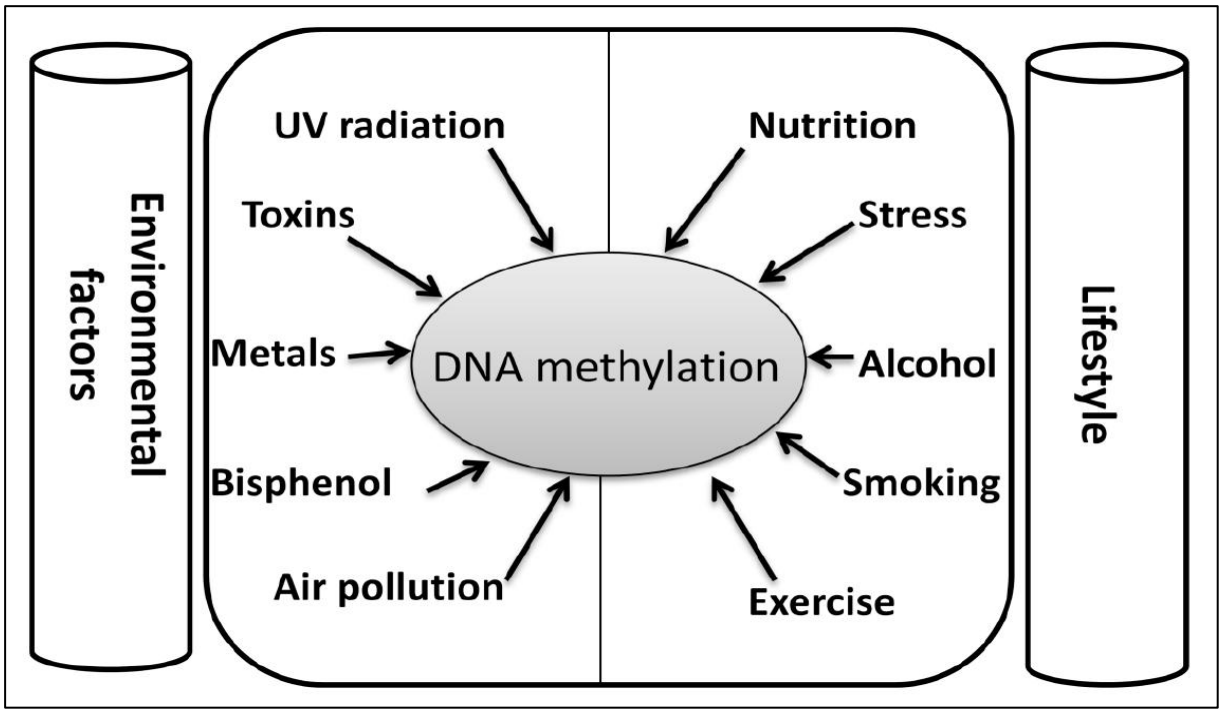

Figure 7: Environmental factors and lifestyle interplay with DNA methylation

Air pollution, dioxin, and environmental metals, such as cadmium, nickel and arsenic, are correlated with various diseases, such as cardiovascular, autoimmune, respiratory disease, neurological disorders, cancer, and fertility problems (**Jirtle & Skinner, 2007; Baccarelli & Bollati, 2009; Marsit & Christensen, 2011; Hou *et al*., 2011**). Furthermore, continuous exposure to different environmental and lifestyle factors, such as UV radiation, sunlight, psychological stress and alcohol, has been correlated with alterations in DNA methylation patterns (**Grönniger *et al*., 2010; Stein, 2011; Alegría-Torres *et al*., 2011**). However, the jury is still out on the effect of smoking on sperm DNA methylation, and that is why it is the main issue in this study.

1.6 Cigarette smoking and DNA methylation

Cigarette smoking is believed to be not only one of the major public health problems causing disease and premature death worldwide but is also an established environmental hazard factor for infertility. According tohe World Health Organization (WHO), almost one-third of populations above 15-years-old are smokers (**Mackay & Eriksen, 2002; Ezzati & Lopez, 2003; Oberg *et al*., 2011**). Smoking contributes to the evolution of communicable diseases and has a negative impact on the genetic and epigenetic mechanisms inside the human cells. (**Breitling, 2013**). Cigarette smoke contains more than 4000 chemical compounds, many of which are harmful and/or carcinogenic such as nicotine, cotinine, benzene, phenols, polyaromatic hydrocarbon, radioactive polonium, tar, tobacco-specific nitrosamines, and cadmium. These toxic chemicals find their way into the respiratory passages through inhalation and are then diffused to all organs of the human body by the blood and consequently causing harmful effects to all tissue compartments within the body. Moreover, these toxins impair the healing power of tissues and impede their capability for renewal (**Borgerding & Klus, 2005; Colagar et al., 2007; Kumar *et al*., 2011; Zhang *et al*., 2013; Rodgman & Perfetti, 2016**). Nicotine, the primary alkaloid and the most toxic ingredient in tobacco products, is an oxidizing substance and a psychoactive drug that is actively addictive. Moreover, it leads to damage of the cellular membranes and causes DNA fragmentation (**Arabi, 2005; Fiore *et al*., 2008**). In addition, cadmium disrupts the spermatogenesis process and decreases the level of zinc in seminal plasma; cotinine also reduces male fertility through affecting various semen parameters **(Martelli *et al*., 2006; Chen & Kuo, 2007**). Cigarette smoking leads to change the epigenetic mechanisms include histone modification, noncoding RNAs, and DNA methylation (**Herceg &Vaissiere., 2011**).

Moreover, cigarette smoke contributes to modify DNA methylation by the impact of nicotine on gene expression (**Lee & D'Alonzo., 1993**), and abnormality in sperm chromatin structure through disruption the ratio between P1/P2 (**Hammadeh *et al*., 2010).** Recent studies have conducted investigations on the relationship between the deleterious effects of smoking and their impact on transcription and epigenetic profiles, such as chromatin modifications and DNA methylation **(Ostrow *et al*., 2013; Zeilinger *et al*., 2013).** Therefore, understanding the disparity between the different effects of smoking and DNA methylation can give us a clue into the molecular mechanisms behind the evolution of diseases such as infertility where smoking is a causing agent.

2. Aims of the PhD thesis

As explained in the introduction, cigarette-smoking is considered as one of the main leading causes of different diseases and has deleterious effects on human fertility. Furthermore, DNA methylation plays a vital role in various biological functions in general and in the early developmental stages of spermatogenesis in particular. It has been postulated that smoking has a detrimental effect on different cellular functions in humans and also on the molecular DNA genome, causing alterations in epigenetic mechanisms. However, this relationship is questionable, still being contradictory and under investigation. Hence, the aim of the present study is to shed a light on the effect of cigarette smoking on human sperm DNA methylation.

Genome-wide array analysis was used to define differential methylated CpGs between different groups of smokers and those who had never smoked and was then validated in more study group populations, using local deep bisulfate sequencing. The aims of the study were as following:

1. To determine the influence of cigarette-smoking on sperm DNA methylation and on sperm parameters between heavy smokers and non-smoker males, and also to evaluate the correlation between the changes in sperm DNA methylation levels and sperm parameters.
2. To assess whether there is an alteration in the methylation levels of sperm DNA obtained from subfertile males compared to that obtained from proven fertile males, as well as to evaluate the association between the changes in sperm DNA levels and sperm parameters in subfertile males.

3. Results

3.1 Impact of cigarette-smoking on sperm DNA methylation and its effect on sperm parameters

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ORIGINAL ARTICLE

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Impact of cigarette-smoking on sperm DNA methylation and its effect on sperm parameters

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Summary

DNA methylation is an epigenetic modification of the genome. The purpose of this study was to determine the influence of cigarette-smoking on sperm DNA methylation from a genomewide survey of sperm samples and to ascertain its effect on sperm parameters. Twenty-eight sperm DNA samples (from 14 fertile smokers as a case study and 14 proven fertile nonsmokers as controls) were subjected to Infinium 450K BeadChip arrays to identify the changes in the DNA methylation level between the two groups. Then, deep bisulphite sequencing was used to validate five CpGs on 78 samples. The results from the Infinium 450K found that only 11 CpGs showed a significant difference in DNA methylation between the case and the control groups. Five CpGs of the eleven (cg00648582, cg0932376, cg19169023, cg23841288 and cg27391564) underwent deep bisulphite sequencing where cg00648582, related to the *PGAM5* gene, and the cg23841288 CpGs, related to the *PTPRN2* gene amplicons, showed a significant increase in their DNA methylation level in more than one CpG in the case group. In contrast, a significant decrease was found at cg19169023 and at its various neighbouring CpGs in the *TYRO3* gene-related amplicons. Furthermore, this study demonstrated a significant correlation between the variation in sperm DNA methylation level and standard sperm parameters in the case group.

KEYWORDS
cigarette-smoking, sperm DNA methylation, sperm parameters

1 | INTRODUCTION

Cigarette-smoking is still a common practice worldwide. Indeed, according to the World Health Organization (WHO), about one-third of populations above 15 years old are smokers (Saleh, Agarwal, Sharma, Nelson, & Thomas, 2002). Several studies have revealed that cigarette-smoke contains no less than 4,000 harmful chemicals (including mutagenic substances and carcinogens) (Bernhard, Rossmann, & Wick, 2005; Kumar, Matthews, Joshi, de Jager, & Aspiras, 2011; Kumosani, Elshal, Al-Jonaid, & Abduljabar, 2008). Moreover, many of these chemicals are poisonous substances, such as nicotine and its metabolite, cotinine, as well as radioactive polonium, and cadmium (Colagar, Jorsaraee, & Marzony, 2007; Järup et al., 2000; Richthoff, Elzanaty, Rylander, Hagmar, & Giwercman, 2008). Cigarette-smoking has many deleterious effects on human health in general and on the reproductive system in particular (Zhang et al., 2013). Results from various publications have documented the adverse effects of cigarette-smoking on human sperm parameters (volume, concentrations, morphology, motility and vitality), which in turn and in time affect male fertility (Hammadeh, Hamad, Montenarh, & Fischer-Hammadeh, 2010; Künzle et al., 2003; Ramlau-Hansen et al., 2007; Zinaman, Brown, Selevan, & Clegg, 2000). Furthermore, the results from tests on cigarette-smokers have shown poor sperm penetration and fertilisation capacity (Sofikitis et al., 1995; Vine, 1996). In addition, there has been shown to be a decrease in embryo implantation rates in smokers compared to nonsmokers (Ramlau-Hansen, Thulstrup, Olsen, & Bonde, 2008;

Soares, Simon, Remohi, & Pellicer, 2007; Zitzmann et al., 2003). Other researchers have found a high level of reactive oxygen species (Agarwal et al., 2014; Hammadeh et al., 2010; Homa, Vessey, Perez-Miranda, Riyait, & Agarwal, 2015) and leucocytes (Zhang et al., 2013) in the seminal plasma of smokers. Moreover, cigarette-smoking has a negative impact on the process of protamination through the disruption of P2, leading to a change in the P1/P2 ratio (Hammadeh et al., 2010). Cigarette-smoking also produces changes in the histone H2B (Hamad, Shelko, Kartarius, Montenarh, & Hammadeh, 2014) and in the sperm DNA methylation level (Marsit, Houseman, Schned, Karagas, & Kelsey, 2007).

DNA methylation is a type of epigenetic modification of the genome, where a methyl group is added to the 5th position of cytosine, located in the cytosine–phosphate–guanine dinucleotide "CpGs" (Portela & Esteller, 2010). The methylation of DNA is catalysed by methyltransferase enzymes (DNMT1, DNMT3a and DNMT3b) (Jeltsch, 2006). DNA methylation plays a vital role in the regulation of gene expression through modulating the transcription of DNA into RNA (Shukla et al., 2011) and is involved in the protection of genome integrity through the inhibition of the movement of the transposal element (Lee & Pausova, 2013; Levin & Moran, 2011). The DNA methylation process can also be altered by many other factors, such as genetic (Bell et al., 2011) and environmental ones, of which cigarette-smoking is a prime example (Breitling, Yang, Korn, Burwinkel, & Brenner, 2011). The interaction of these factors results in changes in the phenotypic identity or can cause diseases such as cancer (Feil & Fraga, 2012). Carcinogenic substances in the cigarette-smoke induce a break in the DNA double-strand; this fracture is considered the ignition point for changes in DNA methylation (Huang et al., 2013). Furthermore, carbon monoxide leads to hypoxia, which then causes a reduction in the methionine adenosyltransferase2A that is responsible for the synthesis of S-adenosyl methionine, a methyl group donor for DNA methylation (Liu et al., 2011). Previous studies conducted on male fertility have suggested that sperm DNA methylation may be used as a biomarker for semen quality and fertility. The mammalian germline undergoes broad epigenetic reprogramming, during which DNA demethylation occurs in the primordial germ cell development stage, and the remethylation (de novo methylation) process ensues before spermatogenesis (Morgan, Santos, Green, Dean, & Reik, 2005). In males, the remodelling of chromatin takes place during spermatogenesis (Flanagan et al., 2006). Disruption in the DNA methylation has been shown to correlate with poor spermatogenesis, which results in compromised sperm parameters and infertility (Aston, Punj, Liu, & Carrell, 2012; Hammoud, Purwar, Pflueger, Cairns, & Carrell, 2010; Houshdaran et al., 2007; Li, 2002; Nanassy & Carrell, 2011; Schaefer, Ooi, Bestor, & Bourc'his, 2007). As a consequence of this, the fertilisation capability of spermatozoa is impaired, affecting embryo development and resulting in poor embryo quality. This will affect the implantation potential or can even have detrimental effects on the embryo, resulting in a poor pregnancy outcome or even the failure of the whole cycle (Anifandis, Messini, Dafopoulos, & Messinis, 2015; Aoki et al., 2006; Oakes, La Salle, Smiraglia, Robaire, & Trasler, 2007).

This study was conducted to determine the influence of cigarette-smoking on sperm DNA methylation and on sperm parameters. The correlation between the changes in sperm DNA methylation and sperm parameters was also evaluated.

2 | MATERIAL AND METHODS

2.1 | Ethics statement

Institutional review board approval (No. PHRC/HC/13/14) was obtained from the Ethics Committee of Helsinki before the initiation of this study. In addition, each participant gave written consent to their inclusion in this study. The study itself was conducted in the laboratory of biochemistry and molecular biology of reproductive medicine within the Department of Obstetrics and Gynecology at the University Hospital of Saarland, Homburg, Germany.

2.2 | Sample collection and semen analysis

One hundred and six semen samples were from participants, who were required to abstain from sexual activity for 3 days before masturbating; the samples were collected in clean, dry, sterile and leak-proof plastic containers; the procedure was carried out in a collection room attached to the laboratory. Following the liquefaction of the semen samples at 37°C for 30 min, the sperm parameters were performed according to the World Health Organization guidelines (WHO., 2010), using a Makler chamber. From each sample included in this study, six smears were prepared (10 μl) for a morphology assessment. Furthermore, 100 μl semen was mixed with 1.0 ml of a hypo-osmotic solution (HOS) to assess the sperm membrane integrity (a vitality test). Moreover, 5 μl semen was mixed with 5 μl of a 0.5% aqueous yellowish Eosin solution (the Eosin test) to assess the sperm viability.

All males were in the reproductive age group, namely 25–50 years old.

2.3 | Smoking estimation

The cigarette-smoking status was evaluated according to guidelines previously established by Anifandis et al. (2014). This basically involved recording the number of cigarettes consumed per day/year for each participant. Cigarette-smokers were categorised into the following two groups: fertile heavy smoker males who had smoked more than 20 cigarettes per day in the last 2 years until their enrolment in this study as a case study (n = 59) and proven fertile non-smoker males who had never smoked or lived with smokers as a control (n = 47).

2.4 | Exclusion criteria

The following parameters were taken into consideration for exclusion: varicocele, antisperm antibodies, Y chromosome microdeletions, males subjected to surgical operation in the reproductive system, heavy body mass index and advanced age > 50 years old.

2.5 | Spermatozoa purification and DNA isolation

Each semen sample was loaded on 45%-90% discontinuous PureSperm gradients (Nidacon international, Sweden) and centrifuged at 2,300 rpm for 20 min at room temperature to remove somatic cells. The pellet was washed twice with a HAMs-F10 medium, supplemented with human serum albumin (5 mg/ml) and penicillin G/streptomycin sulphate (0.1 mg/ml; PAN Biotech, Germany) and carefully overlaid with 0.75 ml of the same medium. The samples were placed in an incubator at 37°C, and after 45 min, the upper layer containing the motile sperm was withdrawn, immediately assessed for sperm count and motility and then stored at −20°C until processing. The sperm DNA was isolated using an isolation genomic DNA kit according to the standard protocol provided by the manufacturer (Cat. #: BIO 52066, Bioline, UK). The concentration of extracted DNA was measured using a Nanodrop spectrophotometer 2000c (Thermo Scientific).

2.6 | Sodium bisulphite treatment

As previously described, 500 ng extracted sperm DNA was treated with sodium bisulphite using an Epitect bisulphite conversion kit (Cat. #: 59104, Qiagen, Germany) that converted unmethylated cytosines into uracil, while the 5-methylcytosine (5MeC) remained unaltered, (Wu, Kang, Zheng, Liu, & Liu, 2015).

2.7 | DNA methylation analysis by Infinium 450K BeadChip array

As a pilot study, twenty-eight samples were subjected to Infinium 450K BeadChip arrays (Illumina, San Diego, CA, USA) at the Life and Brain GmbH Biomedicine and Neuroscience technology centre in Bonn, following the manufacturer's recommendations (Bibikova et al., 2011), to identify any variations in the sperm DNA methylation regions between the fertile heavy smoker and the proven fertile nonsmoker males. The samples were whole-genome amplified and put on the bead arrays, as described in Sandoval et al. (2011). Bioinformatic processing and evaluation were performed with the RnBeads program package (Assenov et al., 2014). The beta values were then generated by analysing the intensities for methylation or for no methylation at each CpG, tiled on the array, using the following calculation: β-value = methylated/ (methylated + unmethylated). The β-value results ranged from 0 to 1 and indicated the methylation level for each CpG. A value of 1 represented a completely methylated CpG and a score of 0 meant a completely unmethylated CpG. The methylation level in each CpG was considered as a differentially methylated CpG (DMC) when the absolute difference of the means of the average beta values between the two groups was above 0.1. Furthermore, a Benjamini–Hochberg-corrected *t* test FDR (false discovery rate) of <0.1, no. of beads per sample ≥ 5 and an absolute log 2 ratio ≥ 0.2 were considered significant findings. $p \leq .01$ was used to select for differentially methylated CpG (DMC) with possible biological as well as statistical significance. According to these results, we selected CpGs not overlapping any common SNP site, according to dbSNP142, and studied 78 samples using local deep bisulphite sequencing (Gries et al., 2013).

2.8 | Bisulphite profiling (Bi-PROF)

Three hundred ng of sperm DNA was bisulphite-treated using an Epitect bisulphite conversion kit (Cat. #: 59104, Qiagen, Germany). PCRs encompassing the differentially methylated CpGs that had been identified by 450K BeadChip array were performed in a 30 μl total volume reaction, using a "MyTaq™ HS Red Mix" with a 2× concentration (Catalog #: BIO-25047, Bioline, UK), according to the manufacturer's protocol. For the amplification, fusion primers were used, consisting of a specific 3′-portion (Listed in Table 1, together with respective annealing temperatures) and a universal 5′-portion, containing the necessary nucleotide sequences for Illumina sequencing. About 5 μl of the PCR product was loaded onto a 2% agarose gel, including

TABLE 1 Identified differentially methylated CpG sites in sperm samples from heavy smokers and proven fertile nonsmokers males (n = 28)

cigd	Chro	Start	Annotated	Nonsmoker	Smoker	Diff meth. *p* value
cg00648582	chr12	133294524	PGAM5, Body/Island	0.43004664	0.797863227	1.18E-05
cg094302376	chr22	36044226	–	0.886717598	0.479951418	1.29E-06
cg19169023	chr15	41853346	TYRO3-Body/S Shore	0.9258933	0.552754264	8.35E-07
cg23841288	chr7	158342105	PTPRN2, Body/N Shore	0.159790018	0.441430882	1.58E-05
cg27391564	chr2	240530497	–	0.862300155	0.51321121	2.23E-06
cg07227024	chr2	202163482	*ALS2CR12, Body/*	0.043273629	0.926757907	4.82E-08
cg01584086	chr11	10373718	-	0.034917006	0.524015616	1.21E-07
cg08108333	chr7	157320662	*-/Island*	0.071346757	0.407840484	8.41E-06
cg15412446	chr2	106886593	*-/Island*	0.060752763	0.269507355	3.49E-06
cg20978247	chr6	32905085	*HLA-DMB-Body/*	0.90991696	0.506085887	4.10E-07
cg23109721	chr2	106886537	*-/Island*	0.039950676	0.251148581	1.94E-06

The CpG sites taken for testing in larger individuals are highlighted in grey.

the DNA ladder (Cat. #: N0467S, Biolabs) and stained with ethidium bromide. The PCR products were purified using the Agencourt® AMPure XP beads (Beckman Coulter, USA) and then measured using the Quant-iT™ DNA Assay Kit (Fisher Scientific, USA), according to the manufacturers' recommendations, and finally 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 the data obtained from the sequencing step were processed, filtered and aligned, using the BiQ Analyzer HT software (Lutsik et al., 2011) and excluding all reads containing more than 10% of missing CpG sites (the maximal fraction of unrecognised sites ≥ 0.1).

2.9 | Statistical analysis

Data were analysed using IBM SPSS for the Windows software package version 23.0 (SPSS Inc., USA). The samples included in this study were non-normal distributed according to the value of the skewness test, the Kurtosis test, the Z-value and the Shapiro test. An independent sample *t* test (Mann–Whitney test) was used to compare the means of the quantitative variables. In addition, Spearman's test was used to assess the correlation coefficient between the methylation levels in different CpG and sperm parameters. The results from all the above-mentioned procedures were accepted as statistically significant when the *p* -value was less than 5% ($p \leq .05$).

3 | RESULTS

3.1 | Cigarette-smoking and sperm DNA methylation

Twenty-eight sperm DNA samples (14 from proven fertile nonsmokers and 14 from fertile heavy smokers) were subjected to an Infinium 450K Bead Chip analysis to detect the DNA methylation level of ~485,000 CpGs covering 99% positions in each sample. As shown in Table 1, the 450k BeadChip analysis revealed that 11 CpGs, namely *cg00648582*, *cg01584086*, *cg07227024*, *cg08108333*, *cg09432376*, *cg15412446*, *cg19169023*, *cg20978247*, *cg23109721*, *cg23841288 and cg27391564* were showing a significant difference of >20% between the two analysed groups. Five CpG sites (*cg00648582*, *cg09432376*, *cg19169023*, *cg23841288 and cg27391564*) of the 11 were selected randomly to validate this observed methylation difference using bisulphite profiling.

The *cg00648582* is located in intron 4 of the phosphoglycerate mutase family member 5 (*PGAM5*) gene within chromosome 12. This CpG (*Cg00648582*) is close to DNaseI hypersensitivity and is related to the transcription factor binding site motif of:

- POLR2A, which is responsible for the production of messenger RNA in eukaryotes as it encodes for the largest portion (subunit) of RNA polymerase II;
- MYC, which is considered as a gene regulator where its encoded proteins contribute to many vital processes, such as apoptosis, cellular differentiation and cell cycle progression (ENCODE Project Consortium, 2012).

The *cg09432376* is related to the transcription factor binding site motifs of:

- WRNIP1, which plays a crucial role in modulation episodes due to the synthesis of DNA polymerase delta-mediated DNA. Furthermore, it has a principal ATPase activity. It joins the DNA repair system where it searches for any missing DNA or inhibited replication forks, thus controlling the process of vital DNA synthesis;
- NFIC has a vital role in the induction of transcription and replication processes;
- SIN3AK20 functions as a corepressor interacting with HDAC1, N-coR, SMRT and MeCP2;
- STAT3 promotes the expression of a variety of genes, and it is essential for multiple cell functions, such as cell growth and differentiation, apoptosis and possibly stem cell self-renewal;
- TBP plays a pivotal role in the formation of the DNA helix complex and in the start of the transcription episode (ENCODE Project Consortium, 2012).

The *Cg19169023* is located in exon 2 of the *TYRO3* gene and does not overlap with the DNaseI hypersensitivity and transcription factor binding site (ENCODE Project Consortium, 2012).

The *Cg23841288* is located in intron 1 of the protein tyrosine phosphatase, receptor type, N polypeptide 2 (*PTPRN2)* gene. This gene encodes a protein with a sequence similarity to the receptor-like protein tyrosine phosphatases (ENCODE Project Consortium, 2012).

The *Cg27391564* is related to the transcription factor binding site motifs of:

GM12878, H1-hESC, K562, HeLa-S3, HepG2 and HUVEC (ENCODE Project Consortium, 2012).

The results of this study show a significant increase in the DNA methylation level of *cg00648582* (0.79 ± 0.004) versus (0.43 ± 0.03), and *cg23841288* (0.44 ± 0.008) versus (0.15 ± 0.01) in the fertile, heavy smoker group compared to the proven fertile nonsmoker group respectively.

By contrast, there was a significant decrease in the DNA methylation level at the following CpGs, *cg09432376* (0.47 ± 0.002) versus (0.88 ± 0.01), *cg19169023* (0.55 ± 0.02) versus (0.92 ± 0.006), and *cg27391564* (0.51 ± 0.02) versus (0.86 ± 0.004) respectively (See Table 1).

Moreover, primers were designed to amplify the target CpGs region. Bisulphite-specific PCRs were performed on a large sample cohort (78 samples but without those that had been subjected to the Infinium 450K Bead Chip analysis) to validate the results obtained by the 450K BeadChip array (See Table 2).

The samples were divided into two groups, 45 from the fertile heavy smoker males with a mean age of (38.7 ± 7.9) as a case study group, and 33 samples from the proven fertile nonsmoker males as a control group with a mean age of (39.2 ± 6.1). When local deep bisulphite sequencing was applied on the larger cohort, different CpG sites were obtained besides the target CpG* which resulted from the 450k bead chip.

TABLE 2 Primers sequences and PCR annealing temperatures used to amplify the region

cgID	Chr	Annealing temperature (°C)	Length of product (bp)	Sequence (5′–3′)	
Cg00648582	Chr12	50.7	396	F	GGTTTTGTTGGGTTTATTAT
				R	TCTTCCTCATACTTTTCTCTAA
Cg09432376	Chr22	52.8	367	F	TGTTGTGTATTTTATAGGGTTGT
				R	CTTTCAATTTCCTTTTTACC
Cg19169023	Chr15	52.8	380	F	AAGAGAATGGGAATTGTTTATAG
				R	CCAAAAAAAAAACATAACCTACC
Cg23841288	Chr7	51.6	369	F	ATGTTGGGAGAGAAGGGAGT
				R	CCCTAAAATTCAAAAATTAAATC
Cg27391564	Chr2	51	392	F	ATGAGGGGAGATGTTAAATTGATAA
				R	CCAAACAAAATAAAAAACACCC

To the 5′-end of the oligos, universal adaptor sequences to perform Illumina sequencing were added in silico and then ordered.

Figure 1 illustrates the significant increase in the DNA methylation levels in the case study males compared to those in the control group. Fifteen CpGs of 25 CpGs in the PGAM5 gene-related amplicon were detected; these were as follows: CpG2, CpG4, CpG6, CpG8*, CpG12, CpG13, CpG15, CpG16, CpG19, CpG20, CpG21, CpG22, CpG23, CpG24 and CpG25 ($p \leq .02$, $p \leq .04$, $p \leq .03$, $p \leq .05$, $p \leq .02$, $p \leq .02$, $p \leq .03$, $p \leq .04$, $p \leq .02$, $p \leq .008$, $p \leq .04$, $p \leq .02$, $p \leq .01$, $p \leq .01$ and $p \leq .009$ respectively).

The *cg09432376* and its four neighbouring CpGs, which were found after deep sequencing, showed a decrease in the sperm DNA methylation level in the case group compared to the control group.

Only four of five CpG sites (CpG1, CpG2, CpG3* and CpG5) were significant ($p \leq .009$, $p \leq .01$, $p \leq .04$ and $p \leq .001$ respectively) (Figure 2).

Similarly, a significant decrease in the sperm DNA methylation level was shown at CpG1*, CpG3 and CpG4 in the *TYRO3* gene-related amplicon ($p \leq .008$, $p \leq .005$, p and $p \leq .02$ respectively) (Figure 3). Furthermore, a significant decrease was found at nine of twelve CpG sites in the *PTPRN2* gene-related amplicon, namely CpG2, CpG3, CpG4, CpG5, CpG6, CpG8, CpG9, CpG10 and CpG 12* in the case group compared to the control group, ($p \leq .006$, $p \leq .004$, $p \leq .01$, $p \leq .01$, $p \leq .01$, $p \leq .01$, $p \leq .03$, $p \leq .006$ and $p \leq .01$ respectively) (See Figure 4).

In addition, a significant decrease was observed in the *cg19455396* amplicons (CpG1, CpG3*, CpG5 CpG6, CpG7, CpG8 and CpG10) in the case group compared to the control group ($p \leq .03$, $p \leq .03$, $p \leq .04$, $p \leq .03$, $p \leq .008$, $p \leq .01$ and $p \leq .005$ respectively) (Figure 5).

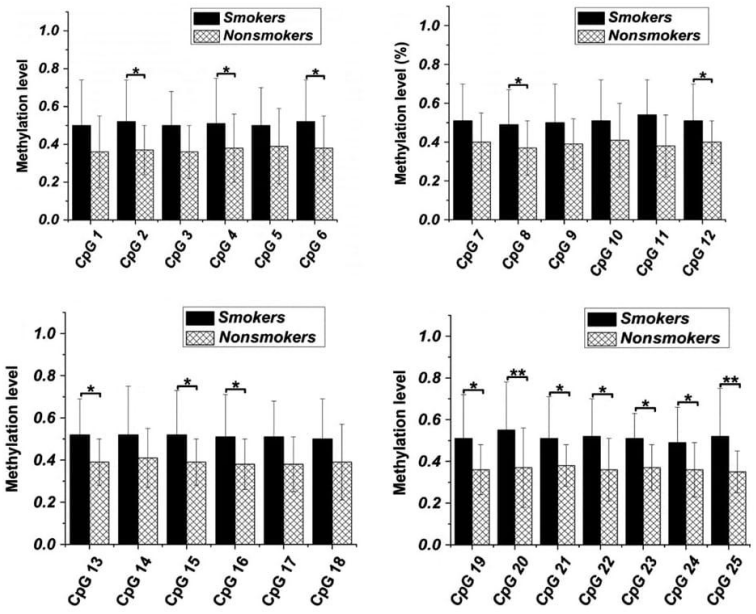

FIGURE 1 DNA methylation levels at (cg00648582) and its different neighbouring CpGs in *PGAM5* gene-related amplicons among smokers ($n = 45$) and nonsmokers ($n = 33$) $p \leq .05$*, $p \leq .01$**

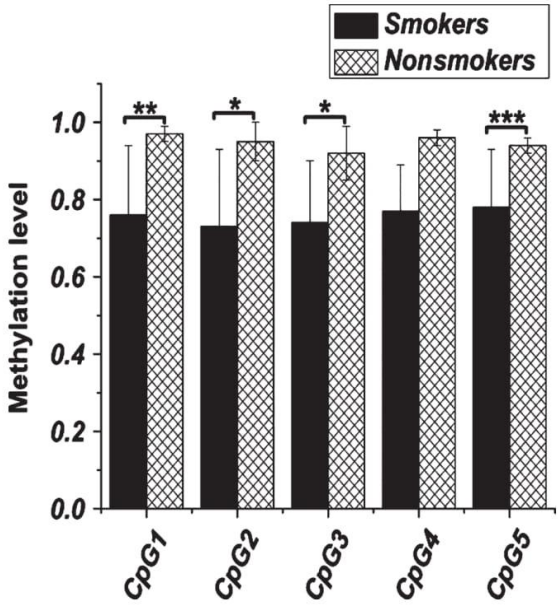

FIGURE 2 DNA methylation levels at cg09432376 and its different neighbouring CpGs-related amplicons $p \leq .05^{*}$, $p \leq .01^{**}$, $.001^{*}$

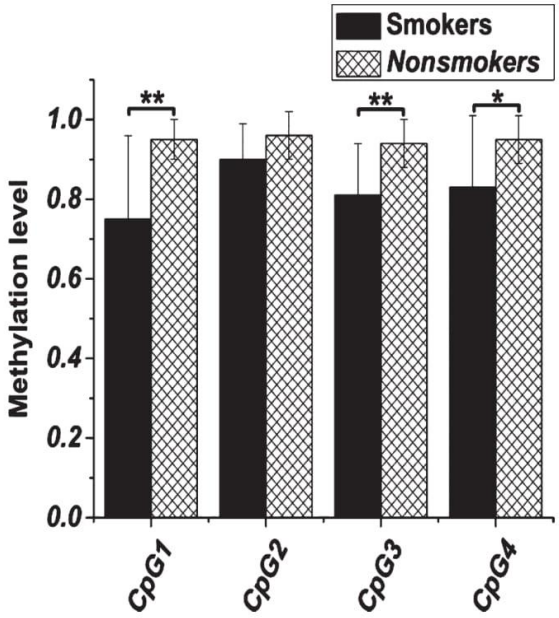

FIGURE 3 DNA methylation levels at (cg19169023) and its different neighbouring CpGs in *TYRO3* gene-related amplicons $p \leq .05^{*}$, $p \leq .01^{**}$

3.2 | Effect of cigarette-smoking on sperm parameters

As illustrated in Figure 6, a comparison of the investigated sperm parameters interestingly revealed that there was a significant difference between the two groups in the following parameters: sperm concentration (57.65 ± 21.00 versus 83.49 ± 8.02; $p \leq .004$), total sperm motility (46.30 ± 23.21 versus 61.93 ± 13.98; $p \leq .002$), number of progressive sperms (32.47 ± 22.31 versus 49.21 ± 15.11; $p \leq .0001$), number of immotile sperms (54.36 ± 24.02 versus 38.06 ± 13.98; $p \leq .002$), sperm morphology (31.02 ± 20.26 versus 35.60 ± 9.59; $p \leq .004$), sperm viability (using an eosin test) (49.95 ± 13.08 versus 59.21 ± 13.10; $p \leq .003$) and vitality (Hos test) (72.10 ± 15.00 versus 80.66 ± 7.33; $p \leq .02$) (See Figure 6).

3.3 | Correlation between sperm DNA methylation and sperm parameters in male smokers

The correlation between the DNA methylation level obtained by Bi-PROF in different CpGs and sperm parameters was assessed.

There was a significant correlation between Cg09432376 and its adjacent CpGs and sperm parameters in the following pattern:

- Sperm concentration: where CPG1, CPG2, CPG3, CPG4 and CPG5 ($p \leq .02$, $p \leq .01$, $p \leq .01$, $p \leq .02$ and $p \leq .03$ respectively) showed a positive correlation;
- Total sperm motility: where CPG1, CPG2, CPG3 and CPG5 ($p \leq .02$, $p \leq .007$, $p \leq .03$ and $p \leq .02$ respectively) were positively correlated;
- Mean percentage of progressive motility: All CpGs were positively correlated (CpG1, CpG2, CpG3, CpG4 and CpG5) showing ($p \leq .01$, $p \leq .004$, $p \leq .03$, $p \leq .04$ and $p \leq .02$ respectively);
- Immotile sperms: where CpG1, CpG2, CpG3 and CpG5 ($p \leq .01$, $p \leq .004$, $p \leq .02$ and $p \leq .01$ respectively) were negatively related;
- Normal sperm forms: where the entire CpGs revealed positive significant correlation (CpG1, CpG2, CpG3, CpG4 and CpG5 respectively) ($p \leq .01$, $p \leq .01$, $p \leq .04$, $p \leq .04$ and $p \leq .02$ respectively);
- Sperm viability (using an Eosin test): CpG1, CpG2, CpG3, CpG4 and CpG5 showed significant correlation ($p \leq .000$, $p \leq .000$, $p \leq .000$, $p \leq .000$ and $p \leq .000$ respectively);
- and sperm membrane integrity (vitality): where CpG1, CpG2, CpG3, CpG4 and CpG5 were intensely correlated ($p \leq .000$, $p \leq .000$, $p \leq .000$, $p \leq .000$ and $p \leq .000$ respectively), as shown in Table S1.

Moreover, a significant correlation between *Cg19169023*—and its adjacent CpGs in the *TYRO3* gene-related amplicon—and the other sperm parameters was observed as follows:

- The mean percentage of progressive motility was correlated with CpG1 ($p \leq .04$);
- Normal sperm forms (morphology) showed a correlation with CpG1 and Cp4 ($p \leq .01$, $p \leq .04$ respectively);
- Both sperm viability and sperm membrane integrity (vitality) showed a similar positive correlation with CpGs 1, 3 and 4 where the correlation of sperm viability was ($p \leq .000$, $p \leq .01$ and $p \leq .01$ respectively) and that of sperm membrane integrity was ($p \leq .000$, $p \leq .000$ and $p \leq .001$ respectively), as shown in Table S1.

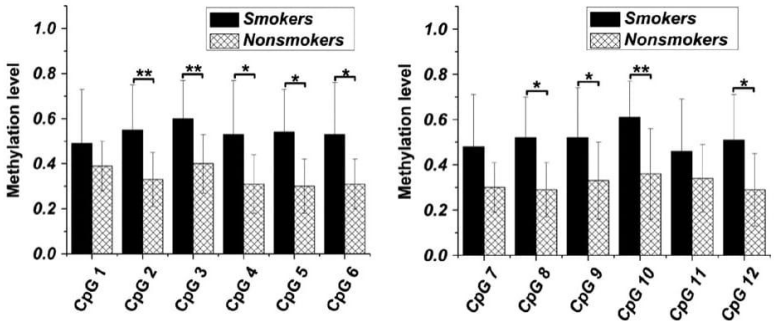

FIGURE 4 DNA methylation level at cg23841288 and its different neighbouring CpGs in *PTPRN2* gene-related amplicons $p \leq .05^{*}$, $p \leq .01^{**}$

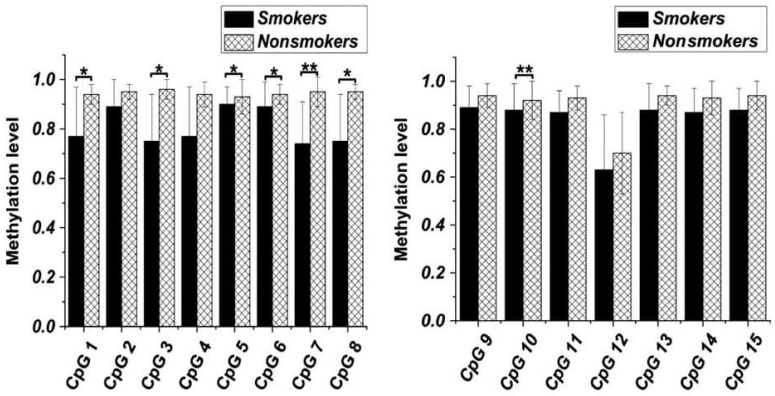

FIGURE 5 DNA methylation levels at cg27391564 and its neighbouring CpGs-related amplicons, $p \leq .05^{*}$, $p \leq .01^{**}$

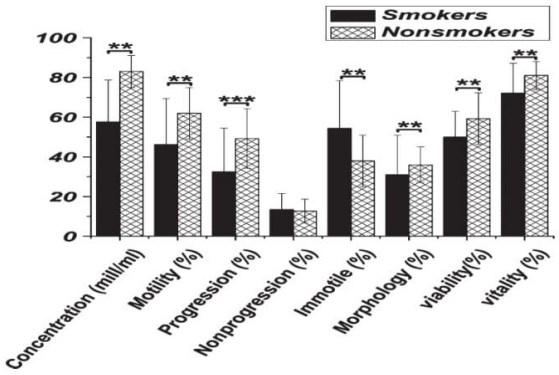

FIGURE 6 Effect of cigarette-smoking on sperm parameters in smokers ($n = 45$) and nonsmokers ($n = 33$) $p \leq .05^{*}$, $p \leq .01^{**}$, $.001^{***}$

A significant correlation was also found between the DNA methylation level at *Cg23841288* and its adjacent CpGs in the *PTPRN2* gene-related amplicon and the sperm parameters in the following distribution:

- The mean percentage of the sperm normal forms (Morphology): a significant correlation was found between the DNA methylation level at the following CpG1, CpG2, CpG3, CpG4, CpG5, CpG7, CpG8, CpG9, CpG11 ($p \leq .01$, $p \leq .04$, $p \leq .04$, $p \leq .02$, $p \leq .03$, $p \leq .005$, $p \leq .04$, $p \leq .04$ and $p \leq .01$ respectively);
- For both viability (using an Eosin test) and vitality (using a Hos test), the sperm parameters showed a negative correlation with almost all the related CpGs except for CpG11, which did not show any correlation. The correlation for viability accounted for ($p \leq .004$, $p \leq .002$, $p \leq .007$, $p \leq .003$, $p \leq .003$, $p \leq .001$, $p \leq .002$, $p \leq .003$, $p \leq .01$, $p \leq .02$ and $p \leq .002$ respectively) and that of vitality was ($p \leq .001$, $p \leq .000$, $p \leq .000$, $p \leq .000$, $p \leq .000$, $p \leq .000$, $p \leq .000$, $p \leq .000$, $p \leq .000$, $p \leq .001$ and $p \leq .000$ respectively);
- The mean percentage of total sperm motility and immotile sperm parameters showed a similar correlation regarding CpG10; however, sperm motility was negatively correlated ($p \leq .02$) in comparison with the immotile sperms, which were positively correlated ($p \leq .01$), as illustrated in Table S1.

The correlation found between the DNA methylation level at *cg27391564*—and its adjacent CpGs resulting from using a Bi-PROF-related amplicon—and the sperm parameters was only restricted to the following CpGs:

- CpG1 was significantly correlated with the mean percentage of sperm vitality and sperm membrane integrity ($p \leq .01$, $p \leq .000$ respectively);
- CpG3 was significantly correlated with the mean percentage of total sperm motility, progressive motility, immotile sperm, normal form, viability and sperm membrane integrity ($p \leq .01$, $p \leq .004$, $p \leq .009$, $p \leq .01$, $p \leq .000$ and $p \leq .000$ respectively);

- CpG4 was significantly correlated with the mean percentage of sperm viability and sperm membrane integrity ($p \leq .01$ and $p \leq .000$ respectively);
- CpG7 was correlated with the mean percentage of progressive sperm, normal form, sperm viability and sperm membrane integrity ($p \leq .04$, $p \leq .01$, $p \leq .000$ and $p \leq .000$ respectively);
- CpG8 was significantly correlated with the mean percentage of morphologically normal spermatozoa, sperm viability and membrane integrity ($p \leq .01$, $p \leq .000$ and $p \leq .000$ respectively), as shown in Table S1.

The remaining CpGs, however, did not show any significant correlation with the sperm parameters.

4 | DISCUSSION

The effects of cigarette-smoking on sperm DNA methylation and the correlation between DNA methylation and sperm parameters remain contradictory issues. Consequently, the main concern of this study was to highlight the various arguments supporting the divergent opinions/results concerning the evaluation of the effect of cigarette-smoking on sperm DNA methylation and its direct consequences on sperm parameters. Furthermore, this study aimed to assess the correlation between the alteration of sperm DNA methylation—as a result of smoking—and sperm parameters. A genome-wide assessment of CpG methylation revealed only 11 sites with more than a 20% difference between the cases and the controls, among which the first five CpGs were randomly selected to be tested by bisulphite sequencing. This result very clearly indicates the influence of cigarette-smoking on DNA methylation. When bisulphite sequencing was applied to a large cohort, the results showed a significant increase in the level of DNA methylation between the cases and the controls at 15 CpG sites in a *PGAM5* gene-related amplicon and nine CpG sites in a *PTPRN2* gene-related amplicon (See Figures 1 and 4). In contrast, the results demonstrated a significant decrease in the level of DNA methylation at three CpG sites in the *TYRO3* gene-related amplicon in the case group compared to the control group (See Figure 3). In addition, a decrease in the level of DNA methylation was shown in *cg09432376* and *cg 27391564* and some of their neighbouring CpGs (See Figures 2 and 5). That this alteration in the sperm DNA methylation level was detected in this study is in agreement with other previous studies that showed that the DNA methylation level is significantly altered by cigarette-smoking (Breitling et al., 2011; Dogan et al., 2014; Sun et al., 2013; Zhu et al., 2016).

The PGMA5 gene is located on chromosome 12 and encodes two protein isoforms, PGAM5s and PGMA5L (phosphoglycerate mutase family member 5), which are mitochondrial proteins (ENCODE Project Consortium., 2012). Both proteins are expressed in the testes and play a vital role in mitophagy, which is a cellular process that eliminates damaged mitochondria (Chen et al., 2014; Wu et al., 2014). Moreover, it is also considered as an antioxidant regulator through binding to the substrate of the E3 ligase cul3 adaptor Keap1 (Lo & Hannink, 2006, 2008).

The TYRO3 gene is part of a 3-member transmembrane receptor kinase family (*TYRO3, Axl and Mer*), located on chromosome 15; it encodes the Tyrosine-protein kinase receptor (*TYRO3*), which is stimulated by the growth arrest-specific gene 6 (Gas6) and protein S genes and plays a vital role in the spermatogenesis process (Godowski et al., 1995; Joseph, 1997; Richard et al., 1994). Moreover, the Sertoli cells are essential for the maturation of germ cells, endocytosis and the degradation of apoptotic and residual bodies, which results from most of the degraded spermatogenic cells (Jégou, 1991). Any defect in TYRO3 leads to failure in the production of mature sperm (Lu et al., 1999).

The PTPRN2 gene is located on chromosome seven and encodes the protein tyrosine phosphatase receptor type, N polypeptide 2. It is also known as an islet cell autoantigen-related protein ICAAR, phogrin, IA2ß (ENCODE Project Consortium, 2012; Leiter et al., 1997; Van den Maagdenberg et al., 1998). This encoded protein (PTPRN2) has a crucial role in insulin-dependent diabetes mellitus through its involvement in insulin secretion (Li et al., 1997; Smith et al., 1996). Disruption of the PTPRN2 gene leads to a slight change in the results of a glucose tolerance test and influences insulin secretion as well (Kubosaki et al., 2004). Previous studies found that diabetic patients have a reduction in semen parameters (a low volume of seminal fluid, sperm concentration, motility and morphology) compared to nondiabetics (Vignera, Condorelli, Vicari, D'Agata, & Calogero, 2012).

The present study has uniquely demonstrated the relationship between these afore-mentioned genes, namely *PGMA5*, *TYRO3* and *PTPRN2*, and the effect cigarette-smoking has on the human sperm DNA methylation level.

Cigarette-smoking adversely affects sperm parameters. The exact mechanism of this negative effect still remains incompletely understood but it might be due to the effect of toxic chemicals in cigarette-smoke, such as carbon monoxide, nicotine, cotinine, cadmium and carcinogens, which interact with gametes and disrupt their functions and viability (Sepaniak et al., 2006; Thompson & Bannigan, 2008). The accumulation of cadmium leads to a reduction of zinc in seminal plasma and to the disruption of spermatogenesis, which causes low sperm concentration, impaired motility, abnormal morphology and disturbed sperm membrane stability (Liu, Sie, Liu, Agresta, & Baker, 2009; Mankad, Sathawara, Doshi, Saiyed, & Kumar, 2006; Martelli, Rousselet, Dycke, Bouron, & Moulis, 2006; Shelko, F Hamad, Montenarh, & E Hammadeh, 2016). Furthermore, smoking leads to an alteration in the balance between oxidants and endogenous antioxidants, through decreasing vitamins, such as ascorbic acid (vitamin C) (Mostafa et al., 2006), Tocopherol (Vitamin E) (Sobczak, Gołka, & Szołtysek-Bołdys, 2004) and enzymatic antioxidants, which all act as useful scavengers. It also has a pivotal role in elevating the harmful oxidants such as reactive oxygen species (ROS) (Aitken & Baker, 2006). Cigarette-smoking leads to a high production of ROS through a myriad of different mechanisms, such as the high level of ROS being present in the cigarette-smoke per se. Furthermore, the inflammatory reaction, induced by smoking metabolites that adversely affect the male genital tract, leads to the release of inflammatory substances, which activate

leucocytes that produce a high amount of ROS. In addition, the elevation of ROS contributes to the destruction of endogenous antioxidants (Cross, Traber, Eiserich, & Van Der Vliet, 1999), which impairs spermatogenesis and induces sperm DNA damage (Aitken & Koppers, 2011). Moreover, ROS induce lipid peroxidation of the polyunsaturated fatty acids in the sperm membrane, and this causes a loss of membrane fluidity and integrity, and subsequently a loss of sperm fertilisation capacity (Arabi & Moshtaghi, 2005; Kao et al., 2008; Velando, Torres, & Alonso Alvarez, 2008). In the present study, the mean percentage of sperm concentration, motility, progressive motility, viability and sperm membrane integrity (vitality) is lower in heavy smokers. In addition, the mean percentage of immotile sperm is higher in heavy smokers (See Figure 6) compared to nonsmokers.

There has been considerable discussion regarding the effect of cigarette-smoking on sperm parameters. Some authors found that cigarette-smoking negatively affects sperm parameters; others found that it does not affect sperm parameters. In the present study, significant differences were found in heavy smokers compared to nonsmoker males. These results were in accordance with those from many previous studies (Hammadeh et al., 2010; Jong, Menkveld, Lens, Nienhuis, & Rhemrev, 2014; Zhang et al., 2013).

Furthermore, the present study showed a correlation between the DNA methylation of some DMCs and sperm concentration, the mean percentage of motility and immotile sperms (See Table S1), which agreed with the results found by Montjean et al. (2015) and Laqqan et al. (2017). However, this study remarkably showed a significant correlation between the DNA methylation of some CpGs and the mean percentage of progressive sperm, abnormal morphology, viability and sperm membrane integrity (vitality) (See Table S1).

5 | CONCLUSION

This study has demonstrated biologically the relevant differences in sperm DNA methylation levels at different CpGs in *PGAM5*, *PTPRN2* and *TYRO3* gene-related amplicons, which could be potentially related to the effects of smoking on the development of spermatozoa. In addition, a correlation was clearly found between the alteration in sperm DNA methylation levels at some CpGs and sperm parameters (concentration, total motility, progressive motility, normal morphology, viability and vitality).

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LIMITATIONS

The sample size was relatively small.

CONFLICT OF INTEREST

None of the authors have any conflict of interest to be declared.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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3.2 Cigarette smoking induces only marginal changes in sperm DNA methylation levels of patients undergoing intracytoplasmic sperm injection treatment

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ORIGINAL ARTICLE

WILEY ANDROLOGIA

Cigarette smoking induces only marginal changes in sperm DNA methylation levels of patients undergoing intracytoplasmic sperm injection treatment

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Summary

DNA methylation plays important roles in genome stability and regulation of gene expression. This study was designed to determine the influence of cigarette smoking on sperm DNA methylation. From a genome-wide survey on sperm samples, differentially methylated target CpGs should be selected and subjected to local deep bisulphite sequencing. Obtained methylation data are compared to sperm parameters and (ICSI) outcome. Similar to pilot study, samples were subjected to Infinium 450K BeadChip arrays to identify alterations in sperm DNA methylation between smokers and nonsmokers males. Routine testing on a significantly altered CpG site was performed on more samples using local deep bisulphite sequencing. Of approximately 485,000 CpG sites analysed, only seven CpGs were found to show a significant DNA methylation difference of >20% with the top six CpGs overlapping common SNP sites. The remaining CpG site (cg19455396) is located in intron 12 of the TAP2 gene. The results of deep bisulphite sequencing showed only a tendency towards hypomethylation in the smoking group. This study could not detect biologically relevant CpG positions that are altered in sperm DNA methylation on the influence of cigarette smoking beyond individual-specific effects that may be caused by other environmental factors.

KEYWORDS
DNA methylation, ICSI, smoking, sperm, TAP2 gene

1 | INTRODUCTION

Epigenetics is defined as stable but reversible modification of the DNA and histone structure without changing the DNA sequence itself, which can be inherited through mitotic or meiotic division (Carrell, 2012). Epigenetic processes include actions such as DNA methylation, histone modifications, and chromatin remodelling (Anway, Cupp, Uzumcu, & Skinner, 2005; Richardson, 2002). DNA methylation plays an important role in the regulation of gene expression and occurs by the action of DNA methyltransferases (DNMTs) mostly on the fifth carbon atom of cytosine (5mC) found in cytosine-phosphate-guanine dinucleotides "CpGs" (Portela & Esteller, 2010). DNA methylation could be modified by genetic, environmental factors, etc. Cigarette smoking is one example of environmental factors and it can potentially integrate the effects of both gene expression and environmental factors on male infertility (Feil & Fraga, 2012). According to the World Health Organization (WHO) and American Society for Reproductive Medicine (ASRM), infertility is defined as failure of couples to get a child during 12 months of unprotected regular intercourse (Bayer, Alper, & Penzias, 2011; Rowe & Comhaire, 2000). Worldwide, about 10%–20% of couples suffer from infertility (Krausz, Quintana-Murci, & McElreavey, 2000). In many cases, the underlying causes are unknown and about 60%–70% of male infertility cases are idiopathic (Filipponi & Feil, 2009). Previous studies revealed that aberrant DNA methylation levels in spermatozoa may be correlated with male infertility (Hammoud, Purwar, Pflueger, Cairns, & Carrell, 2010; Nanassy & Carrell, 2011). Moreover, changes in DNA methylation levels of some genes were related to a decrease in semen parameters (Aston, Punj, Liu, & Carrell, 2012; Houshdaran et al., 2007). Cigarette smoking was considered as a major cause of premature death and many diseases

worldwide. Besides, it leads to serious defects of spermatozoa (Ezzati & Lopez, 2003; Mathers & Loncar, 2006; Thun, DeLancey, Center, Jemal, & Ward, 2010). It has been previously shown that cigarette smoking increases reactive oxygen species (ROS) and decreases the level of antioxidant concentrations, which leads to many changes in semen parameters (Frey, Navarro, Kotelchuck, & Lu, 2008; Soares & Melo, 2008). This is in agreement with other studies (El-Melegy & Ali, 2011; Hammadeh, Hamad, Montenarh, & Fischer-Hammadeh, 2010; Pasqualotto, Sharma, Pasqualotto, & Agarwal, 2008; Saleh, Agarwal, Sharma, Nelson, & Thomas, 2002; Taha, Ez-Aldin, Sayed, Ghandour, & Mostafa, 2012) which found that smoking has a negative effect on sperm parameters, including semen volume, sperm concentration, motility and DNA fragmentation. Contrary to that, some studies failed to find any influence of cigarette smoking on sperm parameters (Jong, Menkveld, Lens, Nienhuis, & Rhemrev, 2014; Ozgur, Isikoglou, Seleker, & Donmez, 2005). Besides, cigarette smoking has an influence on the process of protamination, which results in the change in the ratio of P1 to P2 by the disruption of P2 (Hammadeh et al., 2010). In addition, cigarette smoking leads to changes in the histone H2B and protamine ratio P1/P2 (Hamad, Shelko, Kartarius, Montenarh, & Hammadeh, 2014). Moreover, cigarette smoking has been correlated with poor sperm function in sperm penetration tests (Vine, 1996) as well as a decrease in fertilisation capacity and implantation rate efficiency (Ramlau-Hansen, Thulstrup, Olsen, & Bonde, 2008; Soares, Simon, Remohi, & Pellicer, 2007; Zitzmann et al., 2003). Many studies reported that cigarette smoking affects gene expression and is associated with changes in DNA methylation (Charlesworth et al., 2010; Monick et al., 2012; Shenker et al., 2013; Zeilinger et al., 2013). It has been documented that epigenetics is associated with poor spermatogenesis, decreased male fertility, declined fertilisation potential of spermatozoa and ICSI outcome (Glaser et al., 2009; Oakes, La Salle, Smiraglia, Robaire, & Trasler, 2007). Also, DNA methylation may impact embryo development and consequently may correlate with reduced results of pregnancy, due to the global sperm DNA hypomethylation (Anifandis, Messini, Dafopoulos, & Messinis, 2015; Benchaib et al., 2005). CpG methylation within gene promoter regions is of particular importance because hypermethylation of promoter CpG islands plays a key role in transcriptional suppression, while hypomethylation in that region is associated with elevated levels of gene expression (Bronner, Chataigneau, Schini-Kerth, & Landry, 2007; Zhang et al., 2006).

The purpose of this study was to determine the influence of cigarette smoking on sperm DNA methylation level and its association with sperm parameters and ICSI outcome. Genome-wide array analysis was used to define differential methylated CpGs between smokers and nonsmokers males, which was validated in more patients using local deep bisulphite sequencing.

2 | MATERIAL AND METHODS

2.1 | Ethics statement

Institutional review board approval (No. PHRC/HC/13/14) was obtained before initiation of this study, and informed consent was provided according to the Declaration of Helsinki. Each participant had given a written agreement for inclusion into 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 of Saarland, Germany.

2.2 | Sample collection and semen analysis

Semen samples were collected from partners of couples who underwent assisted reproduction techniques for infertility treatment, at the Department of Obstetrics and Gynecology, Saarland University, Germany. Semen samples were collected by masturbation after 3 days of sexual abstinence in a clean, dry, sterile and leak-proof plastic container in a collection room attached to the laboratory. Following liquefaction of semen at 37°C for 30 min, the sperm parameters such as sperm concentration and motility were determined according to World Health Organization guidelines (World Health Organization, 2010) using Meckler chamber (Sefi-Medica, Haifa, Israel). Briefly, the normal concentration was 15×10^6 mill/ml, and the motility was classified into three categories: (A) progressive motility, (B) nonprogressive motility and (C) immotile spermatozoa. In this study, we included motile sperm categories (A + B) together and excluded immotile sperm category (C). According to smoking status, samples were classified into two groups.

2.3 | Smoking assessment

The cigarette smoking status was evaluated according to the number of cigarettes consumed per day. Smokers had smoked >20 cigarette per day during the last year until the enrolment in this study. Cigarette smoking persons were categorised into two groups: nonsmokers (n = 20) and heavy smokers (n = 19).

2.4 | Embryo quality assessment

Embryo quality was evaluated on day 2 (pronucleus stage) and day 3 after ICSI, according to Scott, Alvero, Leondires, and Miller (2000), Anifandis, Dafopoulos, Messini, Chalvatzas, and Messinis (2010). Briefly, embryos at day 3 were scored based on the following morphological parameters: the number of blastomeres, their regularity, multinucleation (more than one nucleous in each blastomere) and the amount of cytoplasmic fragmentation. Embryos have four same-size non-multinucleated blastomeres, and those that were not fragmented were classified as grade one. Grade two embryos have about 25% fragments or two to four multinucleated blastomeres with different size. Grade three embryos have less than two blastomeres, multinucleated blastomeres, and between 25% and 35% fragmentations.

2.5 | Exclusion criteria

The following parameters were taken into consideration for the exclusion: varicocele, antisperm antibodies, Y chromosome microdeletions,

males subjected to surgical operation in the reproductive system, heavy body mass index and advanced age >55 years.

2.6 | Spermatozoa purification

Each semen sample was loaded on 45%–90% discontinuous PureSperm gradients (Nidacon International, Sweden) and centrifuged at 500× *g* for 20 min at room temperature to separate sperm cells from semen fluid. The pellet was washed twice with Ham-F10 medium supplemented with human serum albumin (5 mg/ml) and penicillin G/streptomycin sulphate (0.1 mg/ml; PAN Biotech, Germany) and carefully overlaid with 0.75 ml of the same medium. Samples were placed in an incubator at 37°C, and after 45 min, the upper layer containing the motile spermatozoa was withdrawn, immediately assessed for sperm count and motility and stored at −20°C until processing (not more than 3 months).

2.7 | Sperm DNA isolation

Sperm DNA was isolated using the Isolate II Genomic DNA Kit according to the standard protocol provided by the manufacturer (Cat. #: BIO 52066, Bioline, UK). The extracted DNA was measured using the NanoDrop Spectrophotometer 2000c (Thermo Scientific).

2.8 | Sodium bisulphite treatment

500 ng of extracted sperm DNA was treated with sodium bisulphite, using the Epitect Bisulfite Conversion Kit (Cat. #: 59104, Qiagen, Germany), that converts unmethylated cytosines to uracil, while the 5-methylcytosine (5MeC) remains unaltered, as previously described (Wu, Kang, Zheng, Liu, & Liu, 2015).

2.9 | DNA methylation analysis by Infinium 450K BeadChip array

Samples were subjected to Infinium 450K BeadChip arrays at Life and Brain GmbH Biomedicine and Neuroscience Technology in Bonn following the manufacturer's recommendations (Bibikova et al., 2011) as a pilot study, to identify alterations in sperm DNA methylation regions between smokers and nonsmokers males with unexplained infertility. Samples were whole-genome-amplified and put on the bead arrays as described (Sandoval et al., 2011). Bioinformatic processing and evaluation were performed with the RnBeads program package (Assenov et al., 2014). DNA methylation differences between smokers and nonsmokers males were found to be significant when $p \leq .01$. According to these results, we selected CpGs not overlapping any common SNP site according to dbSNP142 and located in candidate genes that are related to male infertility to study them in 19 samples using local deep bisulphite sequencing (Bi-PROF, Gries et al., 2013).

2.10 | Bisulphite profiling (Bi-PROF)

Hundred nanogram of genomic DNA was bisulphite-treated using the Epitect Bisulfite Conversion Kit (Cat. #: 59104, Qiagen, Germany). PCRs encompassing the differentially methylated CpGs identified by 450K BeadChip array were performed in a 30 µl total volume reaction using the "MyTaq™ HS Red Mix" with 2× concentration (Catalog #: BIO-25047, Bioline, UK) according to the manufacturer's protocol. For the amplification, fusion primers were used that consist of a specific 3′-portion (listed in Table 1 together with respective annealing temperatures) and a universal 5′-portion containing the necessary nucleotide sequences for Illumina sequencing. Five microlitre of PCR was loaded on a 2% agarose gel, including the DNA ladder (Cat. #: N0467S, Biolabs), and stained with ethidium bromide. PCR products were purified using the Agencourt® AMPure XP beads (Beckman Coulter, USA) and measured by using Quant-iT™ 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 sequencing step were processed, filtered and aligned using BiQ Analyzer HT software (Lutsik et al., 2011), and all reads containing more than 10% of missing CpG sites were excluded (maximal fraction of unrecognised sites ≥0.1).

2.11 | Statistical analysis

Data were analysed using IBM SPSS for Windows software package version 23.0 (SPSS Inc., USA). Samples included in this study were non-normally distributed according to the value of skewness test, Kurtosis test, *Z*-value and Shapiro test. The independent-samples *t*-test (Mann–Whitney *U*-test) was used to compare means of quantitative variables. The results in all the above-mentioned procedures were accepted as statistically significant when the *p*-value was less than 5% ($p < .05$).

3 | RESULTS

Sperm samples obtained from smokers and nonsmokers males were subjected to Infinium 450K BeadChip analysis detecting the DNA

TABLE 1 Primer sequences and PCR annealing temperatures used to amplify the region including cg19455396. To the 5′-end of the oligos, universal adaptor sequences to perform Illumina sequencing were added in silico and then ordered

Gene Name	CpG Position	Chr	Annealing temperature (°C)	Amplicon length (bp)	Sequence (5′–3′)	
TAP2	1,797,050	Chr 6	52.4	167 bp	F	GTAAAATGGGGATAATAATTAA
					R	AAAACAAAAAACAATTCCCT

methylation level of ~485,000 CpGs covering 99% positions in each sample. As shown in Table 2, 450K BeadChip analysis revealed seven CpGs (namely cg08225549, cg01835922, cg13284789, cg06202802, cg13496755, cg04012354, and cg19455396) showing a significant and biologically relevant difference of >20% between the two analysed groups. The six most different CpG sites (cg08225549, cg01835922, cg13284789, cg06202802, cg13496755, and cg04012354) were found to overlap annotated common polymorphisms, so they were excluded from further analysis. The least different CpG site (cg19455396) was found to be located in intron 12 of the TAP2 gene, a member of transporter associated with antigen-processing genes that are located within MHC class II loci on the short arm of human chromosome 6 (Campbell & Trowsdale, 1993; Spies et al., 1990; Trowsdale et al., 1990). CG19455396 did not overlap any enhancer-related histone marks (i.e., H2K4me1 or H3K27ac) or transcription factor binding sites (ENCODE Project Consortium, 2012). There was a significant hypomethylation of cg19455396 in the smokers group (72.7% versus 93.6%) compared to the nonsmokers group. Although we did not have any indication for a regulatory role of this CpG site, primers were designed to amplify the target CpG region and bisulphite-specific PCRs were performed on a larger sample cohort (39 samples) to validate the results obtained by 450K BeadChip array. After local deep bisulphite sequencing obtaining several thousand reads per CpG and sample, we found a noncommon SNP in 20 samples, so these samples were excluded from further evaluation. The remaining 19 samples were grouped into 10 samples nonsmokers (52.6%) with a mean age (40.10 ± 9.09 years), and nine samples smokers (47.4%) with a mean age (37.67 ± 3.71 years).

Although a tendency towards hypomethylation in the smokers group could be observed, the methylation difference at cg19455396 between smokers and nonsmokers was not significant (0.97 ± 0.01 versus 0.88 ± 0.20; $p \leq .28$; Figure 1). This result is mostly due to high individual variation (standard deviation of ~20%) of methylation in the smokers group. Comparison of investigated sperm parameters revealed no significant changes between the two groups in sperm count (10.13 ± 8.18 versus 11.25 ± 3.94; $p \leq .16$), sperm motility (37.13 ± 19.17 versus 25.93 ± 12.12; $p \leq .15$), number of fertilised oocytes (8.10 ± 6.11 versus 4.11 ± 2.52; $p \leq .09$), fertilisation rate (2.3 ± 1.63 versus 1.55 ± 1.58; $p \leq .27$) and ICSI outcome (1.80 ± 0.78 versus 2.11 ± 0.78; $p \leq .38$; Table 3). To assess the influence of age on sperm parameters, the samples of this work were classified according to the reproductive age into younger than or equal to 40 years ($n = 12$) and older than 40 years ($n = 7$; Table 4). Sperm count, sperm motility, fertilisation rate, ICSI outcome and methylation level were similar in both age groups. However, the mean number of fertilised oocytes (4.00 ± 1.90 versus 10.00 ± 6.63) showed significant differences ($p \leq .008$) among the different age groups.

TABLE 2 Identification of differentially methylated CpG sites in sperm samples from three subfertile smokers and three subfertile nonsmokers. The CpG site taken for testing in a larger cohort is highlighted in bold

cigd	Chromosome	Start	Strand	Nonsmoker	Smoker	Diff meth.p.adj.fdr
cg08225549	chr8	80,550161	–	0.919473281	0.54090129	0.005753125
cg01835922	chr11	121,626,930	+	0.046942809	0.481236921	0.005753125
cg13284789	chr3	113,254,986	–	0.506531427	0.893586647	0.005753125
cg06202802	chr4	7,436,239	+	0.392985674	0.884584572	0.005753125
cg13496755	chr9	92,681,430	–	0.912903277	0.481296324	0.006200438
cg04012354	chr4	143,771,325	+	0.910077769	0.53827173	0.012719443
cg19455396	**chr6**	**327,96,056**	**–**	**0.936038247**	**0.726639221**	**0.047734324**

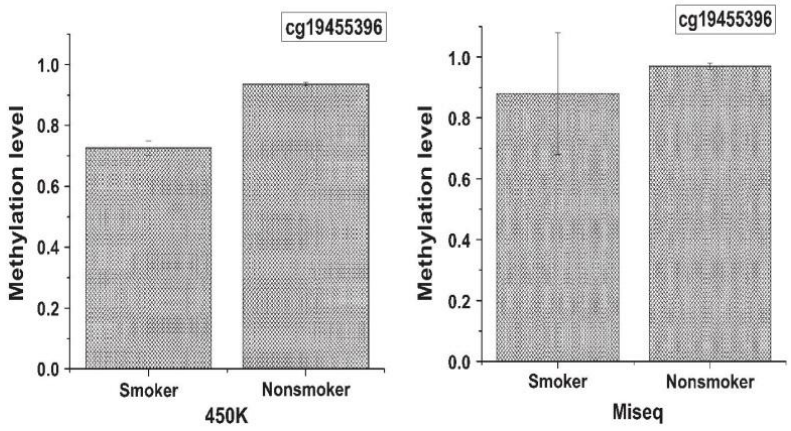

FIGURE 1 DNA methylation level at cg19455396 in smoker versus nonsmoker groups. Left: 450K BeadChip results, right: local deep bisulphite sequencing results

TABLE 3 The main sperm and ICSI result parameters of the studied sample cohort

	Heavy smokers	Nonsmokers	U[a]	p[b]
Concentration (10^6 spermatozoa/ml)	11.25 ± 3.94	10.35 ± 8.18	28.00	.16
Sperm motility	25.93 ± 12.12	37.13 ± 19.17	27.50	.15
Fertilised oocytes	4.11 ± 2.52	8.10 ± 6.11	25.00	.09
Fertilisation rate	1.55 ± 1.58	2.3 ± 1.63	32.50	.27
ICSI results	2.11 ± 0.78	1.80 ± 0.78	35.00	.38
Methylation rate	0.88 ± 0.20	0.97 ± 0.01	32.00	.28

[a]U = Mann–Whitney *U*-test.
[b]p = p value.

TABLE 4 Comparison between sperm parameters and DNA methylation levels at cg19455396 in relation to the study age group

	Age (year)		
Variables	Age ≤40	Age >40	*p* value
Sperm count milli/ml %	10.91 ± 5.66	10.54 ± 7.92	.61
Sperm motility %	29.53 ± 12.16	35.75 ± 23.38	.55
Number of Fertilised oocytes	4.00 ± 1.90	10.00 ± 6.63	.008
Fertilisation rate of oocytes	0.55 ± 0.29	0.73 ± 0.16	.17
ICSI results	2.00 ± 0.73	1.86 ± 0.90	.68
TAP2 Methylation level at cg19455396	0.90 ± 0.20	0.98 ± 0.005	.86

4 | DISCUSSION

The purpose of this study was to investigate the relationship between cigarette smoking and DNA methylation of spermatozoa obtained from males who presented for treatment at the Andrology Laboratory of the Department of Obstetrics; Gynecology & Assisted Reproduction Laboratory, University of Saarland, Germany. Genome-wide assessment of CpG methylation revealed only seven sites with more than 20% difference between smokers and nonsmokers with six CpG sites overlapping common SNPs. This result clearly points to the influence of cigarette smoking on DNA methylation to be minimal and probably not of high biological relevance. Of course, 450K BeadChip arrays only cover ~2% of all CpGs present in the human genome and the selection of CpGs for analysis on this platform was biased towards cancer-related loci. However, most of the loci analysed are located in promoter and known enhancer sequences, so potentially regulatory regions are well covered. The remaining differential methylated CpG site is located in intron 12 of the TAP2 gene with no overlap to known regulatory marks like transcription factor binding sites or enhancer-marking histone modifications (H3K4me1 and H3K27ac (ENCODE Project Consortium, 2012). CG19455396 barely overlaps a weak DNAseI hypersensitivity site, which might point to its accessibility in an at least partially open chromatin region. When local deep bisulphite sequencing on a larger cohort was applied, 18 samples showed an SNP at cg19455396 which was not annotated as common SNP in dbSNP142 database. Excluding those from analysis and looking at the remaining 19 samples revealed no significant difference in the level of DNA methylation at cg19455396 ($p \leq .28$) between smokers and nonsmokers. Still, a tendency towards hypomethylation in smokers could be observed with high individual DNA methylation variation at cg19455396 in the smoker group only. The effect may vanish when a larger cohort is analysed, which may, of course, be beneficial to support our findings.

TAP gene family contains TAP1 and TAP2, which encode TAP1 and TAP2 transporters, that harbour ATP-binding cassettes (ABC) (Higgins, 1992). ABC proteins transport different molecules across the extra- and intracellular membrane linking the TAP2 gene with autoimmune diseases such as insulin-dependent diabetes (Jackson & Capra, 1995). According to the results found in the studies by Garcia-Diez, Corrales Hernandez, Hernandez-Diaz, Pedraz, and Miralles (1991) and Miralles-Garcia and Garcia-Diez (2004), insulin-dependent diabetes mellitus is associated with male infertility (low volume of seminal fluid, sperm concentration, motility, and morphology). Although no obvious regulatory features were found overlapping cg19455396, it cannot be excluded that its hypomethylation influences the expression of the TAP2 gene. At least for some patients in the analysed cohort, this finding could be relevant. The mechanisms of the impact of cigarette smoking on sperm parameters are not fully understood, but a possible explanation is the direct toxic influence of nicotine and other chemical components in the epithelium of the male genital tracts with subsequent release of chemical mediators of inflammation, such as interleukin-6 and interleukin-8, which can recruit and activate leucocytes (Kumosani, Elshal, Al-Jonaid, & Abduljabar, 2008; Saleh et al., 2002; Zenzes, 2000). The activated leucocytes can produce high amounts of reactive oxygen species (ROS) in semen and may decrease the antioxidant capacity resulting in oxidative stress (Aitken, 1995). Furthermore, the number of cigarettes consumed per day was found to associate with the accumulation of cotinine and increase in the ROS, which leads

to damage of sperm membrane, due to their structure with a high proportion of polyunsaturated fatty acids (El-Melegy & Ali, 2011; Sharma & Agarwal, 1996). In the present study, sperm concentration was not significant and also higher in the heavy smokers than in nonsmokers (Table 3). These results are in accordance with the results found by Trummer, Habermann, Haas, and Pummer (2002), Hassa et al. (2006) and Anifandis et al. (2014).

In addition, the fertilisation rate showed no significant changes between nonsmokers and smokers when all patients independently of their age were analysed. However, when grouping the samples into those obtained from patients younger than or equal to 40 years (n = 12) and older than 40 years (n = 7), a highly significant difference in the number of fertilised oocytes among the different age groups ($p \leq .008$) was found. Also, no significant differences were found at this CpG site in the two different age groups, but there was only a tendency of DNA methylation levels to be elevated at advanced age.

5 | CONCLUSION

This study revealed only marginally biologically relevant differences in DNA methylation that could potentially be related to effects caused by smoking on the development of spermatozoa. Hypomethylation of sperm DNA obtained from smokers at a single CpG site in intron 12 of the TAP2 gene was individual specific and cannot be regarded as a general hypomethylation effect. Besides, there was no association between methylation and age in both groups.

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3.3 Aberrant DNA methylation patterns of human spermatozoa in current smoker males

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Aberrant DNA methylation patterns of human spermatozoa in current smoker males

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ABSTRACT

The purpose of this study was to investigate the impact of current cigarette smoking on sperm DNA methylation patterns. A total of 108 males (51 current smokers and 57 never smoked males) were included in the study. Using 450 BeadChip Arrays, the differentially methylated CpGs between current smokers (n = 15) and never smoked males (n = 15) were identified. Out of significantly 11 CpGs identified, 2 CpGs namely cg07869343 and cg19169023, which are located in the *MAPK8IP3* and *TKR* genes were selected for further analysis. Using deep bisulfite sequencing in an independent cohort of current smokers (n = 36) and never smoked males (n = 42), 6 and 1 CpGs showed a significant difference in the *MAPK8IP* (CpG3, CpG5, CpG6, CpG7, CpG8, and CpG21) and in the *TKR* (CpG4) were identified, respectively ($P \leq 0.05$). Our results indicate that cigarette smoking causes biochemical changes in the sperm DNA methylation in many regions and could adversely affect semen parameters.

1. Introduction

Epigenetics defined as alterations in gene expression without changing the DNA sequence or a change in phenotype without a change in genotype [1]. These changes can be transmitted through both mitotic and meiotic cell divisions and occurred through various mechanisms like DNA methylation, histone modifications, and chromatin remodeling [2]. The addition of a methyl (CH3) group to a 5th carbon atom of a cytosine of CpG dinucleotides to form 5-methylcytosine by DNA methyltransferase defined as DNA methylation [3]. In mammals, the CpG islands occur mostly in the promoter region and remain unmethylated, while the majority of all other CpG islands are methylated [4]. When CpG islands in promoter regions are methylated, the transcription of the corresponding gene is usually suppressed [5]. Methylation status of CpGs located in the promoter and intragenic regions play an important role in gene repression and activation, respectively [6]. In addition, the DNA methylation involves the regulation of gene splicing [7], modulates the activity of enhancers [8] and maintains the chromosomal stability [9]. In human, the DNA methylation patterns can be influenced by several factors including environmental and lifestyle factors, like cigarettes [10]. The cigarette smoke is considered one of the environmental factors that affect on the DNA methylation [11,12]. However, there is a lack of information about the effect of cigarette smoking on sperm DNA methylation patterns. The cigarette smoking potentially contributes to the reduction in male fertility, specifically effects sperms motility and morphology [13–15]. Recent studies showed that the cigarette smoking can adversely influence the transcriptome [16], causes chromosomal aberration [15,17] and alter the DNA methylation pattern in sperms [18]. However, the impact of cigarette smoking on sperm DNA methylation remains debatable, and there are several mechanisms have been hypothesized. These mechanisms were concerning the deterioration of spermatogenesis [19], induction of ultrastructural abnormalities, and apoptosis [20,21]. In this study, we aim to identify whether cigarette smoking alters sperm DNA methylation patterns and to determine whether the change in DNA methylation is associated with basic semen parameters like sperm count, sperm motility, and morphology.

2. Material and methods

2.1. Ethics statement

This study was approved by the Institutional Ethics Committee of Saarland University, and consent was provided according to the declaration of Helsinki. All participants gave written consent before participation in this study. All of the samples were analyzed

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in the laboratory of the Department of Molecular Biology, Genetics & Epigenetics at the University of Saarland. Samples were analyzed according to standard operating procedures.

2.2. Samples collection and semen analysis

Semen samples were collected from 108 males, including 51 current smokers and 57 never smoked control males by masturbation after 3 days of sexual abstinence, The samples allowed to liquefy at 37 °C for 30 min and then the sperm count was assessed using Meckler counting chamber (Sefi-Medica, Haifa, Israel). The sperm parameters were analyzed according to World Health Organization guidelines [22]. Somatic cells were removed from semen samples, by loaded the semen samples onto 45%–90% discontinuous Puresperm gradients (Nidacon International) and then centrifuged at 500*g* for 20 min at room temperature. The pure sperm was incubated with Somatic Cell Lysis Buffer (SCLB) on ice for 30 min and washed two-time with phosphate-buffered saline (10 min at 500*g*). The absence of somatic cells contamination has been confirmed by microscopic examination [23]. The samples were stored at −80 °C until processing. All participants who have diabetes mellitus, alcohol drinkers, the presence of anti-sperm antibodies, varicocele, Y chromosome microdeletions, males subjected to surgical operation in the reproductive system, abnormal hormonal parameters, abnormal body mass index, and infertility related to the woman were excluded from this study. In contrast, the included criteria for the current smoker group were as follows: current smokers, duration of smoking about five years at least, and consume at least 20 cigarettes per day. Besides, the inclusion criteria for the study population were the following: age between 20 and 50, proven fertile males, and males of the same nationality, ethnicity, and food supplementation.

2.3. Study design

Human Methylation 450 k BeadChip Arrays was used as screening phase, to determine the differentially methylated CpGs (DMC) between current smokers (n = 15) and never smoked control males (n = 15). Then, the two differentially methylated CpGs were selected for further analysis using deep bisulfite sequencing (validation phase) in an independent cohort of current smokers (n = 36) and never smoked control males (n = 42).

2.4. Sperm DNA isolation and sodium bisulfite treatment

Sperm DNA was extracted using the Isolate II genomic DNA kit according to the standard protocol provided by the manufacturer (Bioline, UK). DNA was eluted with 30 μL of RNase-free water, and the concentration of extracted DNA was measured with by using a Nanodrop spectrophotometer ND-2000c, (Thermo Scientific, USA) and then the integrity of gDNA was assessed using gel electrophoresis (Supplemental Fig. 1). Five hundred nanograms of isolated sperm 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 previously described [24].

2.5. DNA methylation analysis by infinium 450 K BeadChip array (Screening phase)

In the screening phase, 15 current smokers and 15 never smoked control samples were subjected to Human Methylation 450 k BeadChip Arrays (Illumina, USA) screening according to the manufacturer's instructions [25], and the arrays were scanned using the Illumina iScan. β-values were then generated by analyzing the intensities for methylation or no methylation at each CpG tiled on the array using the calculation: β-value = methylated/(methylated + unmethylated). β-values ranged from 0 to 1 and indicate the methylation level for each CpG. A value of 1 represents a completely methylated CpG and 0 represents a completely unmethylated CpG. Raw intensity values obtained from Illumina were used to generate β-values, and the bioinformatic processing and evaluation were performed with the RnBeads program package [26]. The methylation level in each CpG was considered as being differentially methylated CpG (DMC) when the absolute difference of the means of the average β-values between two groups was ≥ 20%. To determine differentially methylated CpGs (DMC) with possible biological and statistical significance, a Benjamini-Hochberg corrected *t*-test FDR (false discovery rate) of 0.05 was applied, and all CpG sites with a coverage (no. of beads) of ≤ 5 were excluded from the analysis. Findings were considered significant when $p \leq 0.01$. Referring to the technical results of hybridization, the gene call rate above 98% per sample and a detection value $p < 0.01$ per CpG site were set as internal quality criteria. All CpGs that covered a common SNP site in the dbSNP137 database were excluded.

2.6. Bisulfite profiling (Bi-PROF) (Validation phase)

In this part of the work and according to the results of screening phase, we took two CpG sites located in *MAPK8IP* and *TKR* gene to study them on 78 samples (36 samples from current smoker males, and 42 samples from never smoked control males), not including the screening phase samples, using local deep bisulfite sequencing [Bi-PROF, [27]]. In the validation phase, an independent cohort of current smokers (n = 36) and never smoked control males (n = 42) were included. Briefly, 500 ng gDNA of each sample was subjected to bisulfite treatment using EpiTect Bisulfite Kits (Qiagen, Germany). PCR reactions were prepared with the MyTaq™ HS Red Mix Kit (Bioline, UK) according to the manufacturer's instructions using 100 nanograms of the bisulfite-converted gDNA template in a total of 30 μL reaction volume. For the amplification, fusion primers consist of a specific 3′-portion (listed in Table 2 together with respective annealing temperatures and a number of CpGs present within the amplicon sequence) and a universal 5′-portion containing the necessary nucleotide sequences for Illumina sequencing were used. Primers were designed to react with DNA after bisulfite treatment using the BiSearch primer design tool (http://bisearch.enzim.hu/?m=search) using the following criteria: max length of PCR 400, primer concentration 0.167 μmol, potassium concentration 50 mmol, magnesium concentration 2.5 mmol, primer length 20–30, max Tm difference 2.0. By using BiSearch, placing primers onto common SNPs could be eliminated. The annealing temperature was adjusted before started, to avoid the formation of primer dimer. Five microliters of PCR product were then loaded on 2% agarose gels stained with Ethidium bromide (Biolabs, USA) (Supplemental Fig. 2 and 3). Using the Agencourt® AMPure XP beads kit (Beckman Coulter, USA) and Quant-iT™ DNA Assay Kit (Fisher Scientific, USA), the PCR products were purified and then measured, respectively, both steps were carried out according to the manufacturer's instructions, then diluted and pooled. Deep sequencing was performed on the Illumina MiSeq according to the manufacturerís protocols aiming at 10,000 reads per amplicon. All data obtained from the deep sequencing step were processed, filtered, and aligned by using BiQ Analyzer HT software [28], excluding all reads containing equal or more than 10% of missing CpG sites (maximal fraction of unrecognized sites ≥ 0.1). The obtained alignments sequence showed an absence of alterations at CpG positions (no SNP was detected).

Table 1
CpG dinucleotides that differ in their methylation levels for sperm DNA between current smokers and never smoked males (n = 30).

cgID	Chr	nt (hg19)	Strand	Mean methylation never smokers (%)	Mean methylation current smokers (%)	Diff. meth P. value	SD. never smokers	SD. current smokers	Annotation
cg01584086	chr11	10373718	+	3.49170	52.40156	1.21E-07	0.000979	0.050596	-/
cg20978247	chr6	32905085	–	90.99170	50.60859	4.1E-07	0.00097	0.018688	*HLA-DMB-Body/*
cg07869343	chr16	1797050	+	93.11966	49.13377	6.7E-07	0.004419	0.049764	*MAPK8IP3-Body*
cg19169023	chr15	41853346	+	92.58933	55.27543	8.35E-07	0.006632	0.023077	*TKR – TYRO3-Body/S_Shore*
cg09432376	chr22	36044226	–	88.67176	47.99514	1.29E-06	0.011979	0.00252	*APOL6-TSS200/*
cg23109721	chr2	1.07E+08	+	3.99507	25.11486	1.94E-06	0.004959	0.002534	*-/Island*
cg27391564	chr2	2.41E+08	–	86.23002	51.32112	2.23E-06	0.004823	0.0215	-/
cg15412446	chr2	1.07E+08	–	6.07528	26.95074	3.49E-06	0.005289	0.008227	*-/Island*
cg08108333	chr7	1.57E+08	–	7.13468	40.78405	8.41E-06	0.016992	0.001209	*-/Island*
cg00648582	chr12	1.33E+08	+	43.00466	79.78632	1.18E-05	0.039169	0.00426	*PGAM5,PGAM5,PGAM5-Body/Island*
cg23841288	chr7	1.58E+08	+	15.97900	44.14309	1.58E-05	0.016273	0.008269	*PTPRN2,PTPRN2,PTPRN2-Body,/N_Shore*

CpGs selected for Bi-PROF are highlighted.

2.7. Statistical analysis

All data obtained from Bi-PROF was analyzed using IBM SPSS for Windows software package version 24.0 (SPSS Inc., USA). Samples included in this study were nonnormal distributed (nonparametric) according to the value of skewness test, Kurtosis test, Z-value and Shapiro test. The independent-sample *t*-test (Mann–Whitney test) and Kruskal-Wallis test were used to compare means of quantitative variables, besides to the Spearman's test, to assess the correlation coefficient between methylation level and other parameters. The results in all the above-mentioned procedures were accepted as statistically significant when the *p*-value was less than or equal 5% ($p \leq 0.05$).

3. Results

To identify the influence of cigarette smoking on sperm DNA methylation patterns, we evaluated the methylation patterns of 15 current smokers, and 15 never smoked control males by using Human Methylation 450 BeadChip Arrays. The screening phase results showed 95 dinucleotides have significant differences (Supplemental Table 1). However, only 11 of those have significant differences in current smokers as compared to never smoked males without overlap common annotation SNPs. Table 1 shows the location and the most DMC based on the mean DNA methylation difference of ≥20% in current smokers as compared to never smoked males. The methylated sites are predominantly located in gene bodies and CpG islands. Out of 11 CpGs identified, 2 CpGs namely cg07869343 and cg19169023, which are located in *MAPK8IP3* and *TKR* genes, respectively, were selected for further analysis using deep bisulfite sequencing in an independent cohort of current smokers (n = 36) and never smoked control males (n = 42). The genes of this CpGs (cg07869343 and cg19169023) are associated with the spermatogenesis [29–31]. In addition to the other CpGs e.g. cg20978247, cg00648582. Based on the position of both CpG sites next to potentially regulatory regions, we decided to validate the Infinium 450 K BeadChip result on a larger cohort samples (78 samples) to evaluate the effect of cigarette smoking on sperm DNA methylation and sperm parameters, whereas the mean age of the patients enrolled in the present study was 39.31 ± 7.86. The study population was divided into two groups; current smokers males (n = 36; age 40.39 ± 7.32 years) and never smoked males (n = 42; mean age 38.38 ± 8.27 years). The descriptive characteristics of groups are presented in Table 3. The percentage of progressive motility and the percentage of sperm normal form in the current smoker group were found significantly lower compared to the never smoked control group ($p \leq 0.0001$ and $p \leq 0.0001$, respectively). In the contrast, the percentage of non-progressive motility was significantly higher in the current smoker males compared to the never smoked control cohort (30.10 ± 10.32 vs. 10.83 ± 3.43 respectively; $p \leq 0.0001$). On the other hand, there were no significant differences between the current smoker and never smoked control groups in age, sperm count and percentage of total motility ($p \leq 0.212$; $p \leq 0.602$ and $p \leq 0.386$, respectively). The deep bisulfite sequencing analysis revealed that not only the CpGs obtained from the screening pahs experiments has a difference in the methylation level, but also neighboring CpGs. As shown in Table 4, a significant difference was observed in six out of 22 CpGs tested in the *MAPK8IP* gene-related amplicon (CpG3, CpG5, CpG6, CpG7*, CpG8, and CpG21) in current smokers as compared to never smoked control males ($P \leq 0.043$; $P \leq 0.005$; $P \leq 0.002$; $P \leq 0.003$*; $P \leq 0.045$ and $P \leq 0.040$, respectively). Similarly, a significant difference in one out of 4 CpGs tested in the *TKR* gene-related amplicon (CpG4, $P \leq 0.002$).

Next, we evaluated the effect of male age on the methylation level considering all CpGs that have been identified by deep bisulfite sequencing in *MAPK8IP3* and *TKR* gene-related amplicons. By dividing males into three groups, a group I (age ≤ 35 year, n = 28), group II (35 < age ≤ 45 year, n = 32), and group III (age > 45 years, n = 18). Three CpGs out of 22 in the MAPK8IP gene-related amplicon

Table 2
Primer sequences, number of DMCs and PCR annealing temperatures used to amplify regions including the target CpGs analyzed by Bi-PROF.

cgID	UCSC RefGene	Chr-	nt (hg19)	At (°C)	Product size (bp)	DMCs	Primer sequence (5′–3′)	
cg07869343	*MAPK8IP3*	chr16	1797050	52	334 bp	22	F	GAGGTAAAGTGTAAAGTATT
							R	CTAAATAACCTATACTTCCA
cg07869343	*TKR*	chr15	41853346	57.6	386 bp	4	F	TTTTAGAAGAGAATGGGAATT
							R	AAATAACCAAATAAAAAAACCAC

DMC: Differentially methylated CpGs.
Chr-: Chromosome.
At: annealing temperature.

Table 3
Descriptive characteristics in cases compared to controls (n = 78).

Variable	Never smoked (n = 42)	Current smokers (n = 36)	p value
Age (Year)	38.38 ± 8.27	40.39 ± 7.32	0.212[a]
Sperm count milli/ml	87.33 ± 80.66	84.30 ± 65.32	0.602 [a]
Percentage of total motility	57.55 ± 12.06	60.71 ± 15.69	0.386 [a]
Percentage of progressive motility	46.71 ± 11.10	30.61 ± 12.81	≤0.0001 [a]
Percentage of non-progressive motility	10.83 ± 3.43	30.10 ± 10.32	≤0.0001 [a]
Percentage of immotile sperm	42.45 ± 12.06	39.29 ± 15.69	0.386 [a]
Percentage of sperm normal form	62.86 ± 11.37	20.33 ± 7.23	≤0.0001 [a]

All values are expressed as mean ± SD.
P > 0.05: not significant.
P ≤ 0.05: significant.
[a] Mann–Whitney test.

Table 4
Methylation levels in the DMCs obtained from local deep bisulfite sequencing results in current smoker compared to never smoked control males (n = 78).

DMCs	Never smoked (n = 42)		Current smokers (n = 36)		p value
	Mean	SD	Mean	SD	
Methylation level at CpGs in *MAPK8IP* gene-related amplicon					
CpG1	0.98	0.02	0.97	0.03	0.518 [a]
CpG2	0.97	0.03	0.98	0.03	0.131 [a]
CpG3	0.98	0.01	0.98	0.02	≤0.043 [a]
CpG4	0.99	0.01	0.99	0.01	0.666 [a]
CpG5	0.98	0.01	0.99	0.01	≤0.005 [a]
CpG6	0.97	0.02	0.99	0.01	≤0.002 [a]
CpG7*	0.97	0.02	0.95	0.03	≤0.003 [a]
CpG8	0.96	0.02	0.97	0.02	≤0.045 [a]
CpG9	0.97	0.02	0.97	0.02	0.904 [a]
CpG10	0.97	0.02	0.98	0.02	0.059 [a]
CpG11	0.98	0.02	0.99	0.02	0.073 [a]
CpG12	0.99	0.01	0.99	0.02	0.103 [a]
CpG13	0.97	0.03	0.98	0.02	0.171 [a]
CpG14	0.98	0.02	0.98	0.02	0.228 [a]
CpG15	0.97	0.03	0.97	0.02	0.984 [a]
CpG16	0.97	0.03	0.97	0.02	0.904 [a]
CpG17	0.97	0.04	0.96	0.05	0.493 [a]
CpG18	0.99	0.01	0.97	0.03	0.295 [a]
CpG19	0.98	0.02	0.97	0.03	0.520 [a]
CpG20	0.99	0.01	0.98	0.03	0.331 [a]
CpG21	0.99	0.01	0.98	0.02	≤0.040 [a]
CpG22	0.99	0.01	0.97	0.03	0.146 [a]
Methylation level at CpGs in *TKR* gene-related amplicon					
CpG1*	0.95	0.06	0.97	0.02	0.825 [a]
CpG2	0.98	0.01	0.98	0.02	0.779 [a]
CpG3	0.96	0.04	0.97	0.02	0.952 [a]
CpG4	0.92	0.11	0.97	0.01	≤0.002 [a]

DMC: Differentially methylated CpGs.
STD: Standard deviation.
P > 0.05: not significant.
P ≤ 0.05: significant.
* Target CpG from the results of 450 K.
[a] Mann-Whitney test.

has a significant variation in methylation level among various groups, CpG5, CpG14 and CpG16 (P ≤ 0.003, P ≤ 0.029, and P ≤ 0.018, respectively). Besides, two out of four CpGs in TKR gene-related amplicon showed a significant difference in the methylation level (CpG2, P ≤ 0.033, and CpG4, P ≤ 0.0001) between various age groups, Table 5.

Finally, we evaluated the correlation between sperm DNA methylation level obtained by Bi-PROF in different CpG (DMC) and other different parameters investigated for current smoker group (Table 6). The results showed a significant positive correlation between the methylation level in CpG4, CpG8, CpG9, CpG10, CpG15, and CpG22 of *MAPK8IP* gene-related amplicon and percentage of total motility. However, a significant negative correlation was showed between the same CpGs and the percentage of sperm immotile. Besides that, a significant positive correlation has been found between the methylation level of CpG8, CpG9, CpG10, CpG14, CpG15, and CpG22 of *MAPK8IP* gene-related amplicon and percentage of progressive motility. Also, we found a significant positive correlation between the methylation level in all CpGs of *TKR* gene-related amplicon and percentage of total motility and percentage of progressive motility. In contrast, a significant negative correlation found between the same CpGs and the percentage of sperm immotile (Table 6). On the other hand, the results showed a negative significant correlation between the methylation level in CpG5 and CpG11 of *MAPK8IP* gene-related amplicon and the age of patients. In addition to a positive significant correlation between the methylation level in CpG13, CpG16, and CpG18 of *MAPK8IP* gene-related amplicon and sperm count. Furthermore, a significant positive correlation was found between the methylation level in All CpGs of *TKR* gene-related amplicon except CpG4 and percentage of sperm normal form (Table 6).

Table 5
Methylation level at DMC in *MAPK8IP* and *TYRO3* for the study population according to age.

DMC	Age ≤ 35 (n = 28)		35 ≤ Age ≤ 45 (n = 32)		Age > 45 (n = 18)		p value
	Mean	SD	Mean	SD	Mean	SD	
Methylation level at CpGs in *MAPK8IP* gene-related amplicon							
CpG1	0.98	0.02	0.98	0.03	0.98	0.02	0.958 [b]
CpG2	0.98	0.03	0.97	0.03	0.98	0.02	0.189 [b]
CpG3	0.98	0.02	0.98	0.01	0.98	0.02	0.800 [b]
CpG4	0.99	0.01	0.99	0.01	0.99	0.01	0.129 [b]
CpG5	0.99	0.01	0.98	0.01	0.98	0.01	≤0.003 [b]
CpG6	0.98	0.02	0.98	0.02	0.98	0.02	0.153 [b]
CpG7*	0.96	0.03	0.96	0.03	0.96	0.02	0.921 [b]
CpG8	0.97	0.02	0.97	0.01	0.96	0.03	0.753 [b]
CpG9	0.98	0.02	0.97	0.03	0.98	0.02	0.388 [b]
CpG10	0.98	0.01	0.97	0.02	0.97	0.03	0.102 [b]
CpG11	0.99	0.02	0.99	0.01	0.98	0.03	0.088 [b]
CpG12	0.99	0.02	0.99	0.01	0.99	0.01	0.193 [b]
CpG13	0.98	0.02	0.97	0.03	0.97	0.03	0.664 [b]
CpG14	0.98	0.02	0.97	0.02	0.99	0.01	≤0.029 [b]
CpG15	0.97	0.03	0.98	0.02	0.97	0.03	0.402 [b]
CpG16	0.98	0.02	0.96	0.03	0.97	0.03	≤0.018 [b]
CpG17	0.96	0.04	0.95	0.05	0.98	0.02	0.096 [b]
CpG18	0.98	0.03	0.99	0.01	0.97	0.03	0.826 [b]
CpG19	0.97	0.03	0.97	0.02	0.98	0.03	0.661 [b]
CpG20	0.98	0.02	0.99	0.01	0.98	0.03	0.977 [b]
CpG21	0.99	0.01	0.98	0.01	0.98	0.03	0.958 [b]
CpG22	0.98	0.03	0.99	0.01	0.97	0.04	0.859 [b]
Methylation level at CpGs in *TKR* gene-related amplicon							
CpG1*	0.95	0.08	0.96	0.03	0.97	0.03	0.207 [b]
CpG2	0.98	0.01	0.98	0.01	0.98	0.02	≤0.033 [b]
CpG3	0.96	0.04	0.97	0.02	0.97	0.02	0.726 [b]
CpG4	0.97	0.01	0.91	0.12	0.96	0.02	≤0.0001 [b]

DMC: Differentially methylated CpGs.
STD: Standard deviation.
P > 0.05: not significant.
P ≤ 0.05: significant.
* Target CpG from the results of 450 K.
[b] Kruskal Wallis Test.

4. Discussion

The relationship between cigarette smoking and sperm DNA methylation remains a controversial and debatable issue. However, many of the previous studies indicated that the cigarette smoking can influence the transcriptome [16] and alters the DNA methylation pattern [[18], and [33–35]]. In the current study, we investigated the impact of cigarette smoking on sperm DNA methylation patterns and sperm parameters. The results obtained from local deep sequencing revealed a significant differences in six DMC (CpG3, $p \leq 0.043$; CpG5, $p \leq 0.005$; CpG6, $p \leq 0.002$; CpG7, $p \leq 0.003$; CpG8, $p \leq 0.045$; and CpG21, $p \leq 0.040$) of *MAPK8IP* gene-related amplicon, and one CpG (CpG4, $p \leq 0.002$) of *TKR* gene-related amplicon in current smoker group compared to never smoked control group (Table 4). Analysis of freely available ENCODE data revealed that the differentially methylated CpG of *MAPK8IP3* is located in exon 6 within a DNase I cluster and close to an H3K27ac-enriched domain where several transcription factors binding motifs can be found [32]. The respective CpG site in the *TKR* gene is located within a DNase I cluster in exon 2 close to a CTCF binding motif [32]. Previous studies reported that the murine mitogen-activated protein kinase 8 interacting protein 3 (*MAPK8IP3*) may interact with, and regulate the activity of numerous protein kinases of the JNK signaling pathway, and a few of the signaling molecules including G proteins, tyrosine kinases, the inositol (1,4,5)-trisphosphate receptor, *MAPKs* (mitogen-activated protein kinases), have been found in the spermatozoa and oocyte [36]; however, the biochemical networks that connect these molecules and their functions are poorly understood. Recently, [37] reported that the extracellular-signal-regulated kinase and, a member of the *MAPK* family, were shown to be associated with human spermatozoa with a direct or indirect function in sperm capacitation. Besides, it should also be mentioned that the tyrosine-protein kinase receptor gene (*TKR*) expressing the tyrosine kinase 3 protein (*TYRO3*) is a member of the *RTK* subfamily that contains Tyro3, Axl, and Mer "TAM" [38,39]. These proteins play very important roles in controlling cell survival, proliferation and differentiation, spermatogenesis, and phagocytosis [40]. The TAM protein is normally expressed by Sertoli cells during postnatal development [30,31]. Sertoli cells have a significant role in regulating male fertility, because more than 75% of developing spermatogenic cells during spermatogenesis undergo apoptosis before they develop into spermatozoa in physiological states [41], and TAM plays a crucial role in the phagocytic clearance of the apoptotic germ cells by professional phagocytes [42,43]. In general, the variation in this study is in line with previous studies that have found that smoker's males have a high level of oxidative stress, and this plays an important role as a potential cause of change in the level of DNA methylation [44–47]. In addition, these results observed highly significant differences between the current smoker and never smoked controls groups in the percentage of progressive motility, the percentage of non-progressive motility and percentage of sperm normal form ($p \leq 0.0001$, $p \leq 0.0001$, and $p \leq 0.0001$, respectively), and this is consistent with other studies reported the same variation in semen parameters in smoker compared to controls [48–50]. However, the results of this study did not observe any significant differences between study groups in the sperm count, percentage of total motility, and these are in agreement with several studies reported the same results [51–57]. Nevertheless, these results are not consistent with previous studies found that cigarette smoking leads to decreased sperm count and percentage of total motility [58–61].

Regarded to the results of deep bisulfite sequencing depending on the reproductive age for the participants included in the

Table 6
Correlation between the methylation levels in the DMCs obtained by Bi-PROF and the semen parameters for current smoker group (n = 36).

DMC		Age (Year)	Sperm Count ml/millio	% of total motility	% of progressive Motility	% of non-progressive motility	% of immotile sperm	% of sperm normal form
Methylation level at CpGs in *MAPK8IP* gene-related amplicon								
CpG1	r	0.020	−0.224	0.212	0.160	0.015	−0.212	−0.175
	p value	0.906	0.188	0.214	0.353	0.933	0.214	0.307
CpG2	r	−0.113	−0.235	0.276	0.324	−0.039	−0.276	0.087
	p value	0.514	0.167	0.103	0.054	0.822	0.103	0.614
CpG3	r	−0.049	0.227	0.278	0.084	0.201	−0.278	−0.043
	p value	0.776	0.182	0.101	0.628	0.239	0.101	0.802
CpG4	r	−0.163	0.009	0.456	0.161	0.372	−0.456	−0.062
	p value	0.343	0.957	≤0.005	0.347	≤0.026	≤0.005	0.722
CpG5	r	−0.464	0.313	0.054	−0.049	0.199	−0.054	0.070
	p value	≤0.004	0.063	0.753	0.776	0.245	0.753	0.687
CpG6	r	0.145	0.140	0.239	0.048	0.142	−0.239	−0.113
	p value	0.400	0.417	0.161	0.781	0.410	0.161	0.510
CpG7*	r	0.158	−0.167	0.108	0.002	−0.038	−0.108	−0.040
	p value	0.359	0.329	0.532	0.990	0.825	0.532	0.817
CpG8	r	−0.095	0.028	0.546	0.435	0.234	−0.546	−0.271
	p value	0.581	0.871	≤0.001	≤0.008	0.169	≤0.001	0.110
CpG9	r	0.072	−0.150	0.409	0.460	0.075	−0.409	0.028
	p value	0.677	0.383	≤0.013	≤0.005	0.664	≤0.013	0.873
CpG10	r	−0.057	0.020	0.554	0.385	0.293	−0.554	−0.211
	p value	0.743	0.909	≤0.0001	≤0.020	0.082	≤0.0001	0.216
CpG11	r	−0.383	0.090	0.223	0.112	0.080	−0.223	−0.055
	p value	≤0.021	0.603	0.191	0.514	0.642	0.191	0.750
CpG12	r	0.127	0.262	0.293	0.300	0.091	−0.293	−0.153
	p value	0.462	0.123	0.083	0.076	0.599	0.083	0.372
CpG13	r	−0.096	0.405	0.116	−0.018	0.028	−0.116	−0.234
	p value	0.577	≤0.014	0.499	0.919	0.873	0.499	0.169
CpG14	r	0.140	0.119	0.283	0.369	−0.082	−0.283	−0.005
	p value	0.415	0.490	0.095	≤0.027	0.636	0.095	0.976
CpG15	r	0.122	−0.319	0.383	0.439	0.011	−0.383	0.183
	p value	0.477	0.058	≤0.021	≤0.007	0.950	≤0.021	0.284
CpG16	r	0.160	0.358	0.271	0.069	0.164	−0.271	0.139
	p value	0.353	≤0.032	0.110	0.689	0.340	0.110	0.418
CpG17	r	0.161	−0.272	0.124	0.237	−0.166	−0.124	0.158
	p value	0.349	0.109	0.473	0.164	0.334	0.473	0.357
CpG18	r	0.109	0.362	0.276	0.200	0.033	−0.276	0.201
	p value	0.528	≤0.030	0.103	0.243	0.848	0.103	0.240
CpG19	r	0.087	0.029	0.320	0.272	0.066	−0.320	0.243
	p value	0.616	0.866	0.057	0.109	0.704	0.057	0.154
CpG20	r	0.008	0.016	0.186	0.079	−0.045	−0.186	0.183
	p value	0.961	0.928	0.278	0.646	0.793	0.278	0.286
CpG21	r	0.030	0.224	0.190	0.227	−0.171	−0.190	0.406
	p value	0.864	0.189	0.268	0.184	0.320	0.268	≤0.014
CpG22	r	0.007	−0.099	0.478	0.593	−0.035	−0.478	0.176
	p value	0.966	0.564	≤0.003	≤0.0001	0.838	≤0.003	0.304
Methylation level at CpGs in *TKR* gene-related amplicon								
CpG1*	r	−0.250	−0.325	0.413	0.472	0.101	−0.413	0.337
	p value	0.142	0.053	≤0.012	≤0.004	0.557	≤0.012	≤0.045
CpG2	r	−0.009	0.143	0.649	0.594	0.386	−0.649	0.343
	p value	0.957	0.406	≤0.0001	≤0.0001	≤0.020	≤0.0001	≤0.040
CpG3	r	−0.036	0.178	0.496	0.402	0.354	−0.496	0.396
	p value	0.836	0.298	≤0.002	≤0.015	≤0.034	≤0.002	≤0.017
CpG4	r	−0.006	−0.027	0.508	0.392	0.363	−0.508	−0.043
	p value	0.974	0.874	≤0.002	≤0.018	≤0.030	≤0.002	0.803

Spearman's test.
DMC: Differentially methylated CpGs.
r: Correlation Coefficient.
P > 0.05: not significant
P ≤ 0.05: significant.
* target CpG according to 450 K.

study population, the results observed that there is a significant difference between the methylation level in DMC (CpG5, CpG14, and CpG16) in the *MAPK8IP* gene-related amplicon and various age groups ($P \leq 0.003$; $P \leq 0.029$ and $P \leq 0.018$, respectively). In addition to CpG2, and CpG4 in the *TKR* gene-related amplicon ($P \leq 0.033$, and $P \leq 0.0001$, respectively), which is in line with several previous studies which is in line with several previous studies they reported that the sperm DNA methylation patterns are stable over short periods of time but changing with advancing age [62,63]. Nevertheless, these results incompatible with other studies that, did not show an association between global sperm DNA methylation and advancing age [23,64]. Besides that, the sperm parameters did not indicate to presence any statistically significant changes between the various age groups, and these results disagree with the results of previous studies which found a decrease in sperm counts, sperm motility, and sperm production during aging [65–68]. However, the results showed a statistically significant change between diverse age group in a percentage of progressive motility, and this agree with the study reported that a decline in the sperm motility associated with advancing age [69].

On the other hand, the results of this study indicate the presence of a significant correlation between methylation levels of CpG4, CpG8, CpG9, CpG10, CpG15 and CpG22 in the *MAPK8IP* gene-related amplicon and percentage of total motility, and percentage of progressive motility. Furthermore, a significant correlation was found between the methylation level at CpG5 and CpG11 in the *MAPK8IP* gene-related amplicon and the age of current smoker group. A significant correlation has been shown between the methylation level at CpG13, CpG16 and CpG18 in the *MAPK8IP* gene-related amplicon and the sperm count. Besides, a significant correlation between the methylation level at all CpG in the *TKR* gene-related amplicon except CpG4 and percentage of sperm normal form (Table 6). Overall, the correlations that shown in this study are in line with previous studies that found an association between alterations in the methylation patterns of sperm DNA methylation and sperm parameters of smokers [70,71]. However, the correlations in this study contradict other studies, which showed no correlation between sperm global DNA methylation level and paternal age, sperm parameters of smokers [72,73].

5. Conclusion

The results of this study indicated that cigarette smoking causes biochemical changes in the sperm DNA methylation in many regions, which related to MAPK8IP and TKR gene amplicon and could adversely affect semen parameters.

Limitations

The yielded amount of gDNA was insufficient to run both assays for all samples.

Conflicts of interest

We have no potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2017.05.010.

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3.4 DNA methylation level of spermatozoa from subfertile and proven fertile and its relation to standard sperm parameters.

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DNA methylation level of spermatozoa from subfertile and proven fertile and its relation to standard sperm parameters

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Summary

The epigenetic mechanism plays an important role in spermatogenesis such as DNA methylation where this episode is represented by either switching genes on or off. Twenty-eight samples (14 case and 14 controls) were subjected to Infinium 450K BeadChip arrays to identify genomic regions that differ in sperm DNA methylation patterns in the subfertile compared to the proven fertile group. Then two CpGs were validated by deep bisulphite sequencing on 82 sperm samples. The results screening study revealed eight CpGs were significantly different in their sperm DNA methylation levels between cases and control group. The results of the validation study for the two CpGs (cg19779893 and cg19406113) showed that a significant variation in the methylation level at 2 CpGs of 3 CpGs related to cg19779893 site amplicon in cases compared to the controls. Moreover, six CpGs related to the cg19406113 site amplicon showed significant differences in sperm DNA methylation between the cases and the control group. Furthermore, there was a significant decrease in the sperm parameters in the cases compared to the control group. This study found two CpGs altered in their sperm DNA methylation levels. In addition, a strong association was found between changes in the sperm DNA methylation levels in these CpGs sites and sperm parameters.

KEYWORDS
CpGs, DNA methylation, sperm parameters, subfertile

1 | INTRODUCTION

Epigenetics is described as stable changes in the DNA and histone structure without affecting the DNA sequence itself. These changes can be inherited through mitotic or meiotic divisions (Carrell, 2012). The epigenetic mechanism involves histone modification or DNA methylation where these episodes are represented by either the switching on or off of genes (Anway, Cupp, Uzumcu, & Skinner, 2005; Richardson, 2002). The mechanism by which DNA methylation takes place is confined to the addition of a methyl group to the C^5 atom of the cytosine pyrimidine ring that is immediately followed by a Guanine base (CpG dinucleotide) known as CpG islands (Talbert & Henikoff, 2006; Portela & Esteller, 2010; CpG islands are defined as groups of CpG base pairs (bp) approximately 1Kbp long, which have a high frequency of CpG sites (Takai & Jones, 2002). These islands are most probably found in the transcription start site (50%), and the remaining per cent is distributed equally, intragenically (between the genes) or intergenically (within the gene body) (Illingworth et al., 2010). DNA methylation at the gene body CpGs is associated with the activation of gene transcription (Ball et al., 2009; Rauch & Pfeifer, 2005), whereas gene silencing is the main event that occurs at the gene promoters (intragenic) causing the prevention of gene expression (Suzuki & Bird, 2008). This repression mechanism is induced by the insertion of the methyl group into the major groove of DNA, preventing the stable binding of a transcription factor (Feng & Zhang, 2001; Nan et al., 1998).

DNA methylation occurs under the influence of DNA methyltransferase (DNMTs), which catalyses the transfer of one methyl

group from S-adenosyl methionine to cytosine (Yi & Goodisman, 2009). There are three main DNMTs: DNMT1 is responsible for the conservation of DNA methylation through the addition of methyl groups to the newly replicated DNA strands(Bird, 2002). DNMT3a and DNMT3b are de novo methyltransferases that exert methylation changes on the genomic DNA throughout early embryonic development (Lei et al., 1996; Okano, Xie, & Li, 1998).

Intriguingly, after fertilisation, the DNA methylation of imprinted genes remains unchanged and is clonally inherited to all somatic cells of the individual. During foetal development, primordial germ cells (PGCs) are subjected to epigenetic reprogramming, where complete erasure of imprint is followed by a DNA methylation reorganisation depending on the gender of the embryo during its migration to the genital ridge (O'Doherty & McGettigan, 2015). In a male foetus, DNA methylation is maintained steadily throughout the process of spermatogenesis and spermiogenesis. Therefore, adequate methylation in spermatozoa is essential for normal fertilisation, early embryo viability, proper implantation and healthy live births (Kobayashi et al., 2009; Carrell and Hammoud, 2010; Dada et al., 2012; Jenkins & Carrell, 2012; Wu, Hauser, Krawetz, & Pilsner, 2015).Previous studies revealed that male infertility may be associated with abnormal DNA methylation in spermatozoa (Hammoud, Purwar, Pflueger, Cairns, & Carrell, 2010; Nanassy & Carrell, 2011). Moreover, changes in DNA methylation of some genes were related to a decrease in semen parameters, contributing to male infertility (Houshdaran et al., 2007; Aston, Punj, Liu, & Carrell, 2012).

Male infertility is considered one of the major concerns of today's healthcare community. According to the American Society for Reproductive Medicine (ASRM) and the World Health Organization (WHO), infertility is defined as the failure to achieve conception despite a regular unprotected intimate relationship between couples for at least one year of intercourse (Bayer, Alper, & Penzias, 2007; Rowe & &Comhaire, 2000). It affects approximately 13%–15% of couples worldwide, among which male factors contribute about 20% (Jarow et al., 2002). Idiopathic causes of infertility account for approximately 60%–70% of all the cases of male infertility (Filipponi & Feil, 2009). Genetic and epigenetic changes may contribute towards idiopathic male infertility (Emery & Carrell, 2006).

The purpose of this study was to assess whether there is an alteration in the methylation levels of sperm DNA obtained from subfertile males compared to that obtained from proven fertile males. It is furthermore planned to evaluate the association between the changes in sperm DNA patterns and semen parameters in subfertile males. Genome-wide array analysis, using local deep bisulphite sequencing, was used to define differentially methylated CpGs between proven fertile and subfertile males.

2 | MATERIAL AND METHODS

2.1 | Ethics Statement

Institutional review board approval (13/14) was obtained before the initiation of this study, and informed consent was provided according to the Declaration of Helsinki. In addition, each participant provided written agreement for inclusion in this study. The study was conducted in the laboratory of the Molecular Biology; Genetics & Epigenetics Departments at the University of Saarland, Germany.

2.2 | Sample collection and semen analysis

One hundred and ten semen samples were collected and categorised into two groups. The first group included proven fertile males as the control group (n = 51). The second group included subfertile males who did not have children after more than three years of regular unprotected intercourse as the case group (n = 59). Semen samples were collected by masturbation after 3 days of sexual abstinence in clean, dry, sterile and leak-proof plastic containers in a collection room attached to the laboratory. Following liquefaction of the semen at 37°C for 30 min, the sperm parameters were performed according to the World Health Organization guidelines (WHO, 2010) using a Makler chamber. From each sample included in this study, six smears were prepared (10 μl) for morphology assessment. Moreover, 100 μl semen was mixed with 1.0 ml of the hypo-osmotic solution (HOS) to assess the sperm membrane integrity (vitality test). Also, 5 μl semen was mixed with 5 μl of 0.5% aqueous yellowish Eosin solution (an Eosin test) to assess sperm viability. The following parameters were taken into consideration for the exclusion: varicocele, anti-sperm antibodies, Y chromosome microdeletions, consumption of alcohol, smoking, abnormal hormone parameters, males subjected to surgical operation in the reproductive system, abnormal body mass index, advanced age >45 years old and infertility problems related to the wives. Furthermore, the inclusion criteria were as the following: all participants were from the same region with demographic differences such as race. The proven fertile (control group) had at least one child, and sperm parameters were normal according to WHO guidelines (2010), whereas the subfertile did not have any children, and their sperm parameters were under the value given by WHO 2010. The wives in both groups were having regular menstrual cycles and had normal medical histories.

2.3 | Sperm purification

All of the semen samples underwent purification through the gradients technique before the DNA extraction from the spermatozoa. Each semen sample was loaded on 45%–90% discontinuous PureSperm gradients (Nidacon International, Sweden) and centrifuged at 500× g for 20 min at room temperature, which effectively separated the spermatozoa from the lymphocytes, the epithelial cells, abnormal or immature spermatozoa, bacteria and seminal fluid. The absence of immature sperm and somatic cell contamination was confirmed by microscopic examination.

2.4 | Sperm DNA isolation and sodium bisulphite treatment

Sperm DNA was isolated using the isolated DNA/RNA/protein kit according to the standard protocol provided by the manufacturer (Cat.

#: BIO- 52085, Bioline, UK). The concentration and purity of the extracted DNA were measured using the Nanodrop spectrophotometer 2000c (Thermo-Scientific). Five hundred nanogram of extracted sperm DNA was treated with sodium bisulphite using the Epitect bisulfite conversion kit (Cat. #: 59104, Qiagen, Germany) that converts unmethylated cytosines to uracil, while the 5-methylcytosine (5MeC) remains unaltered, as previously described (Wu et al., 2015).

2.5 | Screening study by Infinium 450K BeadChip array

Twenty-eight samples were subjected to Infinium 450K BeadChip arrays (Illumina, San Diego, CA, USA) at Life and Brain GmbH Biomedicine and Neuroscience technology in Bonn, following the manufacturer's recommendations (Bibikova et al., 2011), as a screening study, to identify variation in sperm DNA methylation regions between proven fertile and subfertile males. Samples were whole-genome amplified and put on the bead arrays, as described in Sandoval et al. (2011). Bioinformatic processing and evaluation were performed with the RnBeads program package (Assenov et al., 2014). *B*-values were then generated by analysing the intensities for methylation or no methylation at each CpG tiled on the array, using the calculation: β-value = methylated/(methylated + unmethylated). β-values ranged from 0 to 1 and indicated the methylation level for each CpG. The result of the β-value ranges from 0 to 1 and indicates the methylation level for each CpG. A value of 1 represents a completely methylated CpG, and 0 represents a completely unmethylated CpG. Raw intensity values obtained from Illumina were used to generate β-values, and the bioinformatic processing and evaluation were performed with the RnBeads program package (Assenov et al., 2014). The methylation level in each CpG was considered as being differentially methylated CpGs (DMCs) when the absolute difference of the means of the average b-values between two groups was ≥20%. To determine DMCs with possible biological and statistical significance, a Benjamini-Hochberg corrected *t* test FDR (false discovery rate) of 0.05 was applied, and all CpG sites with a coverage (no. of beads) of ≤5 were excluded from the analysis. Findings were considered significant when $p \leq .01$. Referring to the technical results of hybridisation, the gene call rated above 98% per sample and the detection value $p \leq .01$ per CpG site was set as the internal quality criterion. All CpGs that covered a common SNP site in the dbSNP137 database were excluded.

2.6 | Validation study of Bisulfite profiling (Bi-PROF)

The first two CpG sites that resulted from the screening study by Infinium 450K BeadChip arrays were selected in order to be subjected to further analysis using bisulfite sequencing (Bi-PROF) (Gries et al., 2013), according to the manufacturer's instructions. Eighty-two samples were divided into 45 samples from the proven fertile as the case and 37 samples from the subfertile as the control. These two groups were used in the validation study as follows: four hundred ng of genomic DNA were bisulphite treated using the Epitect bisulfite conversion kit (Cat. #: 59104, Qiagen, Germany). PCRs reactions were performed in a 50-μl total volume reaction, using the "MyTaq™ HS Red Mix" with 2× concentration (Catalog #: BIO-25047, Bioline, UK), according to the manufacturer's protocol. For the amplification, primers were designed to amplify the target CpG region, where fusion primers were used that consisted of a specific 3′-portion (listed in Table 1, together with the respective annealing temperatures) and a universal 5′-portion containing the necessary nucleotide sequences for Illumina sequencing. Ten micrograms of PCR reactions was loaded on a 2% agarose gel, including the DNA ladder (Cat. #: N0467S, Biolabs) and stained with ethidium bromide. PCR products were purified using the Agencourt® AMPure XP beads (Beckman Coulter, USA) and measured by using the Quant-iT™ DNA Assay Kit (Fisher Scientific, USA), according to the manufacturers′ recommendations and 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 sequencing step were processed, filtered and aligned, using BiQ Analyzer HT software (Lutsik et al., 2011), and excluding all reads containing more than 10% of missing CpG sites (the maximal fraction of unrecognised sites ≥0.1).

2.7 | Statistical analysis

Data were analysed using IBM SPSS for the Windows software package version 23.0 (SPSS Inc., USA). The samples included in this study were non-normal distributed according to the value of a skewness test, a Kurtosis test, a *Z*-value and a Shapiro test. The independent sample *t* test (Mann–Whitney test) was used to compare the means of the quantitative variables. In addition, Spearman's test was used to assess the correlation coefficient between the methylation level in the different CpG and sperm parameters. The results in all the

TABLE 1 Primer sequences and PCR annealing temperatures used to amplify regions including the target CpGs analysed by Bi-PROF

CgID	Chromosome	Annealing temperature (°C)	Product size (bp)		Primer sequence (5′–3′)
cg19406113	Chr3	57	308 bp	F	ATTAAAGAGTGAGTTGGAAAAGT
				R	AACCCCAAAAAAAATAAAACC
cg19779893	Chr10	55	212 bp	F	AGAGGGATTTTTAATAATTGT
				R	CAAAAAAAATCCAACTAACTAAA

TABLE 2 Differential methylated CpG sites (DMCs) in sperm samples from cases (subfertile) compared to control (proven fertile) group

cgID	Chromo	Annotation	Strand	Mean.Control	Mean.Cases	Diffmeth.p.adj.fdr
cg19779893	chr10	ADAMTS14-Body	+	0.852891163	0.595902571	0.142813812
cg19406113	chr6	-	–	0.667489451	0.904643996	0.15418376
cg23081194	chr21	-	+	0.117367807	0.903151445	0.000369757
cg16338278	chr11	ALDH3B2- Body/Island	–	0.934857616	0.733098693	0.008456327
cg04807108	chr15	-	–	0.725153089	0.918482785	0.043660488
cg08408433	chr19	PTGIR-Body/Island	+	0.069358559	0.370138538	0.110522162
cg25750688	chr7	-	+	0.86493861	0.637849078	0.142070592

above-mentioned procedures were accepted as statistically significant when the *p*-value was less than 5% ($p \leq .05$).

3 | RESULTS

Twenty-eight samples (14 proven fertile and 14 subfertile) were used in this study as a screening study and sent to the Life and Brain GmbH Biomedicine and Neuroscience Technology in Bonn, Germany. These samples were subjected to Infinium 450K BeadChip analysis, which detects the different DNA methylation levels of circa ~485,000 CpGs, covering 99% positions in each sample. Table 2 summarises the location of the detected DMCs, based on the mean DNA methylation difference of ≥20% between the two analysed groups. Only eight CpGs positions were detected depending on the applied criteria (cg19779893, cg19406113, cg23081194, cg16338278, cg04807108, cg08408433, cg25750688 and cg07227024). Two CpG sites (cg19779893 and cg19406113), resulting from Infinium 450K BeadChip array, were selected to validate the difference in DNA methylation levels on more populations. Eighty-two samples were divided into two groups: a case group ($n = 45$) with a mean age of (38.50 ± 6.68) and a control group ($n = 37$) with a mean age of (37.16 ± 5.5).

Cg19779893 is located in intron 2 of *ADAMTS14* (ENCODE Project Consortium, 2012). There was a significant decrease in the DNA methylation level in the case group (0.59 ± 0.03) compared to the control (0.85 ± 0.01).

Cg19406113 is located in intergenic regions, within chromosome 6 (ENCODE Project Consortium, 2012). This CpG showed a significant increase in DNA methylation level in the case group (0.90 ± 0.007), compared to the control group (0.66 ± 0.04).

When a local deep bisulfite was applied, different CpGs appeared close to the target CpGs* that resulted from the screening study. The mean of the DNA methylation levels at six CpGs of eleven related to the cg19406113 site (CpG3, CpG5, CpG6, CpG8, CpG9, and CpG10) showed a significant difference in the case group compared to the control ($p \leq .009$, $p \leq .03$, $p \leq .02$, $p \leq .03$, $p \leq .007$ and $p \leq .01$ respectively) (See Figure 1). Furthermore, a significant difference in two CpGs within the cg19779893 site-related amplicon (CpG2 and CpG3) was revealed in the case compared to the control ($p \leq .03$ and $p \leq .01$ respectively) (Figure 2). As demonstrated in Table 3, sperm parameters showed a significant decrease in count, total motility, progressive, nonprogressive motility, normal form morphology, viability and vitality in the case compared to the control ($p \leq .02$, $p \leq .0001$, $p \leq .0001$, $p \leq .01$, $p \leq .001$ and $p \leq .01$, and .03 respectively).

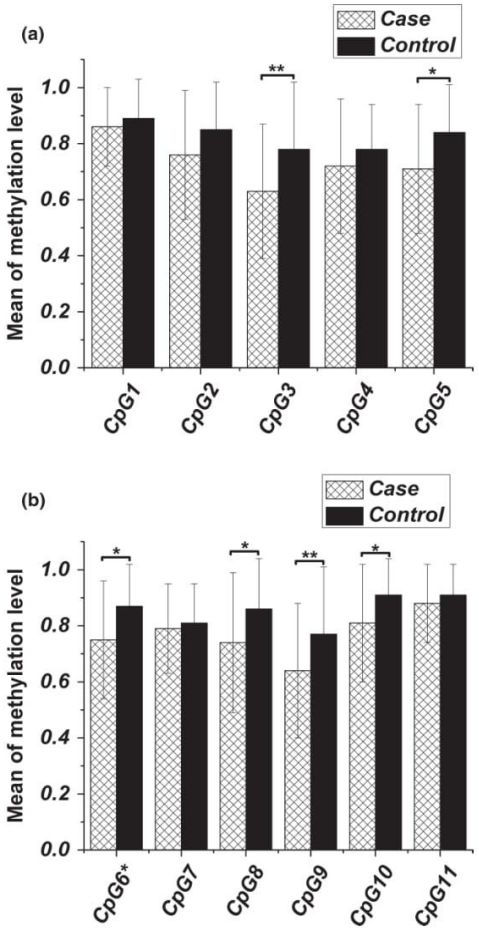

FIGURE 1 DNA methylation levels at (cg19406133) and its different neighboring CpGs related amplicons between proven fertile (Case) $n = 37$, and subfertile (Control) $n = 45$; $p \leq .05^{*}$, $p \leq .01^{**}$

The correlation between the DNA methylation level obtained by Bi-PROF at different CpGs and the sperm parameters of the case group was assessed.

Table 4 illustrates a significant correlation between cg19406113—and its adjacent CpGs—and sperm parameters in the following pattern: the percentage of total sperm motility is associated with CpG6* ($p \leq .04$). In addition, the percentage of progressive motility was significantly correlated with CpG2 and CpG3 ($p \leq .04$ and $p \leq .02$). Moreover, the percentage of nonprogressive motility showed a significant correlation with CpG1.CpG3, CpG5 and CpG6* ($p \leq .05$ and $p \leq .0001$, $p \leq .04$ and $p \leq .009$ respectively). The percentage of immotile spermatozoa showed a significant correlation with CpG3, CpG5, CpG6* and CpG8 ($p \leq .008$ and $p \leq .05$, $p \leq .02$ and $p \leq .003$ respectively). Furthermore, the percentage of viability demonstrated a significant correlation with CpG4 and CpG10 ($p \leq .03$ and $p \leq .007$). The percentage of vitality revealed a significant correlation with CpG2, CpG3, CpG4, CpG5, CpG6, and CpG9 ($p \leq .007$, $p \leq .03$, $p \leq .0001$, $p \leq .001$, $p \leq .02$ and $p \leq .008$ respectively).

In addition, CpG2 adjacent to the cg19779893 site amplicon showed a significant correlation with the mean percentage of sperm viability ($p \leq .008$).(See Table 4.).

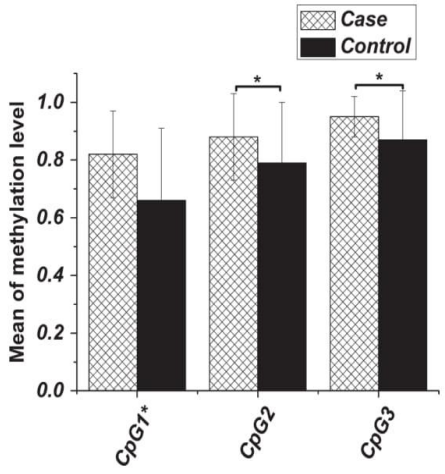

FIGURE 2 DNA methylation levels at (cg19779893) and its different neighboring CpGs in ADAMTS14 gene between proven fertile (Case) $n = 37$, and subfertile (Control) $n = 45$; $p \leq .05$*, $p \leq .01$**.

4 | DISCUSSION

The purpose of the present study was to assess whether there is an alteration in the DNA methylation level of spermatozoa obtained from subfertile males suffering from an inability to father–children due to idiopathic problems, compared to proven fertile males. There was also an attempt to estimate its association with the sperm parameters of the subfertile group. According to previous studies, male fecundity problems are a worldwide issue and recent statistics show that they affect 15% of partners (Krausz, Murci, & Mc Elreavey, 2000). In many cases, the basic causes are unknown and almost 60%–70% of male infertility cases are idiopathic (Filipponi & Feil, 2009). In many cases, epigenetic modifications play an important role in the regulation of biological processes, including spermatogenesis (Anway et al., 2005; Li, Ma, & Wang, 2006; Rajender, Avery, & Agarwal, 2011). Primordial germ cells undergo broad epigenetic reprogramming, including DNA demethylation and re-methylation (Houshdaran et al., 2007); the DNA demethylation occurs in the early stages of evolution, and the DNA re-methylation initiates the maturational process during the prospermatogenetic cycle (Dean, Lucifero, & Santos, 2005; O'Doherty & McGettigan, 2015; Oakes, La Salle, Smiraglia, Robaire, & Trasler, 2007). Therefore, DNA methylation is essential for activation of the spermatogenetic process. Various studies have found that any alteration in the sperm DNA methylation of some genes in the male

TABLE 3 Descriptive characteristics of study populations ($n = 82$)

	Controls		Cases		
Variables	(Mean + *SD*)	Median (Min-Max)	(Mean + *SD*)	Median (Min-Max)	*p*-value
Age (Year)	37.16 ± 5.5	38 (29–45)	38.50 ± 6.68	38 (31–45)	.20
Sperm concentration [Mill/ml]	80.49 ± 54.59	663 (21–210)	51.38 ± 29.74	56 (6.5–125)	.02
Total Sperm motility (%)	52.09 ± 8.99	50 (40–75)	35.06 ± 17.07	30 (24–95)	.0001
Progressive motility (%)	36.64 ± 10.76	37 (16–65)	24.32 ± 15.09	21 (6–73)	.0001
Non Progressive motility (%)	15.67 ± 6.4	15 (5–28)	12.21 ± 6.2	10 (2–24)	.01
Immotile spermatozoa (%)	39.73 ± 11.96	40 (9–60)	60.07 ± 22.39	68 (10–98)	.0001
Normal morphology (%)	35.81 ± 9.14	38 (20–50)	29.59 ± 20.73	25 (10–96)	.001
Sperm viability (%)	60.78 ± 14.20	60 (28–86)	51.99 ± 12.34	55 (22–78)	.01
Sperm vitality (%)	80.41 ± 11.17	85 (45–94)	74.76 ± 12.21	76 (45–93)	.03

SD, Standard deviation.

TABLE 4 Correlation between DNA methylation level in DMCs obtained by Bi-PROF and sperm parameters in the case groups (n = 45)

DMCs		Count	% of total Motility	% of Progressive Motility	% of Non-progressive motility	% of Immotile	Normal form	Viability	Vitality
Methylation level at DMCs CpGs in cg19406113 site related amplicons									
CpG1	*r*	.14	.08	−.06	.29[b]	−.19	−.11	−.19	−.11
	p	.34	.56	.96	.05	.21	.47	.21	.43
CpG2	*r*	.23	−.02	−.29[b]	.18	−.17	−.07	−.21	−.39[a]
	p	.12	.89	.04	.23	.25	.64	.16	.007
CpG3	*r*	−.01	.24	−.32[b]	.5[a]	−.39[a]	−.02	−.09	−.32[b]
	p	.92	.11	.02	.000	.008	.87	.53	.03
CpG4	*r*	.04	.008	−.18	.16	.24	−.19	−.32[b]	−.5[a]
	p	.75	.95	.21	.29	.11	.21	.03	.000
CpG5	*r*	.09	.01	−.23	.31[b]	.29[b]	.01	−.27	−.46[a]
	p	.53	.47	.12	.04	.05	.91	.07	.001
CpG6	*r*	.09	.29[b]	−.11	.38[a]	−.32[b]	−.16	−.16	−.34[b]
	p	.53	.04	.48	.009	.02	.29	.28	.02
CpG7	*r*	.13	.06	−.03	.14	−.004	−.14	−.16	−.17
	p	.38	.65	.81	.34	.97	.36	.28	.24
CpG8	*r*	.24	.22	.01	.25	−.43[a]	−.06	−.06	−.27
	p	.10	.13	.94	.08	.003	.68	.69	.06
CpG9	*r*	.28	.03	−.27	.14	−.16	.14	−.27	−.39[a]
	p	.06	.84	.07	.35	.27	.33	.06	.008
CpG10	*r*	.06	.01	−.09	.20	−.20	−.08	−.39[b]	−.19
	p	.65	.94	.54	.18	.17	.56	.007	.21
CpG11	*r*	.04	.18	.06	−.04	.11	−.10	−.11	−.15
	p	.79	.23	.66	.77	.48	.52	.43	.33
Methylation level at DMCs CpGs in ADAMTS14 gene related amplicons									
CpG1	*r*	−.10	.06	.23	.005	.24	−.21	.07	.19
	p	.93	.66	.12	.97	.11	.17	.62	.19
CpG2	*r*	−.11	.16	.28	−.07	.18	−.14	.39[a]	.21
	p	.45	.28	.06	.63	.22	.35	.008	.15
CpG3	*r*	.008	.17	.11	.19	.05	−.11	.18	.18
	p	.96	.25	.48	.19	.71	.48	.23	.21

[a]Correlation is significant at the .01 level.
[b]Correlation is significant at the .05 level.

reproductive system affects the standard sperm parameters and causes a subsequent reduction in human male fertility (Aston et al., 2012; Grégoire et al., 2013; Houshdaran et al., 2007; Xu et al., 2016). According to the results of 450 K array, 8 CpG sites showed alterations in their DNA methylation level, varying between the case and control group; the following CpGs, cg19779893 and cg19406113, were selected to be validated in further samples. The results of validation, using bisulfite sequencing in more samples, found that a significant variation in the methylation level was related to cg19779893 where two CpGs (CpG2, CpG3) of the 3 CpGs site amplicon were detected in the case group compared to the control one. Moreover, six CpGs (CpG3, CpG5, CpG6*, CpG8, CpG9 and CpG10) of eleven, related to the cg19406113 site amplicon, showed a significant difference in DNA methylation between the case and control groups. These findings are in accordance with previous ones noted by other researchers (Aston et al., 2015; Jenkins et al., 2016; Laqqan, Solomayer, & &Hammadeh, 2017), who found an alteration in the sperm DNA methylation levels between subfertile and fertile males.

Interestingly, cg19779893 is located in intron 2 of the *ADAMTS14* gene, which is a member of the ADAMTS (a disintegrin and metalloproteinase with a thrombospondin motif) that play a vital role in the spermatogenesis cycle, spermatozoon–egg interaction and embryo implantation. ADAMTS14 is located on the human chromosome 10q2 and is expressed highly in the testes (Dun et al., 2012; ENCODE Project Consortium, 2012; Shi-Wu et al., 2001).

The present study showed a strong significant difference in the following sperm parameters, namely in sperm concentration, the percentage of spermatozoa with progressive and nonprogressive motility, the percentage of normal forms and sperm vitality between the case and control groups. These results are in accordance with the results from Jenkins et al. (2016), who also found significant differences in the standard sperm parameters among fertile and subfertile males.

Furthermore, according to the results of the present study, there is a correlation between the alterations in sperm DNA methylation levels of some differentially methylated CpGs and the mean percentage of total sperm motility, progressive and nonprogressive motility, viability and vitality in subfertile males (See Table 4). This is similar to the results found by Houshdaran et al. (2007) and Montjean et al. (2015), who found a significant correlation between sperm DNA methylation and conventional sperm parameters (in concentration and motility).

In conclusion, the results of the present study demonstrated alterations in two CpGs in the sperm DNA methylation levels of the proven fertile control group compared to the subfertile male case group. In addition, a strong significant association was found between the changes in sperm DNA methylation levels in these CpGs (and in their neighbouring CpGs sites) and in sperm parameters.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to be declared.

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3.5 Alterations in sperm DNA methylation patterns of oligospermic males

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Original article

Alterations in sperm DNA methylation patterns of oligospermic males

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ABSTRACT

Aberrant in sperm DNA methylation patterns and histone modification play a role in male fecundity decline. This study was prepared to determine whether sperm DNA methylation at CpG dinucleotides is different in oligospermic males compared to proven fertile males and then to evaluate the correlation between the changes in sperm DNA methylation patterns and semen parameters of oligospermic males. A total of 165 males (64 proven fertile males "controls" and 101 oligospermic males "cases") were included in the study. Three CpG sites have the highest difference in methylation levels (cg23081194, cg25750688, and cg04807108) were underwent to further analysis using deep bisulfite sequencing in 125 samples (44 controls and 81 cases). The results of a validation study showed that variation in methylation levels was found in more than one CpG site: there was a significant alteration in methylation levels at all CpGs tested within the *UBE2G2* and cg25750688 site related amplicon ($p \leq 0.0001$), and at eight CpGs (CpG1, CpG3, CpG6, CpG8, CpG11, CpG13, CpG14, and CpG15) within the cg04807108 site related amplicon ($p \leq 0.0001$) in cases compared to controls. Besides, a significant correlation was found between the changes in the methylation levels at different CpGs and semen parameters of case group. In conclusion, this study showed that these sites have a significant alteration in sperm DNA methylation levels in oligospermic males compared to proven fertile males, and these changes correlated with semen parameters.

1. Introduction

Infertility is defined as the inability of a sexually active couple to achieve a successful pregnancy after 12 months of unprotected intercourse, and about 15% of couples around the world suffer from this problem [1,2]. The male factor considered as the common cause in about 40% of infertility cases [3,4]. Further, approximately 15–30% of couples are diagnosed with unexplained infertility after a routine analysis [5,6]. A common source of infertility problems in human couples is male subfertility or oligospermia [7,8] and genetic abnormalities such as karyotype anomalies [9]; Y chromosome microdeletions [10]. Current studies revealed a strong correlation between aberrant DNA methylation in spermatozoa and male subfertility [11,12], and indicate that epigenetics might have a strong impact on the spermatogenesis and sperm quality. Epigenetics is defined as alterations to DNA that turn genes "on" or "off" without changes to the DNA sequence [13]. Epigenetics involves modifications to histone proteins and DNA methylation; here, we focused on DNA methylation and subfertility in males. In mammals, DNA methylation is an epigenetic mechanism that occurs by the addition of a methyl (CH3) group to the 5 position of cytosine by DNA methyltransferase to form 5-methylcytosine (5-mC) [14], thereby often altering the function of the genes, and it is involved in the establishment and maintenance of genetic imprinting and regulation of tissue-specific gene expression [15–18]. The regulations of the gene expression process occur either by changing the binding site of transcriptional factors or by attracting proteins that modify chromatin structure around the gene transcriptional start site like methyl-DNA-binding proteins and histone deacetylases [19,20], ultimately both mechanisms block transcription and cause gene silencing [21]. During a man's lifetime, the reprogramming of DNA methylation takes place shortly after fertilization in the zygote and then in primordial germ cells (PGCs) which are the embryonic progenitors of sperm or oocytes [22–24]. One of the most concerning aspects of sperm epigenetic anomalies is that under experimental conditions, these defects can be transmitted to the offspring and can influence the offspring's susceptibility to disease [25–27]. Therefore, alterations in sperm DNA methylation patterns of subfertile men may potentially contribute to the increased risk of imprinting disorders, perinatal mortality, and several other pregnancy-related complications that are seen in assisted reproductive technology (ART) babies [28–30]. Until now the

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relationship between alteration in sperm DNA methylation levels and male subfertility is still unclear. Therefore, this study is designed (I) to evaluate the variation in sperm DNA methylation level at CpG dinucleotides between oligospermic males, and proven fertile males, (II) and to assess the relationship between the alterations in sperm DNA methylation levels and semen parameters in oligospermic males, in order to further understand the role of sperm DNA methylation in male fertility.

2. Material and methods

2.1. Sample collection and sperm isolation

This study was approved by the Institutional Ethics Committee of Saarland University (13/14), and consent was provided according to the Declaration of Helsinki. Besides, all participants gave written consent before participation in this study. All samples were analyzed in the laboratory of the Molecular Biology, Genetics & Epigenetics Department at the University of Saarland. Samples were analyzed according to standard operating procedures. Briefly, one hundred and sixty-five samples were collected from males with a mean age of 35.66 ± 3.75 years (64 samples from proven fertile "controls" males and 101 samples from oligospermic males "cases"). The exclusion criteria for participation in the study were as follows: diabetes mellitus, the consumption of alcohol, the presence of anti-sperm antibodies, varicocele, and Y chromosome microdeletions, karyotype abnormalities, smoking, abnormal hormonal parameters, abnormal body mass index, and infertility problems related to the female partner. In contrast, the participants included in this study have no direct contact with environmental pollutants (e.g. toxic metals, radioactive pollutants, lead, and other heavy metals); males had the same nationality, ethnicity, and food supplementation. Briefly, Semen samples were collected by masturbation after three days of sexual abstinence, allowed to liquefy at 37 °C for 30 min, and then the sperm concentration was assessed immediately by using a Makler counting chamber (Sefi-Medica, Haifa, Israel). The sperm parameters were analyzed according to World Health Organization guidelines [31]. All of the semen samples were underwent to the protocol of Somatic Cell Lysis Buffer (SCLB), to remove the somatic cells before DNA extraction step from spermatozoa. The semen samples were loaded onto 45%–90% "two-layer" discontinuous Puresperm gradients (Nidacon International) and then centrifuged at 500g for 20 min at room temperature, which effectively separates normal sperm from lymphocytes, epithelial cells, abnormal or immature sperm, bacteria and seminal fluid. Then the pure sperm was incubated with SCLB on ice for 30 min and washed two-time with phosphate-buffered saline (10 min at 500 g) [32]. The absence of immature sperm and somatic cells contamination has been confirmed by microscopic examination.

2.2. DNA extraction from spermatozoa and sodium bisulfite treatment

Sperm DNA was extracted by using the Isolate II genomic DNA kit according to the standard protocol provided by the manufacturer (Bioline, UK). The quantity and purity of extracted DNA were determined with the use of a Nanodrop spectrophotometer ND-2000c (Thermo Scientific). 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, 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. Five hundred nanograms from extracted sperm DNA were treated with sodium bisulfite using the Epitect bisulfite conversion kit (Qiagen, Germany) that converts unmethylated cytosines to uracil, while the 5-methylcytosine (5MeC) remains unaltered, as previously described [33].

2.3. DNA methylation analysis by infinium 450 K BeadChip array (Screening study)

Forty semen samples from males with a mean age of 36.40 ± 3.24 years were used in the screening study; this included 20 samples from oligospermic males "cases" and 20 from proven fertile males "controls". After bisulfite treatment, the DNA of these samples was subjected to Infinium 450 K BeadChip arrays (Illumina, San Diego, CA, USA) following the manufacturer's recommendations [34], and the arrays were scanned using the Illumina iScan. Beta-values were then generated by analyzing the intensities for methylation or no methylation at each CpG tiled on the array using the calculation: β-value = -methylated/(methylated + unmethylated). The result of β-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 mean a completely unmethylated CpG. Raw intensity values obtained from Illumina were used to generate β-values, the bioinformatic processing and evaluation were performed with the RnBeads program package [35]. The methylation level in each CpG was considered as being differentially methylated CpG (DMC) when the absolute difference of the means of the average β-values between two groups was ≥ 20%; a Benjamini–Hochberg corrected *t*-test FDR (false discovery rate) of 0.05, and all CpG sites with a coverage (no. of beads) of ≤ 5 were excluded from the analysis. Findings were considered significant when $p \leq 0.01$. Referring to the technical results of hybridization, the gene call rate above 98% per sample and a detection value $p < 0.01$ per CpG site were set as the internal quality criteria. All CpGs that covered a common SNP site in the dbSNP137 database were excluded.

2.4. Bisulfite profiling (Validation study)

In this study, and according to the results of screening study, three CpG sites that have the greatest difference in methylation level between the oligospermic males and proven fertile males were subjected to further analysis using deep bisulfite sequencing (Bi-PROF) [36], according to the manufacturer's instructions. In the validation study, 125 samples (independent samples) were used and distributed as follows: 44 proven fertile males "controls" and 81 oligospermic males "cases". Briefly, 500 nanograms of sperm DNA from each sample were subjected to bisulfite treatment using the Epitect bisulfite conversion kit (Qiagen, Germany). PCR reactions were performed in a 50 µl total volume reaction using the "MyTaqTM HS Red Mix" with 2 x concentration (Bioline, UK), according to the manufacturer's protocol. For amplification, fusion primers that consisted of a specific 3′-portion (listed in Table 1, together with respective annealing temperatures and a number of CpGs present within the amplicon sequence) and a universal 5′-portion containing the necessary nucleotide sequences for Illumina sequencing were used. Primers were designed using the BiSearch primer design tool (http://bisearch.enzim.hu/?m=search) with the following criteria: max length of PCR 400, primer concentration 0.167 µmol, potassium concentration 50 mmol, magnesium concentration 2.5 mmol, primer length 20–30, max Tm difference 2.0. By using BiSearch placing primers onto common SNPs could be eliminated. Five microliters of PCR products were loaded on a 2% agarose gel stained with ethidium bromide, including the DNA ladder (Biolabs, NE), PCR products were purified using the Agencourt® AMPure XP beads (Beckman Coulter, USA), measured by using Quant-iT™ DNA Assay Kit (Fisher Scientific, USA) according to the manufacturerś recommendations, and then diluted and pooled. Deep bisulfite sequencing was performed on the Illumina MiSeq according to the manufactureŕs protocols aiming at 10.000 reads per amplicon. All data obtained from the sequencing step

were processed, filtered, and aligned by using BiQ Analyzer HT software [37], excluding all reads containing equal or more than 10% of missing CpG sites (maximal fraction of unrecognized sites ≥ 0.1). The obtained alignments sequence showed an absence of alterations at CpG positions.

2.5. Statistical analysis

Data obtained from the validation study were analyzed using IBM SPSS for Windows software package version 23.0 (SPSS Inc., USA). Samples included in this study were non-normally 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) was used to compare means of quantitative variables. In addition to Spearman's test to assess the correlation coefficient between methylation level and other sperm parameters. The results in the above-mentioned procedures were accepted as statistically significant when $p \leq 0.05$.

3. Results

In a screening study, forty DNA samples from spermatozoa (20 cases, and 20 controls) were subjected to Infinium 450 K BeadChip analysis, to assess the differences in methylation patterns between study groups. The screening phase results showed 50 dinucleotides have significant differences. However, only 5 of those have significant differences in the case compared to control groups without overlap common annotation SNPs. Supplementary Table S1 summarizes the location of the most differentially methylated CpGs (DMC), based on the mean DNA methylation difference of ≥ 20% between the case and control groups. Out of five CpGs identified, 2 were found in gene bodies and CpG islands (cg23081194 and cg19779893) and 3 were found in intergenic regions (cg04807108, cg25750688, and cg19406113). From the results of screening study, three CpG sites have the highest difference in methylation level were subject to validation study in 125 samples: I) cg23081194 was located in intron 2 of the *UBE2G2* gene, and II) cg04807108 and cg25750688 were located in intergenic sequences. Table 2 illustrates that the age of the cohort study population was between 23.0-40.0 years, with a mean age of 34.93 ± 4.25 years, further the descriptive characteristics of the study population. As indicated in Table 3, the study population consisted of two groups: the proven fertile group was composed of 44 males with a mean age of 34.73 ± 3.32 years as the “controls”, and the oligospermic group was composed of 81 males, with a mean age 35.04 ± 4.70 years as the “cases”. The semen parameters revealed a significant decrease in sperm concentration, the percentage of total sperm motility, the percentage of progressive motility, the percentage of non-progressive motility, and the percentage of sperm normal form in the case group compared to the control group ($p \leq 0.00001$). Conversely, a significant increase in the percentage of immotile sperm ($p \leq 0.0001$) was found in cases compared to controls (Table 3). According to the results of the validation, the study found that more than one CpG showed a difference in the methylation level compared to neighboring CpGs obtained from the screening study. The mean DNA methylation levels at all CpGs related to the *UBE2G2* gene amplicon (CpG1, CpG2, and CpG3) and acg25750688 site related amplicon (CpG1 and CpG2) showed a significant difference in the case compared to the control group ($p \leq 0.0001$) (Figure 1, S1 and Figure 2, S2). Furthermore, the results showed that eight of the fifteen CpGs within the cg04807108 site related amplicon (CpG1, CpG3, CpG6, CpG8, CpG11, CpG13, CpG14, and CpG15) had a significant difference in the mean methylation level ($p \leq 0.0001$) in cases compared to controls (gFigure 3, S3).

Finally, this study assessed the correlation between sperm DNA methylation levels obtained by Bi-PROF at different CpGs and other sperm parameters for the case group (Supplementary Table S2). A significant positive correlation was found between the methylation levels at CpG2 in the *UBE2G2* gene-related amplicon and sperm concentration ($p \leq 0.029$). Besides, a significant correlation was found between the methylation levels at CpG1 and CpG3 in the *UBE2G2* gene-related amplicon and the percentage of sperm normal form ($p \leq 0.005$ & $p \leq 0.027$, respectively). Moreover, a significant correlation has been shown between the percentage of total sperm motility, a percentage of sperm progressive motility, a percentage of immotile sperm and the methylation levels at CpG2 in the cg25750688 site-related amplicon ($p \leq 0.006$, $p \leq 0.00001$, and $p \leq 0.002$, respectively). Furthermore, a significant correlation has been shown between the methylation at two CpGs (CpG1, CpG15) related to the cg04807108 site amplicon and the sperm concentration ($p \leq 0.003$ & $p \leq 0.005$, respectively). Moreover, a significant negative correlation was found between the between the methylation at seven CpGs related to the cg04807108 site amplicon (CpG2, CpG4, CpG5, CpG7, CpG9, CpG10 and CpG12) and a percentage of non-progressive motility ($p \leq 0.023$, $p \leq 0.00001$, $p \leq 0.00001$, $p \leq 0.002$, $p \leq 0.019$, $p \leq 0.024$, and $p \leq 0.005$, respectively). On the other hand, a significant negative correlation was observed between the methylation level at two CpGs (CpG4 and CpG5) related to the cg04807108 site amplicon and the percentage of total sperm motility ($p \leq 0.022$ & $p \leq 0.007$, respectively).

4. Discussion

The present study evaluated the methylation levels at DMC in the cg23081194, cg25750688 and cg04807108 site related amplicons in spermatozoa obtained from oligospermic males compared to proven fertile males. It is noteworthy that these sites are associated with male fertility, sperm concentration, and sperm function [38–40]. According to previous studies, the DNA methylation plays a crucial function in the spermatogenesis process and the decline in the reproductive potential of men could be caused by abnormal methylation in genes expressed in the testes [41–44]. Furthermore, other studies revealed that changes in the sperm DNA methylation levels may affect male fertility and semen parameters [29,45]. According to the results of the screening study, three CpGs (cg23081194, cg25750688, and cg04807108) have the highest difference in methylation level were validated on 125 independent samples, and this CpGs are located in the gene body and intergenic regions [46]. The results of validation study showed a significant difference in the methylation level at all CpG in *UBE2G2* gene-related amplicon and in cg25750688 site related amplicon. In addition, significant differences were found in eight of the fifteen CpGs in the cg04807108 site-related amplicon in sperm samples from males from the cases group compared to samples from the control group. These results indicate that differences in sperm DNA methylation at DMC might be among the mechanisms that lead to semen parameters reduction. These changes in the sperm DNA methylation level are in line with the studies previously reported by other authors [47–50] who found alterations in the sperm DNA methylation levels from subfertile males compared to the proven fertile males. In addition to another study prepared by Jenkins et al. who found two genomic regions with statistically significantly decreased methylation and three genomic regions with statistically significantly increased methylation in the failure-to-conceive group. Besides, a decrease in the methylation levels at two sites closely related genes, HSPA1L and HSPA1B, known to be expressed in sperm and correlated with infertility problems [12]. Also, other studies found an association between the change in the level of sperm DNA methylation and reduction in the sperm concentration and fertility potential [51–53]. Furthermore, the Aston and his colleagues pointed that the spermatozoa DNA methylation patterns differ significantly in infertile vs. fertile, normozoospermic males [54]. With regard to the genes of this study, the UBE2G2 gene maps to the region of human chromosome 21q22.3 encode the ubiquitin-conjugating enzyme E2G2 and its transcripts are ubiquitously expressed in human tissues [55]. In spermatozoa, a particularly high level of E2G2

expression was found [38,39]. Ubiquitination involves at least three classes of enzymes: ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin-protein ligases (E3s) [56]. It is linked with the (I) non-proteolytic functions such as the regulation of protein interactions, signal transduction, DNA repair, kinase activation, endocytosis, protein trafficking, cell cycle progression [57,58], (II) intercellular communication [59], (III) cell proliferation [60] and (IV) apoptosis [40,51]. Also, the human ubiquitin-conjugating enzyme E2G2 (*UBE2G2/UBC7*) is involved in protein degradation, including a process known as endoplasmic reticulum-associated degradation (ERAD) [61,62]. Previous studies have shown that both, ubiquitination and deubiquitination, play a role in later phases of spermatogenesis [63], and many recent studies have indicated the importance of ubiquitination in spermatogenesis and fertilization. The dysfunction of different ubiquitin systems results in impaired sperm maturation with abnormal organelle morphology and capacity, which in turn is highly associated with male infertility [40]. In the present study, a highly significant change was observed in cases compared to control groups in each of the following parameters: sperm concentration, percentage of total sperm motility, percentage of progressive motility, percentage of non-progressive motility, percentage of immotile sperm, and percentage of sperm normal form, and these matching with the results of previous studies [51,64,65].

Finally, the results showed a significant correlation between methylation levels at CpG1 and CpG3 in the *UBE2G2* gene-related amplicon and the percentage of sperm normal form. A significant correlation was found between the methylation level at CpGs in the cg25750688 site-related amplicon and semen parameters. Moreover, a similar correlation has been found between the methylation levels at more one CpGs in the cg04807108 site related amplicon and semen parameters. These correlations are consistent with the results of other researchers who found a strong association between sperm concentration, sperm motility, sperm normal form, and sperm DNA methylation levels [66–68].

5. Conclusions

In conclusion, the results of this study show that there are significant differences in sperm DNA methylation levels between oligospermic males and proven fertile males. Moreover, a correlation between the methylation levels at these CpGs and semen parameters in oligospermic males have been found.

Conflict of interest

The authors confirm that they have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.repbio.2017.10.007.

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3.6 Spermatozoa from males with reduced fecundity exhibit differential DNA methylation patterns

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Spermatozoa from males with reduced fecundity exhibit differential DNA methylation patterns

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ABSTRACT

Infertility affects 10–15% of couples, and approximately 50% of cases are linked to male factor infertility. The purpose of this study was to evaluate the DNA methylation patterns in spermatozoa from males who are suffering from a reduction in fecundity. Thirty samples were subjected to 450K arrays as a screening study to evaluate the variation in sperm DNA methylation levels between cases and controls groups, and then four CpG sites (cg05799088, cg07227024, cg16338278, and cg08408433) underwent to deep bisulfite sequencing to validate the observed methylation differences in 111 samples (56 proven fertile males as 'controls' and 55 males suffering from a reduction in fecundity as 'cases'). A significant difference in the mean methylation level was found between cases and controls in the CpGs of *PRICKLE2* gene-related amplicon (CpG1, $p \leq 0.002$, and CpG2, $p \leq 0.004$) and CpG of *ALS2CR12* gene-related amplicon (CpG1, $p \leq 0.015$, and CpG2, $p \leq 0.009$). Besides, a significant difference was found at seven from thirteen CpGs tested in the *ALDH3B2* gene amplicon CpG2, CpG6, CpG9, CpG10, CpG11, CpG12, and CpG13 ($p \leq 0.005$, $p \leq 0.004$, $p \leq 0.012$, $p \leq 0.028$, $p \leq 0.012$, $p \leq 0.009$, and $p \leq 0.001$, respectively). In addition, the results showed that nine CpGs out of the twenty-six within the *PTGIR* gene-related amplicon (CpG4, CpG6, CpG8, CpG9, CpG11, CpG15, CpG19, CpG23, and CpG26) had a significant difference in their mean methylation level ($p \leq 0.006$, $p \leq 0.009$, $p \leq 0.003$, $p \leq 0.003$, $p \leq 0.007$, $p \leq 0.002$, $p \leq 0.018$, $p \leq 0.018$, and $p \leq 0.040$, respectively) in the case vs. control group. In conclusion, an alteration in the methylation levels of sperm DNA from males with reduced fecundity was observed. In addition, an association between changes in the methylation level for these CpGs and different semen parameters has been found.

INTRODUCTION

Infertility affects 10–15% of couples, and approximately 50% of cases are linked to male factor infertility (Nieschlag *et al.*, 2011; Mascarenhas *et al.*, 2012; Raheem *et al.*, 2012; Inhorn & Patrizio, 2015; Kumar & Singh, 2015). In recent decades, many studies have found that approximately 60% of infertility cases are classified as unexplained infertility (Erenpreiss *et al.*, 2006; Anawalt, 2013; Yassin *et al.*, 2017). Besides genetic causes, aberrant patterns of DNA methylation and histone modification have been shown to play a role in male infertility or fecundity decline, and have been identified in 15–30% of infertile males (Amor & Halliday, 2008; Pliushch *et al.*, 2010; Esteves, 2013; Soubry, 2015; Jenkins *et al.*, 2016a). The molecular mechanisms of these defects need more clarification and explanation since male infertility has been clarified as a phenotype of the deficiency in the transcription of various single genes (Matzuk & Lamb, 2002). DNA methylation is an epigenetic mechanism used by cells to regulate gene expression, and which occurs by the addition of a methyl (CH3) group to fifth carbon atom of cytosine (Schübeler, 2015). DNA methylation plays a critical role in the regulation of gene transcription during sperm development (Portela & Esteller, 2010; Ankolkar *et al.*, 2012; Hackett & Surani, 2013). In human, DNA methyltransferases (DNMTs) are responsible for the transfer of a methyl group (CH3) to 5-cytosine of CpG dinucleotides to form 5-methylcytosine (Smith & Meissner, 2013; Celik *et al.*, 2016). The methylated state of CpGs has a critical impact on gene transcription during embryonic growth, genomic imprinting, X-chromosome inactivation, and tumor development (Chaligné & Heard, 2014; Liyanage *et al.*, 2014; Heyn *et al.*, 2016) based on CpG islands found in the 5′ regulatory and promoter regions of genes. Recent findings have shown that epigenetic regulation plays a crucial role in neurodevelopment,

aging, and many human diseases (Tan & Shi, 2012; Kaas *et al.*, 2013; Feinberg *et al.*, 2015; Jones *et al.*, 2015). Several studies have illustrated that changes in the DNA methylation of specific genes in germ cells are linked with oligozoospermia, reduced progressive sperm motility, and abnormal sperm morphology (Marques *et al.*, 2008; Hammoud *et al.*, 2010). Likewise, a recent study detected aberrant DNA methylation levels for genes involved in the spermatogenic program and located outside of imprinted regions in poor quality spermatozoa (Navarro-Costa *et al.*, 2010). Aberrant sperm DNA methylation is one of the most relevant aspects of epigenetic abnormalities in spermatozoa; these defects can be transmitted to children and can affect the child's susceptibility to disease (Wei *et al.*, 2014). However, in a number of cases, the cause of infertility is still unknown because of a lack of knowledge of the genetic and molecular mechanisms that play a role in the control of sperm cell production and maturation. Therefore, the discovery of new genes that may participate in spermatogenesis and studying their functions and methylation status may help to understand fertility problems in males.

The purpose of this study was (i) related to determine whether sperm DNA methylation at CpG dinucleotides is different in males suffering from a reduction in fecundity compared to proven fertile males, and (ii) to assess the relationship between changes in sperm DNA methylation levels, especially in the *PRICKLE2*, *ALS2CR12*, *ALDH3B2*, and *PTGIR* genes, and abnormalities in semen parameters.

MATERIALS AND METHODS

Patient recruitment and spermatozoa purification

This study was approved by the Institutional Ethics Committee of Saarland University (13/14). All participants gave written consent before participation in this study. All of the samples were analyzed in the laboratory of the Department of Molecular Biology, Genetics & Epigenetics at the University of Saarland. Samples were analyzed according to standard operating procedures. Briefly, semen samples were collected from proven fertile males (having at least two children) as a control group, and males who suffered from reduced fecundity (unable to have children after 5 years of unprotected sexual intercourse) as the case group. The exclusion criteria for participation in the study were as follows: diabetes mellitus, alcohol drinkers, the presence of anti-sperm antibodies, varicocoele, Y chromosome microdeletions, smokers, abnormal hormonal parameters, abnormal body mass index, and infertility related to the woman. In contrast, the inclusion criteria for the study population were the following: the participants included in this study have not a direct contact with environmental pollutants, males of same nationality, ethnicity, and food supplementation. Semen samples were collected by masturbation after 3 days of sexual abstinence, allowed to liquefy at 37 °C for 30 min, sperm count was assessed using Meckler counting chamber (Sefi-Medica, Haifa, Israel). Thereafter, the semen was processed through gradient centrifugation. The sperm parameters were analyzed according to World Health Organization guidelines (WHO, 2010). Before DNA extraction from spermatozoa, somatic cells have been removed from all the semen samples through the use of somatic cell lysis buffer (SCLB) which are used widely for sperm cell purification (Goodrich *et al.*, 2007; Johnson *et al.*, 2011; Sun *et al.*, 2016). First, pure populations of spermatozoa were obtained through a 50% gradient (20 min at 300 ***g***), then the pure spermatozoa were incubated with SCLB on ice for 30 min, and washed two times with phosphate-buffered saline (10 min at 500 ***g***). The absence of somatic cells contamination has been confirmed by microscopic examination.

Sperm DNA isolation and sodium bisulfite treatment

Sperm DNA was extracted using the Isolate II genomic DNA kit according to the standard protocol provided by the manufacturer (Cat. #: BIO 52066; Bioline, London, UK). The concentration and purity of extracted DNA were determined with the use of a Nanodrop spectrophotometer (ND-2000c; Thermo Scientific, Waltham, MA, USA). Five hundred nanograms of extracted sperm DNA was treated with sodium bisulfite using the Epitect bisulfite conversion kit (Cat. #: 59104; Qiagen, Venlo, Netherlands) that converts unmethylated cytosines to uracil, while 5-methylcytosine (5MeC) remains unaltered, as described previously (Wu *et al.*, 2015).

DNA methylation analysis by the Infinium 450K BeadChip array

Thirty semen samples were used as a screening study with a mean subject age of 38.0 years, range 23–45 years (15 samples from cases and 15 from controls). After the bisulfite treatment process, the DNA of these samples was subjected to the Infinium 450K BeadChip array (Illumina, San Diego, CA, USA) following the manufacturer's recommendations (Bibikova *et al.*, 2011), 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: β-value = methylated/(methylated + unmethylated). The β-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 β-values; the bioinformatic processing and evaluation were performed with the RNBEADS program package (Assenov *et al.*, 2014). 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 ≥20% with a Benjamini–Hochberg corrected *t*-test FDR (false discovery rate) of 0.05. We excluded all CpG sites from the analysis with a coverage (number of beads) less than or equal to 5, and all CpG sites with a coverage (number of beads) of ≤5 were excluded from the analysis. Findings were considered significant when $p \leq 0.01$. Referring to the technical results of hybridization, the gene call rate above 98% per sample and a detection value $p < 0.01$ per CpG site were set as internal quality criteria. All CpGs that covered a common SNP site in the dbSNP137 database were excluded. In this work and according to the 450K results, we assessed four CpG sites and validated them using 111 samples, not including the screening study samples, using gene-specific deep bisulfite sequencing (Bi-PROF; Gries *et al.*, 2013).

Bisulfite profiling

Five hundred nanograms of genomic DNA were bisulfite treated using the Epitect bisulfite conversion kit (Cat. #: 59104;

Qiagen). PCRs encompassing the differentially methylated CpG (DMC) identified by the 450K BeadChip array were performed in a 50 μL total volume reaction using MyTaq™ HS Red Mix 2x concentrated (Cat. #: BIO-25047; Bioline) according to the manufacturer's protocol. For the amplification, fusion primers were used that consisted of a specific 3′ portion (listed in Table 1, together with respective annealing temperatures and number of CpGs present within the amplicon sequence) and a universal 5′ portion containing the necessary nucleotide sequences for Illumina sequencing. Primers were designed using the BiSearch primer design tool (http://bisearch.enzim.hu/?m=search) using the following criteria: maximum length of PCR (400), primer concentration = 0.167 μM, potassium concentration = 50 mM, magnesium concentration = 2.5 mM, primer length 20–30, and maximum difference in melting temperature = 2.0 °C. Using BiSearch, placing primers onto common SNPs could be eliminated. For this assay, 5 μL of each PCR reaction was loaded onto a 2% agarose gel stained with ethidium bromide (Cat. #: N0467S; New England Biolabs, Ipswich, MA, USA). The PCR products were purified using Agencourt® AMPure XP beads (Cat. #: A63880; Beckman Coulter, Berry, CA, USA) and measured using Quant-iT™ DNA Assay Kit (Cat. #: Q33120; Fisher Scientific, Hampton, NH, 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 (Lutsik *et al.*, 2011), excluding all reads containing equal or more than 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).

Statistical analysis

Data obtained from Bi-PROF were analyzed using IBM SPSS for Windows software package version 24.0 (SPSS, Inc., Chicago, IL, USA). Samples included in this study were non-normally 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) was used to compare the means of quantitative variables, and Spearman's test was used to assess the correlation coefficient between methylation level in DMC and sperm parameters. The results in the above-mentioned procedures were accepted as statistically significant when $p \leq 0.05$.

RESULTS

In this study, 30 sperm DNA samples (15 samples as cases and 15 samples as controls) were taken as a screening study and subjected to Infinium 450K BeadChip analysis, obtaining the DNA methylation levels of approximately 485,000 CpG positions in each sample. Table S1 summarizes the location of the most differentially methylated CpGs (DMC) based on the mean DNA methylation difference of ≥20% between the males with reduced fecundity (cases) and the proven fertile males (controls). Overall, we found only nine CpG positions meeting the criteria. Six out of nine differentially methylated regions (cg05799088, cg07227024, cg16338278, cg08408433, cg23081194, and cg19779893) are located in gene bodies and CpG islands, while three were found in intergenic regions (cg04807108, cg25750688, and cg19406113). From the results of this genome-wide study, we selected the following CpG sites to validate the observed methylation differences in 111 samples.

1 cg07227024: located in intron 2 of the *ALS2CR12* gene, within a DNase I hypersensitivity cluster and a region slightly enriched for H3K4me1 (ENCODE, 2012).
2 cg16338278: located in exon 7 of the *ALDH3B2* gene, within a strong DNase I hypersensitivity cluster and the transcription factor binding site motif CTCF (ENCODE, 2012).
3 cg05799088: located in exon 8 of the *PRICKLE2* gene, located within the transcription factor binding sites of (i) FOSL2, which plays a critical role in the regulation of cell proliferation, differentiation, and transformation, (ii) MAX, which may repress transcription via the recruitment of a chromatin remodeling complex with H3K9 histone methyltransferase activity, and (iii) POU5F1, which plays a role in early embryogenesis and is necessary for embryonic stem cell pluripotency. All of them supposed to be binding sites (ENCODE, 2012). Similarly, the CpG is located within a DNase I cluster and in a region slightly enriched for H3K4me1.
4 cg08408433: located in exon 2 of the *PTGIR* gene, neighboring a DNase I hypersensitivity cluster and a region slightly enriched for H3K4me1 (ENCODE, 2012).

Table 2 illustrates the descriptive characteristics of the study population. The age of the males included in the study population was between 24.0 and 45.0 years, with mean age of 34.93 ± 5.84 years. The study population was divided into two groups: the control group was composed of 56 males with a mean age of 33.98 ± 5.53 years, and the case group was composed of 55 males with reduced fecundity and who had been unable to have children after 5 years of attempting pregnancy with mean age 35.89 ± 6.03 years. The semen parameters

Table 1 Primer sequences, number of DMCs, and PCR annealing temperatures used to amplify regions including the target CpGs analyzed by Bi-PROF

cgID	Annotation	Chr-	nt (hg19)	At (°C)	Product size (bp)	DMCs	Primer sequence (5′–3′)	
cg05799088	*PRICKLE2*	chr3	64080488	51.6	369	2	F	TGAGGTATTAGAGGGATAAATTA
							R	CAAAAAACAAATACTTAAACCT
cg07227024	*ALS2CR12*	chr2	202163482	50.7	285	2	F	TGTTTGGGTTTTAAGATAGAA
							R	CTCTAAATATACCTCACACATA
cg16338278	*ALDH3B2*	chr11	67432957	52.8	321	13	F	GGGTAGTAGTTTTTTTTGTA
							R	ACTAACTAACCACTCTAATT
cg08408433	*PTGIR*	Chr19	47127358	51.6	388	26	F	AGGTTTAGTAGGGAGTTGTT
							R	AATAAACTAAATAATAAAAATAACC

DMC, differentially methylated CpGs; Chr-, chromosome; At, annealing temperature.

showed highly significant differences in semen volume ($p \leq 0.001$), sperm count ($p \leq 0.0001$), the percentage of total sperm motility ($p \leq 0.0001$), the percentage of sperm with progressive motility ($p \leq 0.0001$), the percentage of spermatozoa with non-progressive motility ($p \leq 0.0001$), and the sperm vitality ($p \leq 0.0001$) between the case and control groups. Moreover, a statistically significant difference between cases and controls in the percentage of spermatozoa with a normal form ($p \leq 0.027$) was observed (Table 2). According to the results of local deep bisulfite sequencing, the study revealed that not only the target CpGs obtained from the 450K bead array experiments showed a difference in the methylation level, but also neighboring CpGs. A significant difference in the mean methylation level was found between cases and controls in all CpGs of the *PRICKLE2* gene-related amplicon (CpG1, $p \leq 0.002$, and CpG2, $p \leq 0.004$) (Fig. 1) and the *ALS2CR12* gene-related amplicon (CpG1, $p \leq 0.015$, and CpG2, $p \leq 0.009$) (Fig. 2). Besides, a significant difference was found at seven of thirteen CpGs tested in the *ALDH3B2* gene-related amplicon CpG2, CpG6, CpG9, CpG10, CpG11, CpG12, and CpG13 ($p \leq 0.005$, $p \leq 0.004$, $p \leq 0.012$, $p \leq 0.028$, $p \leq 0.012$, $p \leq 0.009$, and $p \leq 0.001$, respectively) (Fig. 3). In addition, the results showed that nine CpGs out of the twenty-six within the *PTGIR* gene-related amplicon (CpG4, CpG6, CpG8, CpG9, CpG11, CpG15, CpG19, CpG23, and CpG26) showed a significant difference in their mean methylation level ($p \leq 0.006$, $p \leq 0.009$, $p \leq 0.003$, $p \leq 0.003$, $p \leq 0.007$, $p \leq 0.002$, $p \leq 0.018$, $p \leq 0.018$, and $p \leq 0.040$, respectively) in the case vs. the control group (Fig. 4).

Finally, the study assessed the correlation between the methylation levels obtained by Bi-PROF at different CpGs (DMC) and other semen parameters of the cases (Table S2). A positive significant correlation was found between the mean methylation levels in (CpG2, CpG3, CpG10, CpG11, CpG12, and CpG13) of the *ALDH3B2* gene-related amplicon and sperm count ($p \leq 0.046$, $p \leq 0.001$, $p \leq 0.002$, $p \leq 0.004$, $p \leq 0.004$, and $p \leq 0.012$, respectively). Additionally, a positive significant correlation was found between the methylation level of CpG2 and the percentage of spermatozoa with non-progressive motility ($p \leq 0.032$) and the percentage of immobile spermatozoa ($p \leq 0.024$) for the same gene amplicon. On the other hand, a significant positive correlation was observed between the mean methylation levels of CpG10, CpG13, CpG14, CpG16, and CpG22 within the *PTGIR* gene-related amplicon, and the percentage of immobile sperm ($p \leq 0.029$, $p \leq 0.008$, $p \leq 0.023$, $p \leq 0.026$, and $p \leq 0.032$, respectively). Furthermore, a significant negative correlation was shown between the mean methylation level of a number of CpGs (CpG1, CpG2, CpG6, CpG9, CpG10, CpG12, CpG13, CpG14, CpG16, CpG18, CpG21, CpG22, CpG24, and CpG25) obtained by Bi-PROF for the *PTGIR* gene-related amplicon and the sperm vitality ($p \leq 0.017$, $p \leq 0.050$, $p \leq 0.040$, $p \leq 0.029$, $p \leq 0.005$, $p \leq 0.050$, $p \leq 0.013$, $p \leq 0.016$, $p \leq 0.017$, $p \leq 0.024$, $p \leq 0.037$, $p \leq 0.016$, $p \leq 0.029$, and $p \leq 0.016$, respectively).

Figure 1 Methylation level at CpGs in *PRICKLE2* gene-related amplicon. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

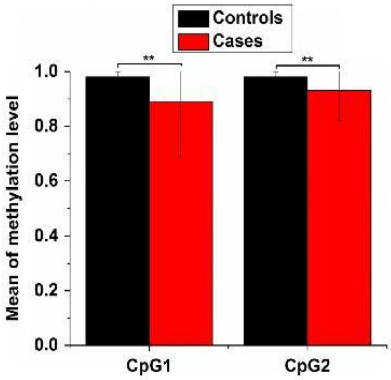


Figure 2 Methylation level at CpGs in *ALS2CR12* gene-related amplicon. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

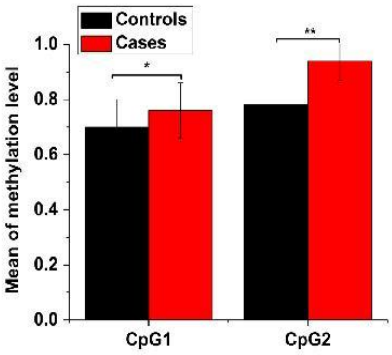


Table 2 Descriptive characteristics for the study population and for cases compared to controls ($n = 111$)

Variables	Mean ± SD	Minimum	Maximum	Controls ($n = 56$) Mean ± SD	Cases ($n = 55$) Mean ± SD	*p* Value
Age (years)	34.93 ± 5.84	24.0	45.0	33.98 ± 5.53	35.89 ± 6.03	0.076[a]
Semen volume (mL)	3.84 ± 3.97	1.0	40.0	4.02 ± 2.06	3.65 ± 5.26	≤0.001[a]
Sperm count (mill/mL)	66.37 ± 67.93	1.5	286.0	94.23 ± 70.83	38.00 ± 51.63	≤0.0001[a]
Percentage of total sperm motility	45.15 ± 20.83	1.0	91.0	60.22 ± 12.09	29.81 ± 16.11	≤0.0001[a]
Percentage of progressive motility	32.14 ± 18.80	0.0	80.0	43.73 ± 14.93	20.33 ± 14.56	≤0.0001[a]
Percentage of non-progressive motility	13.72 ± 9.37	0.0	47.0	16.50 ± 8.68	10.88 ± 9.27	≤0.0001[a]
Percentage of sperm immotile	54.28 ± 21.17	9.0	99.0	39.77 ± 12.08	69.06 ± 17.99	≤0.0001[a]
Sperm vitality	40.68 ± 22.94	5.8	80.0	57.23 ± 13.75	23.83 ± 17.51	≤0.0001[a]
Percentage of sperm normal form	26.23 ± 12.21	5.0	54.0	28.48 ± 11.86	23.93 ± 12.23	≤0.027[a]

SD, standard deviation. All values are expressed as mean ± SD. [a]Mann–Whitney test. $p > 0.05$: not significant. $p \leq 0.05$: significant.

Figure 3 Methylation level at CpGs in *ALDH3B2* gene-related amplicon. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

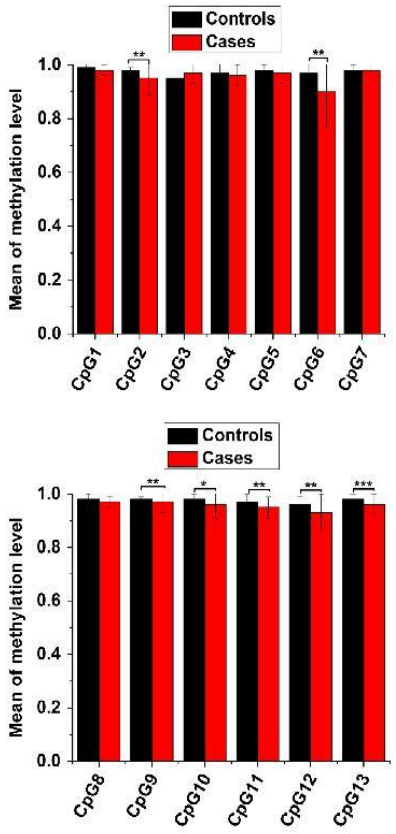
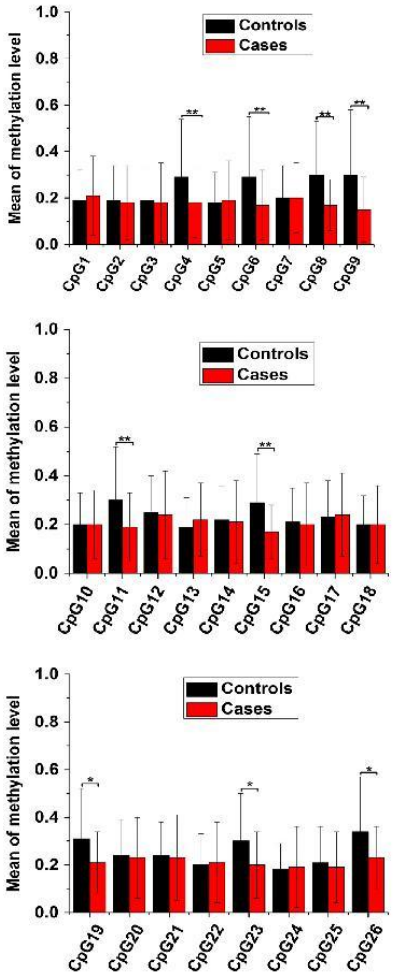


Figure 4 Methylation level at CpGs in *PTGIR* gene-related amplicon. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.



DISCUSSION

The present study evaluated the association between changes in sperm DNA methylation levels and semen parameters, which were obtained from males with reduced fecundity and proven fertile males. The results obtained from local deep sequencing showed a significant decrease in the methylation level between the case and control groups in all CpGs for the *PRICKLE2* amplicon (CpG1, $p \leq 0.002$; CpG2, $p \leq 0.004$) and *ALS2CR12* gene (CpG1, $p \leq 0.015$; CpG2, $p \leq 0.009$). Additionally, a significant decrease in methylation at CpG2, CpG6, CpG9, CpG10, CpG11, CpG12, and CpG13 for the *ALDH3B2* amplicon, and at CpG4, CpG6, CpG8, CpG9, CpG11, CpG15, CpG19, CpG23, and CpG26 for the *PTGIR* amplicon was found in the case group compared to the control group (Fig. 1). DNA methylation plays a critical role in the spermatogenesis process (Carrell, 2012; Bao & Bedford, 2016), and several previous studies have reported that one of the most common reasons that lead to a reduction in male reproductive potential and defects in spermatogenesis may be related to abnormal methylation at specific genes that are expressed in the testes (Krausz *et al.*, 2012; Grégoire *et al.*, 2013; Niederberger, 2013; Jenkins *et al.*, 2016b). Also, different studies have found a strong relationship between a change in the level of DNA methylation in spermatozoa and infertility in males (Hammoud *et al.*, 2010; Poplinski *et al.*, 2010; Sato *et al.*, 2011; Ramasamy *et al.*, 2014; Urdinguio *et al.*, 2015). The results obtained from the study of Aston *et al.* showed that some CpGs in the sperm genome of patients who suffered from a reduction in fecundity and underwent assisted reproductive technologies had alterations in the DNA methylation pattern. They proposed performing a genome-wide evaluation of sperm DNA methylation and hypothesized that DNA methylation may help to predict male fertility status as well as embryo quality (Aston *et al.*, 2015). Overall, our results are consistent with studies that have reported a change in DNA methylation in normal spermatozoa compared to poor spermatozoa (El Hajj *et al.*, 2011; Montjean *et al.*, 2013). Other studies reported that males suffering from a reduction in fecundity have a low level of sperm DNA methylation compared with control males (Aoki *et al.*, 2006; Boissonnas *et al.*, 2010; Ferfouri *et al.*, 2013). However, the results of this study contradict other studies (Houshdaran *et al.*, 2007; Jenkins *et al.*, 2016b) because no association was observed between changes in global sperm DNA methylation profiles and reduced semen parameters or poor sperm quality. It is worth mentioning that all analyzed genes, that is, *ALS2CR12*, *ALDH3B*, *PRICKLE2*, and *PTGIR* play a critical role in the process of spermatogenesis and sperm capacity (Curry

et al., 2009; Fuchs *et al.*, 2010; Choi & Cho, 2011; Matzkin *et al.*, 2012; Gong *et al.*, 2013). *ALS2CR12* transcripts are present in different stages of sperm development and associated with the fibrous sheath in the sperm flagellum (Choi & Cho, 2011). The fibrous sheath has been shown to be important for mechanical flagellar function (Chemes, 2000; Rawe *et al.*, 2001) and plays a crucial role in regulating key processes leading up to fertilization (Eddy *et al.*, 2003). *ALDH3B2* plays unique roles in the cellular defense against oxidative stress and aldehyde toxicity (Marchitti *et al.*, 2010), and the protein family *ALDH3B2* has been implicated in the etiology of some human pathologies such as cancer and male infertility (Shiraishi & Naito, 2007; Fuchs *et al.*, 2010). *PRICKLE2* is part of the non-canonical Wnt signaling pathway, which is involved in the planar cell polarity (PCP) pathway, a major signaling pathway during testis development (Bassuk *et al.*, 2008; Kerr *et al.*, 2014). Based on previous studies, it is very probable that Wnt signaling functions appear in late spermatogenesis and mainly regulates morphological changes in spermatids (Ma *et al.*, 2006; Nicol & Guiguen, 2011). The expression of *PTGIR* has been found in Sertoli cells, and this prostaglandin and its receptors are required for fertility and reproduction (Ishikawa & Morris, 2006; Matzkin *et al.*, 2012). Further studies on larger sample cohorts will show if the identified CpGs are responsible and potential predictors for transcriptional abnormalities in spermatozoa obtained from males suffering from infertility or reduced fecundity. According to the values of semen parameters between the case and control groups, the results show a strongly significant difference in semen volume, sperm count, the percentage of spermatozoa with full motility, the percentage of spermatozoa with progressive motility, sperm vitality, and the percentage of spermatozoa with a normal form ($p \leq 0.001$, $p \leq 0.0001$, $p \leq 0.0001$, $p \leq 0.0001$, $p \leq 0.0001$, and $p \leq 0.027$, respectively). This is consistent with previous studies (Poplinski *et al.*, 2010; Nanassy & Carrell, 2011; Türk *et al.*, 2014).

Finally, this study showed a positive correlation between methylation levels in some DMCs related to the *ALDH3B2* and *PTGIR* gene amplicons, and some semen parameters like sperm count, the percentage of non-progressive spermatozoa, and the percentage of immobile spermatozoa (Table S2). This agrees with a previous study showing a positive correlation between the sperm DNA methylation level, sperm count, and sperm motility (Montjean *et al.*, 2015). In the end, all the results of this study indicate the presence of a strong association between changes in sperm DNA methylation levels and fecundity decline in males. Together with other studies, it also indicates that epigenetics might influence the quality and quantity of spermatogenesis (Minor *et al.*, 2011; Kläver & Gromoll, 2014).

CONCLUSION

This study identified different CpGs related to *ALS2CR12*, *ALDH3B2*, *PRICKLE2*, and *PTGIR* with consistently altered methylation levels in sperm DNA from males with reduced fecundity. In addition, an association between changes in the methylation level for these CpGs and different semen parameters was found. The observed variations may have an influence on sperm phenotype. More studies are needed to clarify the mechanisms relating to these alterations and to discover their significance and functional consequences for male fecundity.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. CpG dinucleotides that differ in their methylation levels for sperm DNA between cases (males suffering from a reduction in fecundity) and controls (proven fertile) (n = 30).

Table S2. Correlation between the methylation level in the DMCs obtained by Bi-PROF and the semen parameters for case samples (n = 55).

4. Discussion

4.1 Cigarette-smoking and sperm DNA methylation

The impact of cigarette-smoking on sperm DNA methylation and the correlation between DNA methylation and sperm parameters remain contradictory and debatable issues. Consequently, the main concern of this thesis is to highlight the various arguments supporting the divergent opinions and results concerning the evaluation of the effect of cigarette-smoking on sperm DNA methylation and its direct consequences on sperm parameters. The mechanisms of the adverse effects of cigarette-smoking on sperm parameters are not fully understood, but a possible explanation is the direct toxic influence of nicotine and other chemical components on the epithelium of the male genital tracts with the subsequent release of chemical mediators of inflammation, such as interleukin-6 and interleukin-8, which can recruit and activate leucocytes (**Kumosani *et al.*, 2008; Saleh *et al.*, 2002**). Furthermore, the number of cigarettes consumed per day was found to be associated with an accumulation of cotinine and an increase in reactive oxygen species (ROS), which leads to sperm membrane damage. ROS causes severe damage to sperm membrane due to the presence of high proportions of polyunsaturated fatty acids in the cell membrane (**El-Melegy & Ali, 2011**).

In the present study, the harmful impacts of cigarette-smoking were investigated, with particular reference to the changes in DNA methylation levels as well as to the association between the alteration of DNA methylation levels and standard sperm parameters in male smokers. Infinium 450K BeadChip arrays were used to identify the alteration in the sperm DNA methylation patterns between the smoker and non-smoker males. A genome-wide assessment revealed differences of more than 20% between the case and control groups in the following CpGs (cg00648582, cg0932376, cg19169023, cg23841288, cg27391564, cg19455396, and cg07869343). This result already provides some clues to the potential effect of cigarette-smoking on DNA methylation. The cg00648582, cg19169023, cg23841288, cg19455396, cg07869343 are located in the gene body and are related to the *PGAM5, TYRO3, PTPRN2, TAP2* and *MAPK8IP3* genes respectively whereas the cg0932376, and cg2739156 genes are located in the intergenic regions (**ENCODE Project Consortium, 2012**).

All the seven CpGs mentioned were subjected to bisulfite sequencing in more populations. Only six CpGs showed a significant difference between the case and the control groups. Moreover, when local deep bisulfite sequencing was applied to the larger cohort, different CpG sites were obtained besides the target CpG* which resulted from the Infinium 450k beadchip array. The following CpG sites showed significant differences in the case compared to the control cases. These sites are related to specific genes and were demonstrated as follows: Fifteen of the twenty-five CpG sites in a *PGAM5* gene-related amplicon, three out of four CpG sites in gene *TYRO3* related amplicon, nine out of twelve CpG sites in gene *PTPRN2* related amplicon and six out of twenty two CpG sites in gene *MAPK8IP3* related amplicons. Furthermore, a significant difference was observed in four out of five CpG sites adjacent to cg09432376, and seven out of fifteen CpG sites neighbouring the cg27391564-related amplicon. The alterations detected in the sperm DNA methylation levels - in the current thesis - concur with those found in other previous studies. These studies found that the DNA methylation patterns were adversely afftected by cigarette-smoking (**Breitling *et al*., 2011; Dogan *et al*., 2014; Sun *et al*., 2013; Zhu et al., 2016**). This present study, however, has uniquely demonstrated the relationship between these afore-mentioned genes, namely *PGMA5*, *TYRO3* and *PTPRN2*, and the effect of cigarette-smoking on the human sperm DNA methylation level. Furthermore, the present thesis clearly showed an association between the sperm DNA methylation at some different methylation CpGs (DMCs) and sperm concentration, the mean percentage of sperm motility, and immotile sperms. Similar findings were found in the studies conducted by **Montjean *et al*. (2015)** and **Laqqan *et al*. (2017)**. However, the current study remarkably showed a significant correlation between the sperm DNA methylation at some DMCs and the mean percentage of progressive sperm motility, the mean percentage of abnormal morphology, viability, and sperm membrane integrity (vitality). On the other hand, the present study found significant differences in sperm parameters between smokers and non-smoker males, and these results were in accordance with those from previous studies (**Hammadeh *et al*., 2010; Jong *et al*., 2014; Zhang *et al*., 2013**).

Toxic substances in cigarette smoke have drastic effects on the sperm DNA methylation process. The hazardous consequences can be seen in the damage caused to various mechanisms. To give a few examples: (I) the cigarette-smoke induces a break in the DNA double-stranded helix where this fracture then leads to the recruitment of DNMTs that change the DNA methylation at CpGs neighbouring the repaired nucleotides (**Huang *et al*., 2013);** (II) carbon monoxide leads to hypoxia, which then causes a reduction in the methionine

adenosyltransferase2A that is responsible for the synthesis of S-adenosyl methionine (SAM), a methyl group donor for DNA methylation (**Liu *et al*., 2011**); (III) cigarette-smoking increases the oxidative stress which alters the DNA methylation level (**Tunc & Tremellen., 2009**); (IV) Sp1 is a transcription factor which binds to CpG sites in the gene promoters, but cigarette smoke leads to an increase in Sp1 and thus prevents *de novo* methylation of these CpG sites during early development (**Han *et a*l., 2001**).

4.2 DNA methylation and male subfertility.

On the other hand, we also assessed whether there is an alteration in the methylation levels of sperm DNA obtained from sub-fertile males compared to that obtained from proven fertile males. We subsequently investigated the correlation between the sperm DNA methylation and the sperm parameters of the subfertile males. After applying the Infinium 450K beadchip array, the following CpG sites (cg19779893, cg19406113, cg23081194, cg04807108, cg25750688, cg07227024, cg16338278, cg05799088, and cg08408433) showed significant differences among the case and control groups. The same CpG sites were validated by bisulfate sequencing on more cohort studies, where cg19779893, cg23081194, cg07227024, cg16338278, cg05799088, and cg0840843 were found to be located in the gene body of *ADAMTS14, UBE2G2, ALS2CR12, ALDH3B2, PRICKLE2, and PTGIR* genes respectively, and the three remaining cg19406113, cg04807108, and cg25750688 were located in the intergenic regions (**ENCODE Project Consortium., 2012**). When local deep bisulfite sequencing was applied, different CpGs were detected close to the target CpGs* that resulted from the screening study. The following CpG sites showed significant differences in the case compared to the control groups. These sites are related to specific genes and were demonstrated as follows: three CpGs related to the *ADAMTS14* gene, six out of eleven CpG site related to cg19406113, three CpGs in the UBE2G2 gene, two CpG sites related to cg25750688, and eight out of fifteen related to cg04807108. In addition, two CpGs related to the *PRICKLE2 gene*, two CpGs related to *ALS2CR12*, seven CpGs related to the *ALDH3B2* gene, and nine CpGs related to the *PTGIR* gene also demonstrated a significant difference in the case compared to the control groups. These variations in the sperm DNA methylation levels are in accordance with previous ones noted by other researchers (**Ferfouri *et al*., 2013**; **Aston *et al*., 2015; Jenkins *et al*., 2016; Laqqan *et al*., 2017**). Moreover, significant differences were observed between the alteration in sperm DNA methylation levels in some CpGs adjacent to those afore-mentioned CpG sites (cg19779893, cg19406113, cg23081194, cg04807108, cg25750688, and cg07227024, cg16338278, cg05799088, and cg08408433) and

standerd sperm parameters. These correlations are consistent with the results that were found by Houshdaran *et al*. (2007) and Montjean *et al.* (2015) where a significant association between sperm DNA methylation and conventional sperm parameters (in concentration and motility) was mentioned. DNA methylation is essential during the spermatogenic process. Diverse studies have found that any alteration in the sperm DNA methylation of some genes in the male reproductive system has an impact on the sperm parameters and causes a subsequent reduction in human male fecundity (**Navarro-Costa *et al*., 2010; Krausz *et al*., 2012; Xu *et al*., 2016**).

In conclusion, the present study revealed biological differences in the DNA methylation levels of CpGs located in the gene body (cg00648582, cg19169023, and cg23841288) and those located in the intergenic regions (cg0932376, and cg27391564). These alterations could potentially be related to the effects caused by smoking on the developmental stages of spermatozoa. In addition, a variation in the sperm DNA methylation patterns between proven fertile and subfertile males was also detected. Furthermore, an association between the changes in sperm DNA methylation and semen parameters was found in the case groups.

In view of the results presented in this Ph.D. thesis, there is clear evidence that cigarette-smoking produces changes in sperm DNA methylation levels. These changes were also found to be correlated with sperm parameters. As a consequence of these findings, it is advised that further studies should be conducted to investigate the influence of altered DNA methylation levels on gene expression, fertilization rates, embryo quality, and the ICSI outcome of male smokers.

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6. Appendix

Chapter 3.1

Supplementary Table 1: Table shows the correlation between DNA methylation pateerns in DMCs with the neighbouring CpGs and sperm parameters

DNA Methylation level at (Cg09432376) and adjacent CpGs related amplicon									
CPGs		**Sperm concentration (mill/mL)**	**Motility (%)**	**Progressive (%)**	**Non-progressive (%)**	**Immotile (%)**	**Normal form (%)**	**Eosin (%)**	**Hostest (%)**
CpG1	r	0.259*	0.253*	0.284*	-0.037	-0.277*	0.275*	0.458**	0.496**
	P value	0.022	0.026	0.012	0.753	0.014	0.015	0.000	0.000
CpG2	r	0.284*	0.304**	0.319**	0.001	-0.323**	0.282*	0.514**	0.570*
	P value	0.012	0.007	0.004	0.993	0.004	0.013	0.000	0.000
CpG3*	r	0.278*	0.246*	0.247*	0.044	-0.268*	0.228*	0.518**	0.524**
	P value	0.014	0.030	0.029	0.704	0.018	0.046	0.000	0.000
CpG4	r	0.274*	0.201	0.232*	-0.048	-0.221	0.230*	0.437**	0.445**
	P value	0.015	0.078	0.041	0.680	0.052	0.044	0.000	0.000
CpG5	r	0.241*	0.249*	0.263*	0.007	-0.270*	0.248*	0.430**	0.461**
	P value	0.033	0.028	0.020	0.949	0.017	0.029	0.000	0.000
DNA Methylation level at (cg19169023) and its adjacent CpGs in TYRO3 gene-related amplicon									
CpG1*	r	0.154	0.183	0.229*	-0.088	-0.203	0.273*	0.406**	0.606**
	P value	0.179	0.108	0.044	0.449	0.074	0.016	0.000	0.000
CpG2	r	0.088	-0.075	-0.102	0.071	0.076	0.045	0.018	0.036
	P value	0.442	0.512	0.374	0.540	0.511	0.700	0.873	0.757
CpG3	r	0.106	0.105	0.114	0.022	-0.131	0.219	0.288*	0.391**
	P value	0.354	0.362	0.320	0.849	0.251	0.056	0.011	0.000
CpG4	r	0.106	0.121	0.125	0.050	-0.155	0.225*	0.292**	0.374**
	P value	0.355	0.291	0.275	0.663	0.176	0.049	0.010	0.001

DNA Methylation level at (cg23841288) and its adjacent CpGs in PTPRN2 gene-related amplicon									
CpG1	r	-0.033	-0.011	-0.004	-0.035	0.021	-0.289*	-0.326**	-0.367**
	P value	0.774	0.923	0.969	0.765	0.858	0.011	0.004	0.001
CpG2	r	-0.085	-0.067	-0.067	-0.051	0.096	-0.227*	-0.343**	-0.427**
	P value	-0.033	-0.011	-0.004	-0.035	0.021	-0.289*	-0.326**	-0.367**
CpG3	r	-0.100	-0.096	-0.093	-0.055	0.122	-0.247*	-0.299**	-0.412**
	P value	0.258	0.350	0.352	0.666	0.244	0.040	0.007	0.000
CpG4	r	-0.058	-0.061	-0.058	-0.056	0.088	-0.254*	-0.333**	-0.399**
	P value	0.613	0.597	0.614	0.628	0.444	0.026	0.003	0.000
CpG5	r	-0.054	-0.065	-0.067	-0.038	0.091	-0.241*	-0.332**	-0.436**
	P value	0.639	0.574	0.558	0.740	0.429	0.035	0.003	0.000
CpG6	r	-0.042	-0.102	-0.121	0.006	0.128	-0.218	-0.356**	-0.467**
	P value	0.712	0.372	0.290	0.957	0.263	0.057	0.001	0.000
CpG7	r	-0.021	-0.096	-0.100	-0.044	0.127	-0.317**	-0.349**	-0.439**
	P value	0.858	0.403	0.381	0.705	0.266	0.005	0.002	0.000
CpG8	r	-0.051	-0.099	-0.109	-0.020	0.126	-0.225*	-0.337**	-0.473**
	P value	0.660	0.391	0.341	0.862	0.270	0.049	0.003	0.000
CpG9	r	-0.012	-0.075	-0.079	-0.038	0.103	-0.228*	-0.267*	-0.431**
	P value	0.916	0.514	0.492	0.742	0.371	0.047	0.018	0.000
CpG10	r	-0.169	-0.252*	-0.211	-0.157	0.268*	-0.165	-0.256*	-0.383**
	P value	0.139	0.026	0.064	0.173	0.018	0.152	0.023	0.001
CpG11	r	-0.084	-0.139	-0.077	-0.167	0.131	-0.283*	-0.119	-0.173
	P value	0.467	0.223	0.501	0.146	0.253	0.013	0.299	0.130
CpG12*	r	-0.057	-0.135	-0.143	-0.026	0.161	-0.222	-0.341**	-0.491**
	P value	0.622	0.238	0.210	0.825	0.159	0.052	0.002	0.000
DNA Methylation level at (cg27391564) and its adjacent CpGs-related amplicon									
CpG1	r	0.174	0.120	0.177	-0.118	-0.143	0.176	0.290**	0.491**
	P value	0.128	0.294	0.121	0.307	0.210	0.126	0.010	0.000
CpG2	r	0.010	0.165	0.158	0.011	-0.155	0.101	-0.004	-0.067
	P value	0.791	0.257	0.243	0.855	0.295	0.403	0.787	0.819

CpG3*	r	0.167	0.275*	0.319**	-0.084	-0.293**	0.269*	0.416**	0.591**
	P value	0.143	0.015	0.004	0.470	0.009	0.018	0.000	0.000
CpG4	r	0.167	0.111	0.172	-0.124	-0.137	0.162	0.284*	0.489**
	P value	0.144	0.332	0.133	0.281	0.232	0.158	0.012	0.000
CpG5	r	-0.067	-0.070	-0.003	-0.198	0.074	-0.002	0.018	0.006
	P value	0.562	0.542	0.976	0.085	0.517	0.988	0.878	0.961
CpG6	r	-0.035	-0.024	0.039	-0.186	0.029	0.075	-0.006	-0.020
	P value	0.761	0.834	0.735	0.105	0.801	0.515	0.959	0.862
CpG7	r	0.154	0.183	0.229*	-0.088	-0.203	0.273*	0.406**	0.606**
	P value	0.179	0.108	0.044	0.449	0.074	0.016	0.000	0.000
CpG8	r	0.143	0.166	0.212	-0.086	-0.189	0.274*	0.398**	0.596**
	P value	0.211	0.145	0.062	0.460	0.097	0.016	0.000	0.000
CpG9	r	-0.036	-0.029	-0.020	-0.032	0.033	0.071	0.045	-0.083
	P value	0.711	0.770	0.949	0.484	0.747	0.356	0.130	0.134
CpG10	r	-0.096	0.004	0.002	-0.004	0.002	0.071	0.028	-0.092
	P value	0.493	0.859	0.676	0.077	0.820	0.655	0.722	0.762
CpG11	r	-0.193	-0.139	-0.073	-0.194	0.140	-0.005	-0.089	-0.194
	P value	0.546	0.890	0.625	0.070	0.873	0.637	0.827	0.903
CpG12	r	-0.193	-0.164	-0.099	-0.207	0.174	-0.177	-0.040	0.056
	P value	0.090	0.152	0.390	0.072	0.129	0.124	0.730	0.623
CpG13	r	-0.161	-0.098	-0.088	-0.040	0.102	0.052	-0.034	-0.139
	P value	0.933	0.976	0.683	0.252	0.982	0.741	0.893	0.582
CpG14	r	-0.018	0.108	0.082	0.071	-0.103	0.101	0.033	-0.051
	P value	0.936	0.959	0.677	0.160	0.916	0.724	0.974	0.744
CpG15	r	-0.187	-0.076	-0.051	-0.079	0.079	-0.052	-0.213	-0.196
	P value	0.996	0.994	0.689	0.226	0.960	0.746	0.977	0.574

Chapter 3.3

Supplementary Table 1: Table shows all CpG dinucleotides that differ in their methylation levels for sperm DNA between current smokers and never smoked males (n=30)

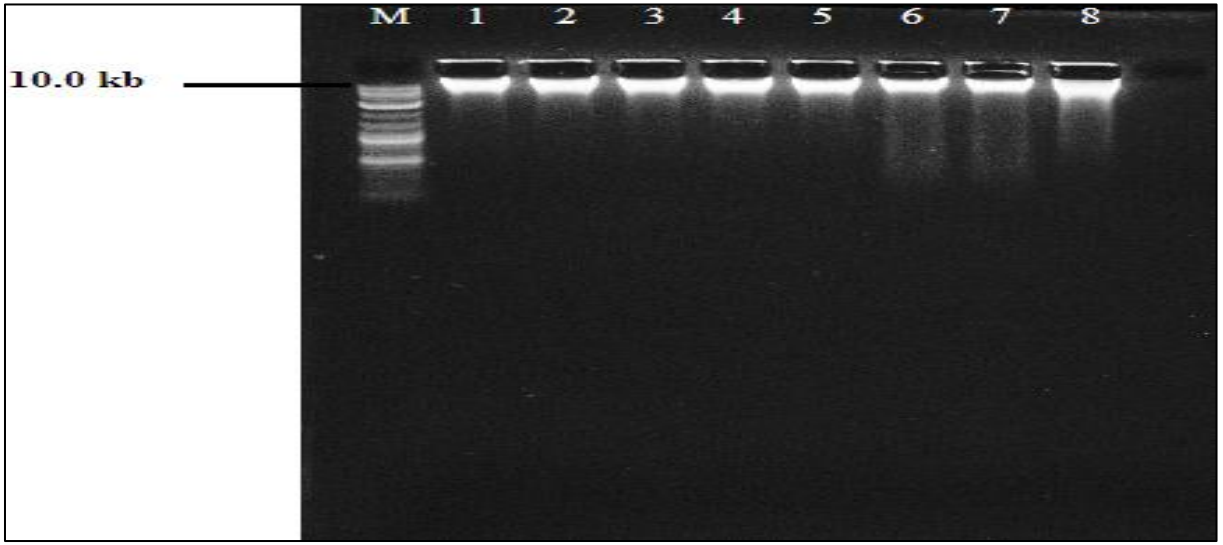
Cgid	Chromosome	Start	Strand	Mean Methylation (Never smokers)	Mean Methylation (Current smokers)	Mean difference	Absolute difference	Differential Methylation (P value)	SD (Never smokers)	SD (Current smokers)
cg21575308	chr15	29763183	-	0.0365	0.9008	-0.8643	0.8643	9.34E-09	0.0056	0.0034
cg23081194	chr21	46219184	+	0.1174	0.9164	-0.7990	0.7990	1.94E-08	0.0048	0.0131
cg22280068	chr11	285037	-	0.8492	0.0993	0.7498	0.7498	1.81E-08	0.0049	0.0077
cg05813498	chr17	78093353	+	0.8298	0.1444	0.6854	0.6854	4.16E-08	0.0072	0.0053
cg07227024	chr2	202163482	-	0.0433	0.9268	-0.8835	0.8835	4.82E-08	0.0116	0.0081
cg26337497	chr3	31935279	+	0.5445	0.0583	0.4862	0.4862	1.01E-07	0.0168	0.0015
cg01584086	chr11	10373718	+	0.0349	0.5240	-0.4891	0.4891	1.21E-07	0.0010	0.0506
cg09998151	chr14	32926900	-	0.0545	0.5341	-0.4796	0.4796	1.92E-07	0.0065	0.0035
cg02730303	chr11	103480630	+	0.4948	0.0511	0.4437	0.4437	1.87E-07	0.0156	0.0063
cg02507579	chr3	97887864	-	0.5624	0.0465	0.5160	0.5160	2.07E-07	0.0133	0.0097
cg24147543	chr6	32554481	-	0.0363	0.3473	-0.3110	0.3110	2.43E-07	0.0029	0.0102
cg10881162	chr3	55613558	-	0.9190	0.5478	0.3712	0.3712	4.15E-07	0.0027	0.0042
cg13740636	chr11	93754223	+	0.5242	0.0611	0.4630	0.4630	3.36E-07	0.0205	0.0092
cg22963027	chr19	49527060	+	0.0954	0.6027	-0.5073	0.5073	4.04E-07	0.0101	0.0180
cg17455348	chr2	213406960	-	0.9091	0.4859	0.4231	0.4231	3.79E-07	0.0053	0.0056
cg08720028	chr2	716366	-	0.9713	0.4778	0.4935	0.4935	3.45E-07	0.0065	0.0011
cg01188578	chr2	26464058	-	0.0644	0.5298	-0.4654	0.4654	3.22E-07	0.0077	0.0098
cg06833981	chr6	31597708	+	0.9241	0.5236	0.4005	0.4005	3.17E-07	0.0035	0.0110
cg20978247	chr6	32905085	-	0.9099	0.5061	0.4038	0.4038	4.10E-07	0.0010	0.0187
cg27639199	chr15	81666528	-	0.0612	0.5156	-0.4544	0.4544	2.75E-07	0.0024	0.0401
cg00017157	chr1	167127353	-	0.0729	0.5831	-0.5102	0.5102	4.54E-07	0.0106	0.0068
cg23622129	chr1	214328927	-	0.9210	0.5575	0.3634	0.3634	4.88E-07	0.0032	0.0113

cg07869343	chr16	1797050	+	0.9312	0.4913	0.4399	0.4399	6.70E-07	0.0044	0.0498
cg26805113	chr16	86112141	-	0.5623	0.9129	-0.3506	0.3506	6.92E-07	0.0125	0.0022
cg08996597	chr6	130747460	-	0.4758	0.8786	-0.4028	0.4028	6.87E-07	0.0069	0.0055
cg27086157	chr13	114872606	+	0.8877	0.4077	0.4800	0.4800	6.63E-07	0.0124	0.0054
cg09663736	chr11	131554122	+	0.5645	0.9179	-0.3534	0.3534	6.60E-07	0.0152	0.0026
cg22984586	chr3	46411541	-	0.0658	0.5531	-0.4873	0.4873	6.99E-07	0.0119	0.0082
cg03457281	chr4	48912027	-	0.9019	0.4579	0.4440	0.4440	7.24E-07	0.0109	0.0036
cg12648811	chr1	17053886	-	0.0696	0.3857	-0.3161	0.3161	8.23E-07	0.0051	0.0071
cg04315227	chr2	106886503	-	0.0444	0.3173	-0.2729	0.2729	8.32E-07	0.0033	0.0266
cg26303777	chr1	230311676	-	0.9212	0.5488	0.3724	0.3724	8.20E-07	0.0063	0.0234
cg19169023	chr15	41853346	+	0.9259	0.5528	0.3731	0.3731	8.35E-07	0.0066	0.0231
cg13386926	chr5	156890399	+	0.8898	0.4628	0.4270	0.4270	8.86E-07	0.0104	0.0121
cg01397495	chr2	136496804	-	0.8532	0.4471	0.4061	0.4061	8.84E-07	0.0046	0.0012
cg17240976	chr2	242248764	-	0.8643	0.4370	0.4273	0.4273	9.20E-07	0.0079	0.0167
cg17671604	chr19	41032399	+	0.4267	0.8380	-0.4114	0.4114	9.94E-07	0.0095	0.0002
cg08429705	chr19	2583601	+	0.9200	0.5618	0.3582	0.3582	1.05E-06	0.0080	0.0139
cg04245870	chr16	1722957	+	0.9073	0.4811	0.4262	0.4262	1.12E-06	0.0119	0.0018
cg09432376	chr22	36044226	-	0.8867	0.4800	0.4068	0.4068	1.29E-06	0.0120	0.0025
cg16122736	chr22	49635337	-	0.4528	0.8825	-0.4297	0.4297	1.39E-06	0.0153	0.0175
cg08338478	chr2	197104914	+	0.9179	0.5532	0.3647	0.3647	1.39E-06	0.0026	0.0447
cg14230280	chr9	132502800	-	0.8920	0.5145	0.3775	0.3775	1.43E-06	0.0027	0.0360
cg09737095	chr11	128787688	-	0.1306	0.5024	-0.3718	0.3718	1.54E-06	0.0078	0.0106
cg11627968	chr7	139351108	+	0.9107	0.5151	0.3956	0.3956	1.63E-06	0.0093	0.0368
cg16380681	chr16	66155275	-	0.5082	0.9039	-0.3956	0.3956	1.61E-06	0.0311	0.0089
cg22816462	chr8	6633067	-	0.9838	0.6294	0.3544	0.3544	1.62E-06	0.0021	0.1007
cg04698472	chr19	52000825	+	0.9196	0.5234	0.3962	0.3962	1.79E-06	0.0131	0.0068
cg23109721	chr2	106886537	+	0.0400	0.2511	-0.2112	0.2112	1.94E-06	0.0050	0.0025
cg10650398	chr10	11443364	+	0.8947	0.5050	0.3898	0.3898	2.01E-06	0.0102	0.0317
cg09762182	chr1	1046164	+	0.9331	0.5287	0.4044	0.4044	2.13E-06	0.0128	0.0291
cg14741888	chr10	3240019	+	0.7771	0.3789	0.3981	0.3981	2.18E-06	0.0096	0.0054

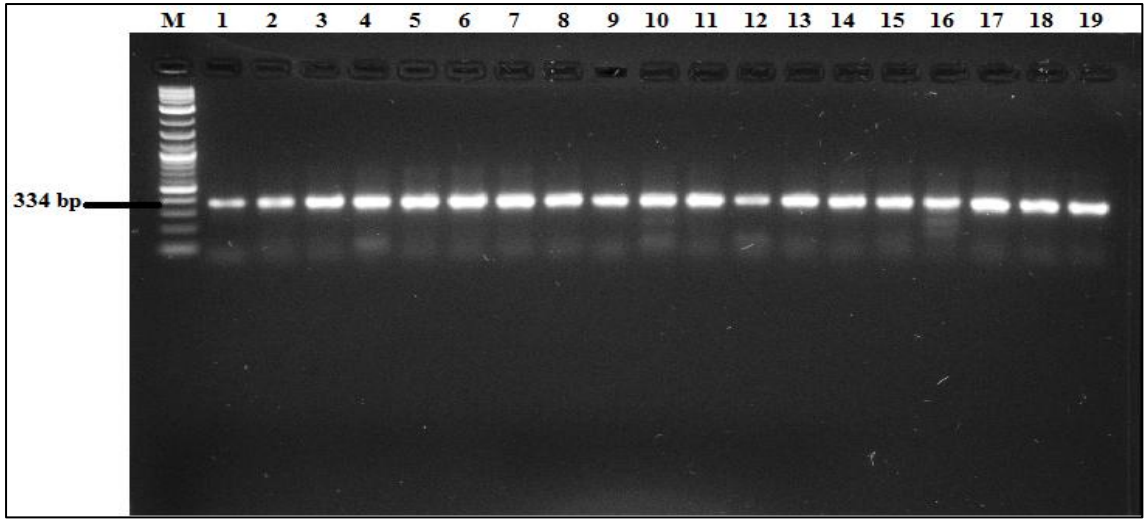
cg27391564	chr2	240530497	-	0.8623	0.5132	0.3491	0.3491	2.23E-06	0.0048	0.0215
cg23667563	chr12	131258209	+	0.9282	0.6236	0.3046	0.3046	2.28E-06	0.0035	0.0421
cg10280035	chr2	98827491	+	0.9099	0.4755	0.4344	0.4344	2.52E-06	0.0172	0.0039
cg22508145	chr19	17015427	-	0.5094	0.9189	-0.4094	0.4094	2.68E-06	0.0441	0.0111
cg27049594	chr11	124439146	-	0.8888	0.5236	0.3652	0.3652	2.86E-06	0.0132	0.0080
cg16810310	chr11	66104993	+	0.4728	0.8407	-0.3678	0.3678	2.84E-06	0.0047	0.0170
cg24432632	chr10	3935314	-	0.8900	0.5260	0.3640	0.3640	2.77E-06	0.0131	0.0099
cg09471659	chr15	28753787	+	0.0537	0.3654	-0.3117	0.3117	3.19E-06	0.0084	0.0388
cg27079096	chr11	4389638	-	0.8903	0.5867	0.3036	0.3036	3.33E-06	0.0081	0.0173
cg02151632	chr8	23310132	-	0.9040	0.5149	0.3890	0.3890	3.35E-06	0.0154	0.0095
cg15412446	chr2	106886593	-	0.0608	0.2695	-0.2088	0.2088	3.49E-06	0.0053	0.0082
cg09015880	chr17	47207658	+	0.8693	0.5245	0.3448	0.3448	3.65E-06	0.0129	0.0004
cg16099169	chr2	106886729	+	0.0696	0.3037	-0.2341	0.2341	4.64E-06	0.0040	0.0322
cg17830140	chr19	626119	-	0.9404	0.5646	0.3758	0.3758	4.80E-06	0.0138	0.0201
cg18137779	chr1	56163457	-	0.8984	0.4832	0.4152	0.4152	5.07E-06	0.0173	0.0368
cg02745784	chr2	178970574	-	0.8758	0.5391	0.3367	0.3367	5.27E-06	0.0142	0.0196
cg27160141	chr4	8473051	-	0.9066	0.5822	0.3244	0.3244	5.37E-06	0.0135	0.0255
cg23837945	chr13	112806830	+	0.4807	0.8257	-0.3450	0.3450	6.13E-06	0.0261	0.0082
cg22270863	chr8	129839439	+	0.0296	0.2982	-0.2686	0.2686	7.17E-06	0.0029	0.0845
cg24100357	chr7	1311498	+	0.9033	0.5756	0.3277	0.3277	7.75E-06	0.0166	0.0122
cg08108333	chr7	157320662	-	0.0713	0.4078	-0.3365	0.3365	8.41E-06	0.0170	0.0012
cg06813297	chr12	132537251	-	0.7205	0.9493	-0.2288	0.2288	8.27E-06	0.0337	0.0080
cg23436081	chr3	190594456	+	0.0663	0.2926	-0.2264	0.2264	8.34E-06	0.0074	0.0309
cg00125893	chr15	71747687	+	0.4800	0.8345	-0.3545	0.3545	8.53E-06	0.0224	0.0236
cg08335702	chr12	107381175	-	0.0509	0.2114	-0.1605	0.1605	9.26E-06	0.0036	0.0258
cg11680857	chr1	152635200	-	0.5601	0.8522	-0.2921	0.2921	9.90E-06	0.0219	0.0124
cg08603678	chr8	109235928	-	0.5727	0.9153	-0.3426	0.3426	1.10E-05	0.0375	0.0210
cg22037249	chr6	33037690	+	0.8905	0.5573	0.3332	0.3332	1.13E-05	0.0094	0.0619
cg00648582	chr12	133294524	+	0.4300	0.7979	-0.3678	0.3678	1.18E-05	0.0392	0.0043
cg09785377	chr15	60644157	+	0.9073	0.5738	0.3335	0.3335	1.19E-05	0.0203	0.0079

cg12474013	chr6	33095865	+	0.8191	0.4938	0.3253	0.3253	1.31E-05	0.0191	0.0167
cg05127924	chr17	18647524	+	0.6374	0.8723	-0.2348	0.2348	1.35E-05	0.0196	0.0034
cg09975044	chr14	104007538	+	0.5337	0.9259	-0.3922	0.3922	1.38E-05	0.0749	0.0088
cg23841288	chr7	158342105	+	0.1598	0.4414	-0.2816	0.2816	1.58E-05	0.0163	0.0083
cg14302130	chr6	33032830	-	0.9342	0.7446	0.1896	0.1896	1.66E-05	0.0063	0.0347
cg07469546	chr16	86927990	+	0.1081	0.4632	-0.3551	0.3551	1.77E-05	0.0132	0.0735
cg05407200	chr11	93586725	+	0.5206	0.8587	-0.3382	0.3382	1.83E-05	0.0214	0.0320
cg05531409	chr3	131637172	-	0.8985	0.5711	0.3274	0.3274	1.88E-05	0.0226	0.0074
cg19885539	chr2	1358283	-	0.8949	0.4218	0.4731	0.4731	1.95E-05	0.0264	0.0774
cg21584493	chr7	158342721	+	0.2113	0.4984	-0.2871	0.2871	1.98E-05	0.0160	0.0139
cg26311454	chr12	49520648	-	0.8392	0.5077	0.3315	0.3315	2.03E-05	0.0238	0.0163
cg27392904	chr19	50400714	+	0.5832	0.8651	-0.2818	0.2818	2.07E-05	0.0399	0.0013
cg08470742	chr6	169653905	-	0.1005	0.4088	-0.3083	0.3083	2.13E-05	0.0110	0.0676

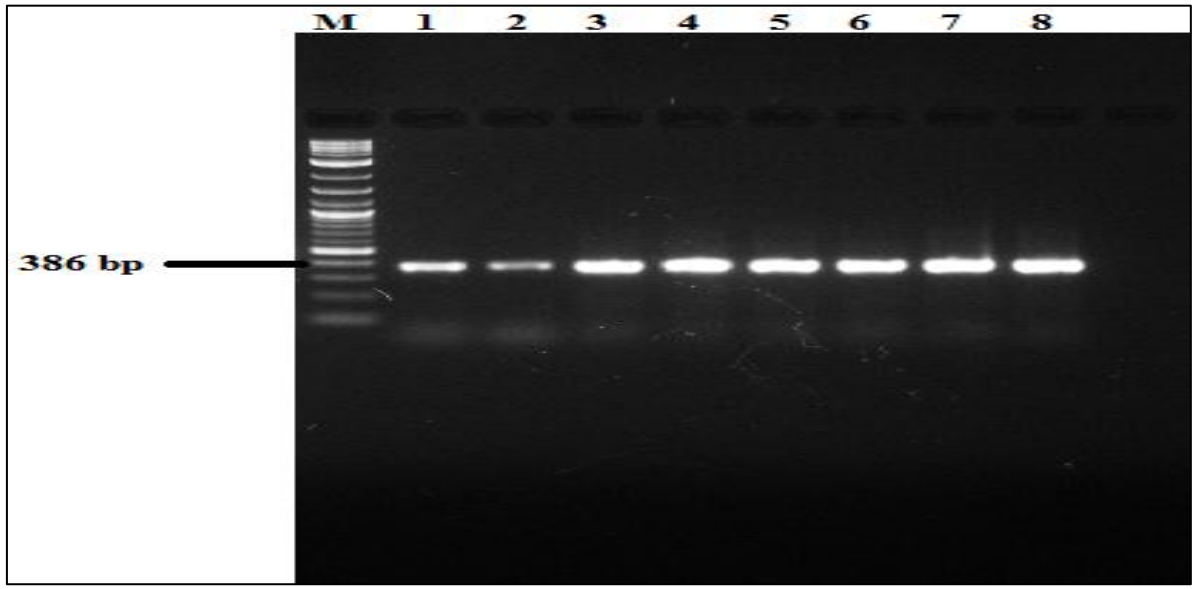
SD: Standard deviation



Supplementary Figure 1: Agarose gel electrophoresis of genomic DNA obtained from sperm samples. Briefly, genomic DNA was extracted from sperm samples by using an Isolate II genomic DNA kit. The whole genomic DNA obtained was run on 1.5% agarose gel electrophoresis (lines 2-8), highly purified sperm DNA as a positive control from Qiagen (Germany) was used (line 1), and M corresponds to DNA ladder (0.1-10.0 kb) (NE Biolabs, UK) was used. The DNA was separated in a constant electric field of 45 V for about 60 minutes. After the separation was completed, the DNA bands were visualized and photographed using a gel documentation system (BIO-RAD, Germany). The data showed that the molecular weight of the extracted DNA was greater than 10 kbp.



Supplementary Figure 2: Agarose gel electrophoresis of PCR products. Briefly, various sperm samples were collected and genomic DNA was extracted. The whole genomic DNA obtained was used as the template for PCR analysis. The products of PCR that are related to *MAPK8IP* gene amplification showed in 334 bp as described in lines 1-19. M corresponds to DNA ladder (0.1-10.0 kb) from NE Biolabs was used. The DNA was separated in a constant electric field of 65V for about 90 minutes, on the basis of molecular weight. After the separation was completed, the DNA bands on the gel were visualized and photographed using a gel documentation system (BIO-RAD, Germany).



Supplementary Figure 3: Agarose gel electrophoresis of PCR products. Briefly, various sperm samples were collected and genomic DNA was extracted. The whole genomic DNA obtained was used as the template for PCR analysis. The products of PCR that are related to *TKR* gene amplification shown in 386 bp, as described in lines 1-8. M corresponds to the DNA ladder (0.1-10.0 kb) from NE Biolabs, which was used. The DNA was separated in a constant electric field of 65V for about 90 minutes, on the basis of its molecular weight. After the separation was completed, the DNA bands on the gel were visualized and photographed using a gel documentation system (BIO-RAD, Germany).

Chapter 3.5

Supplementary Table 1: CpG dinucleotides that differ in their methylation levels for sperm DNA between the oligospermic male "case" and the proven fertile male "control" (n = 40).

CgID	Chromosome	Start	Strand	Mean Proven fertile males	Mean Oligospermic males	Mean difference	Difference Methylation (P value)	Annotation
cg23081194	chr21	46219184	+	0.117	0.953	-0.83	7.93E-10	UBE2G2
cg25750688	chr7	63037165	+	0.964	0.437	0.527	5.79E-06	/
cg04807108	chr15	78697933	-	0.325	0.918	-0.593	1.50E-06	/
cg19779893	chr10	72451501	+	0.852	0.595	0.256	6.13E-06	ADAMTS14
cg19406113	chr6	170558063	-	0.667	0.904	-0.237	7.28E-06	/

Supplementary Table 2: Correlation between the methylation level in the DMCs obtained by Bi-PROF and the different semen parameters investigated for the case group.

CpG		Sperm concentration (mill/mL)	Total of sperm motility (%)	Progressive Motility (%)	Non-progressive motility (%)	Immotile Sperm (%)	Normal form of sperm (%)
Methylation level at CpGs in UBE2G2 gene-related amplicon							
CpG1	r	-0.094	-0.112	-0.156	0.014	0.106	0.307
	P value	0.404	0.322	0.164	0.898	0.345	≤0.005
CpG2	r	0.243	-0.056	-0.064	-0.111	0.046	-0.116
	P value	≤0.029	0.620	0.573	0.325	0.682	0.301
CpG3	r	0.199	-0.133	-0.115	0.030	0.035	-0.246

	P value	0.075	0.238	0.305	0.792	0.760	≤0.027
Methylation level at CpGs in cg25750688 site related amplicon							
CpG1	r	0.125	0.084	0.121	0.025	-0.112	0.160
	P value	0.266	0.458	0.282	0.827	0.318	0.154
CpG2	r	0.114	0.305	0.494	0.087	-0.341	-0.112
	P value	0.313	≤0.006	≤0.00001	0.438	≤0.002	0.321
Methylation level at CpGs in cg04807108 site related amplicon							
CpG1	r	-0.324	0.017	0.061	0.025	-0.101	0.089
	P value	≤0.003	0.882	0.589	0.827	0.368	0.431
CpG2	r	-0.039	-0.065	0.062	-0.253	0.055	0.184
	P value	0.732	0.565	0.583	≤0.023	0.628	0.100
CpG3	r	-0.213	0.158	0.247	-0.001	-0.200	-0.031
	P value	0.056	0.158	≤0.026	0.995	0.073	0.784
CpG4	r	-0.013	-0.254	-0.117	-0.415	0.254	0.267
	P value	0.906	≤0.022	0.297	≤0.00001	≤0.022	≤0.016
CpG5	r	0.039	-0.295	-0.140	-0.460	0.272	0.220
	Pvalue	0.732	≤0.007	0.211	≤0.00001	≤0.014	≤0.049
CpG6	r	-0.196	-0.069	-0.090	-0.104	0.053	0.196
	P value	0.080	0.543	0.425	0.357	0.636	0.079
CpG7	r	-0.091	-0.217	-0.046	-0.345	0.189	0.190
	P value	0.419	0.052	0.681	≤0.002	0.092	0.090
CpG8	r	-0.091	0.015	0.079	0.033	-0.136	-0.065
	P value	0.417	0.893	0.481	0.769	0.226	0.564
CpG9	r	-0.056	-0.054	0.068	-0.261	0.077	0.122
	P value	0.619	0.632	0.549	≤0.019	0.496	0.276
CpG10	r	-0.066	-0.079	0.065	-0.251	0.077	0.212
	P value	0.559	0.482	0.565	≤0.024	0.493	0.058
CpG11	r	-0.004	-0.079	-0.060	0.124	-0.004	0.262
	P value	0.972	0.483	0.594	0.271	0.975	≤0.018
CpG12	r	-0.061	-0.149	0.068	-0.311	0.126	0.108
	P value	0.589	0.185	0.545	≤0.005	0.261	0.338

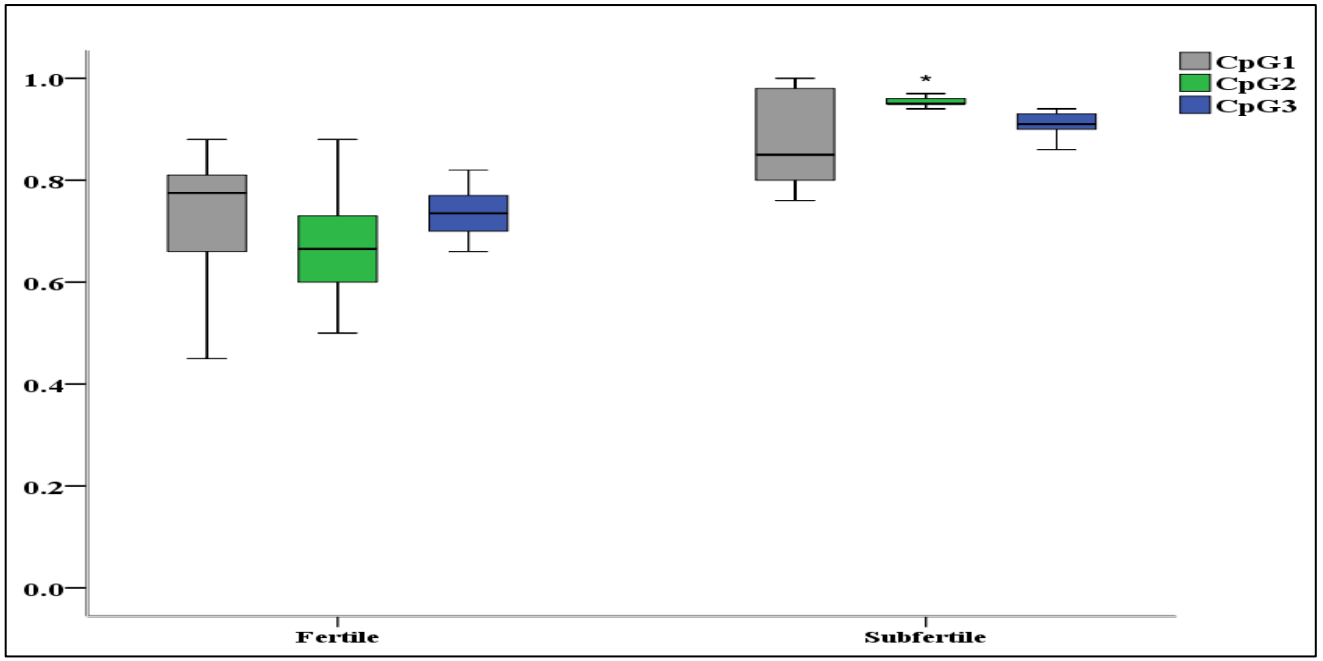
CpG13	r	-0.135	0.043	0.025	-0.034	-0.086	0.043
	P value	0.230	0.706	0.823	0.766	0.445	0.703
CpG14	r	0.092	0.173	0.188	0.020	-0.213	0.060
	P value	0.412	0.123	0.092	0.862	0.056	0.592
CpG15	r	0.308	0.118	0.137	0.056	-0.141	0.304
	P value	≤0.005	0.296	0.222	0.621	0.210	≤0.006

DMC, Differentially methylated CpGs,

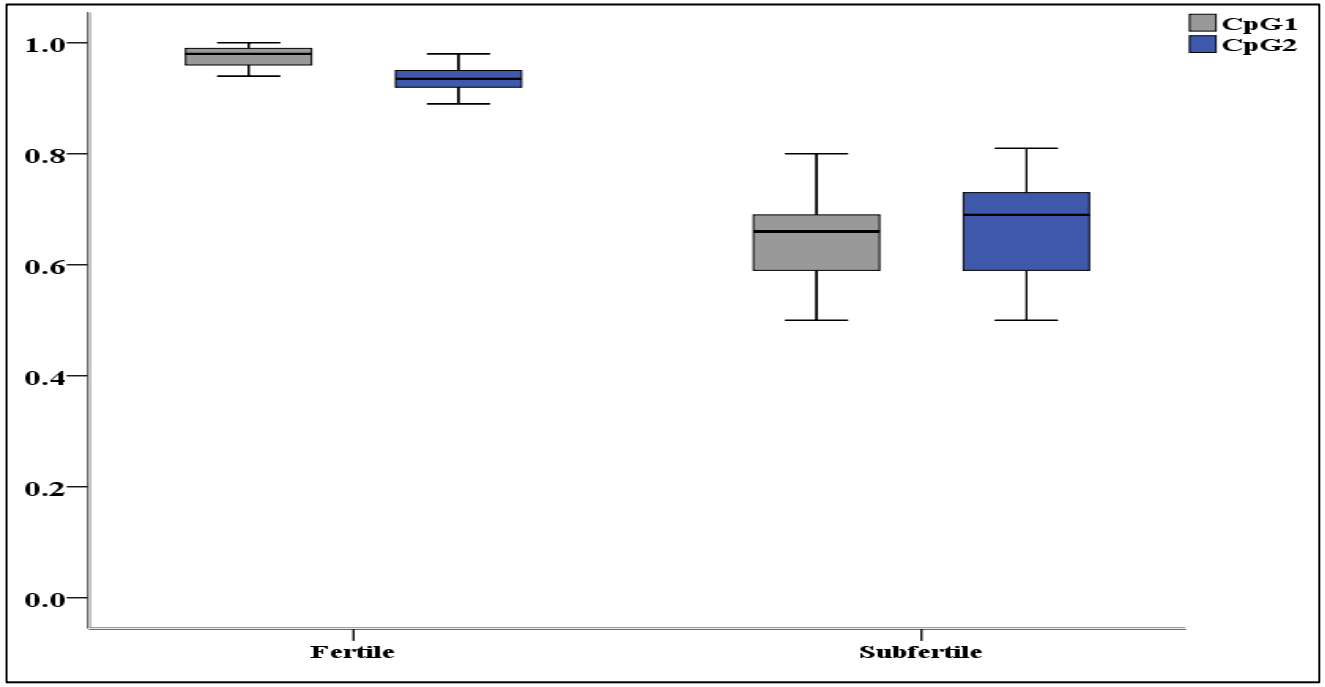
R, Correlation Coefficient,

P value ≤ 0.05: Significant,

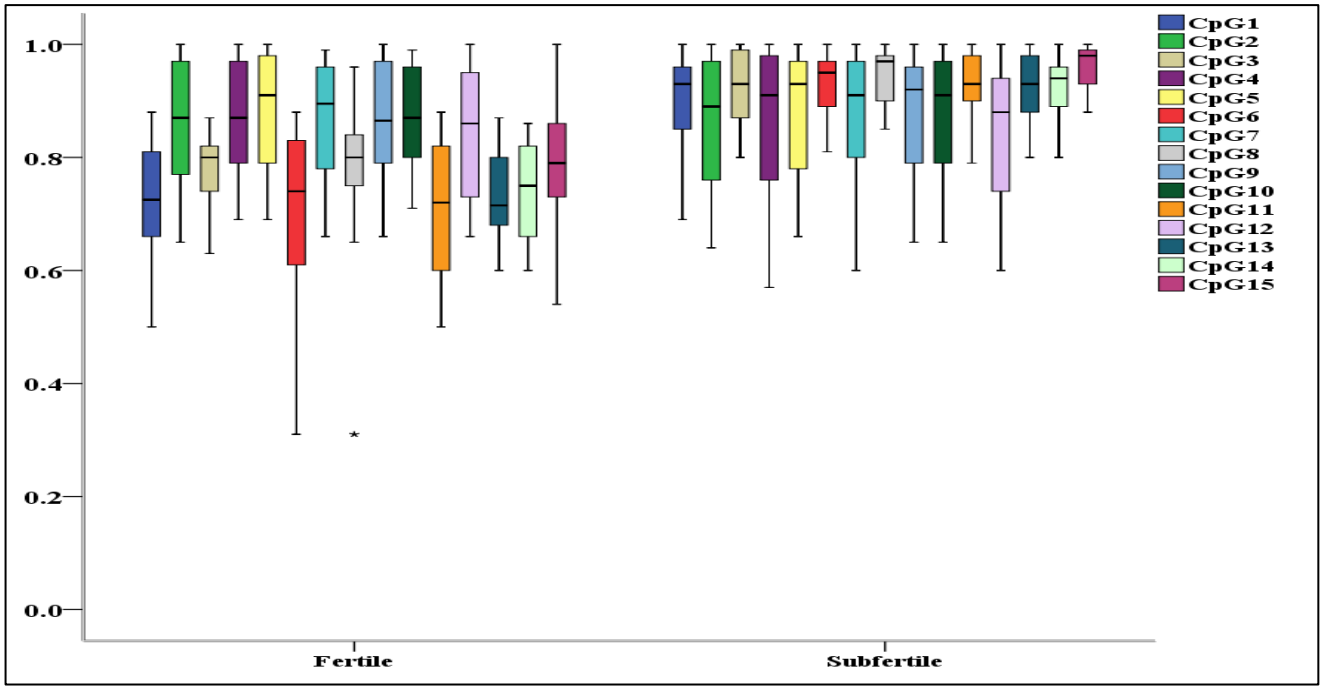
P value > 0.05: Not significant.



Supplementary Figure 1: Methylation levels at DMC in the *UBE2G2* gene-related amplicon in cases compared to controls



Supplementary Figure 2: Methylation levels at DMC in the cg25750688 site-related amplicon in cases compared to controls



Supplementary Figure 3: Methylation levels at DMC in the cg04807108 site-related amplicon in cases compared to controls

Chapter 3.6

Supplementary Tables 1: CpG dinucleotides that differ in their methylation levels for sperm DNA between cases (Males suffering from a reduction in fecundity) and controls (Proven fertile) (n=30)

CgID	Chromosome	Start	Strand	Mean Controls	Mean Cases	Mean difference	Difference Methylation (P value)	Annotation
cg05799088	chr3	64080488	-	0.916	0.545	0.371	3.67E-07	PRICKLE2
cg07227024	chr2	202163482	-	0.043	0.908	-0.865	7.33E-09	ALS2CR12
cg16338278	chr11	67432957	-	0.935	0.733	0.202	2.00E-07	ALDH3B2
cg08408433	chr19	47127358	+	0.370	0.069	-0.301	4.27E-06	PTGIR
cg04807108	chr15	78697933	-	0.725	0.918	-0.203	1.50E-06	/
cg23081194	chr21	46219184	+	0.117	0.903	-0.786	7.93E-10	UBE2G2
cg25750688	chr7	63037165	+	0.865	0.638	0.227	5.79E-06	/
cg19779893	chr10	72451501	+	0.853	0.596	0.257	6.13E-06	ADAMTS14
cg19406113	chr6	170558063	-	0.667	0.905	-0.237	7.28E-06	/

Supplementary Tables 2: Correlation between the methylation levels in the DMCs obtained by Bi-PROF and the semen parameters for the case samples (n=55)

DMC		Semen Volume (mL)	Sperm count (mill/mL)	Total sperm motility (%)	Progressive motility (%)	Non-progressive motility (%)	Sperm immotile (%)	Sperm vitality (%)	Sperm normal form (%)
Methylation level at CpGs in PRICKLE2 gene-related amplicon									
CpG1	r	-0.059	0.026	-0.194	-0.244	-0.084	0.185	0.204	-0.092
	P value	0.671	0.849	0.155	0.073	0.543	0.176	0.135	0.503
CpG2	r	0.255	0.153	0.021	0.229	-0.086	-0.073	0.090	-0.037
	P value	0.061	0.266	0.881	0.092	0.532	0.595	0.516	0.790
Methylation level at CpGs in ALS2CR12 gene-related amplicon									

CpG1	r	-0.017	0.088	-0.274	-0.282	-0.170	0.278	0.147	0.027
	P value	0.901	0.522	≤ 0.043	≤ 0.037	0.214	≤ 0.040	0.283	0.844
CpG2	r	0.135	-0.019	-0.173	0..266	-0.137	0.181	0.023	0.019
	P value	0.325	0.889	0.207	≤ 0.049	0.319	0.185	0.869	0.891
Methylation level at CpGs in ALDH3B2 gene-related amplicon									
CpG1	r	-0.107	-0.002	0.206	0.042	0.290	-0.184	-0.101	-0.026
	P value	0.435	0.986	0.131	0.763	≤ 0.032	0.180	0.465	0.850
CpG2	r	0.204	0.270	-0.269	-0.258	-0.213	0.304	-0.132	-0.089
	P value	0.136	≤ 0.046	≤ 0.047	0.057	0.119	≤ 0.024	0.337	0.518
CpG3	r	0.177	0.433	-0.101	-0.161	-0.041	0.115	0.077	-0.182
	P value	0.197	≤ 0.001	0.463	0.240	0.767	0.403	0.578	0.184
CpG4	r	0.167	0.119	0.054	-0.020	0.252	-0.104	0.079	-0.164
	P value	0.222	0.386	0.697	0.884	0.064	0.452	0.567	0.232
CpG5	r	0.030	0.077	0.127	0.015	0.159	-0.069	-0.068	-0.018
	P value	0.829	0.578	0.355	0.911	0.247	0.614	0.622	0.894
CpG6	r	-0.046	0.075	-0.026	-0.173	-0.080	0.083	0.116	0.172
	P value	0.740	0.588	0.853	0.206	0.559	0.547	0.398	0.209
CpG7	r	-0.039	0.113	0.006	0.051	0.102	-0.048	-0.022	-0.204
	P value	0.775	0.411	0.964	0.713	0.458	0.725	0.876	0.135
CpG8	r	0.070	0.185	0.059	0.092	-0.032	-0.035	-0.027	-0.092
	P value	0.610	0.177	0.667	0.502	0.814	0.801	0.848	0.503
CpG9	r	-0.052	0.019	0.113	-0.059	0.167	-0.055	-0.010	-0.033
	P value	0.709	0.889	0.412	0.668	0.222	0.689	0.944	0.808
CpG10	r	0.196	0.400	0.037	-0.054	0.112	-0.011	0.126	-0.146
	P value	0.152	≤ 0.002	0.787	0.696	0.414	0.934	0.360	0.289
CpG11	r	-0.134	0.385	0.077	-0.117	0.246	-0.030	0.045	-0.048
	P value	0.328	≤ 0.004	0.578	0.397	0.070	0.826	0.745	0.727
CpG12	r	-0.235	0.378	-0.065	-0.268	0.204	0.120	0.159	0.027
	P value	0.085	≤ 0.004	0.637	≤ 0.048	0.135	0.384	0.246	0.846
CpG13	r	-0.075	0.335	0.234	0.128	0.154	-0.172	0.192	-0.032
	P value	0.584	≤ 0.012	0.086	0.350	0.262	0.210	0.159	0.819
Methylation level at CpGs in PTGIR gene-related amplicon									
CpG1	r	0.024	0.204	-0.239	-0.242	-0.115	0.368	-0.472	0.003
	P value	0.908	0.329	0.249	0.243	0.585	0.070	≤ 0.017	0.990
CpG2	r	0.059	0.236	-0.232	-0.230	-0.144	0.367	-0.394	0.184

	P value	0.778	0.255	0.264	0.269	0.492	0.071	0.050	0.379
CpG3	r	-0.051	0.347	-0.203	-0.246	-0.088	0.339	-0.386	0.143
	P value	0.809	0.089	0.330	0.235	0.676	0.097	0.057	0.496
CpG4	r	-0.081	0.305	-0.167	-0.293	-0.006	0.298	-0.310	0.207
	P value	0.701	0.139	0.424	0.155	0.976	0.148	0.131	0.320
CpG5	r	-0.094	0.272	-0.164	-0.270	0.025	0.299	-0.363	0.055
	P value	0.655	0.188	0.433	0.192	0.905	0.146	0.075	0.792
CpG6	r	-0.101	0.299	-0.227	-0.262	-0.123	0.362	-0.413	0.199
	P value	0.630	0.147	0.275	0.206	0.558	0.075	≤ 0.040	0.340
CpG7	r	0.071	0.195	-0.312	-0.206	-0.031	0.324	-0.365	0.023
	P value	0.735	0.351	0.128	0.324	0.884	0.115	0.073	0.913
CpG8	r	0.114	0.332	-0.075	-0.037	0.068	0.077	-0.218	0.206
	P value	0.586	0.105	0.723	0.861	0.748	0.715	0.295	0.323
CpG9	r	-0.044	0.172	-0.201	-0.234	-0.144	0.336	-0.437	0.188
	P value	0.833	0.411	0.336	0.260	0.493	0.101	≤ 0.029	0.369
CpG10	r	-0.041	0.153	-0.339	-0.309	-0.128	0.437	-0.542	0.017
	P value	0.847	0.466	0.097	0.133	0.543	≤0.029	≤ 0.005	0.934
CpG11	r	0.022	0.162	-0.158	-0.228	-0.030	0.295	-0.325	0.197
	P value	0.916	0.439	0.451	0.273	0.887	0.152	0.112	0.344
CpG12	r	0.027	0.194	-0.162	-0.175	-0.138	0.303	-0.395	0.193
	P value	0.897	0.353	0.439	0.403	0.512	0.141	0.050	0.355
CpG13	r	-0.008	0.069	-0.398	-0.395	-0.195	0.518	-0.491	0.091
	P value	0.970	0.742	≤ 0.049	0.050	0.350	≤ 0.008	≤ 0.013	0.665
CpG14	r	0.010	0.111	-0.325	-0.337	-0.206	0.453	-0.477	0.307
	P value	0.960	0.599	0.113	0.100	0.323	≤ 0.023	≤ 0.016	0.135
CpG15	r	0.066	0.317	-0.080	-0.129	-0.058	0.224	-0.282	0.167
	P value	0.755	0.123	0.702	0.537	0.783	0.283	0.172	0.426
CpG16	r	-0.119	0.217	-0.312	-0.337	-0.194	0.443	-0.474	0.206
	P value	0.571	0.297	0.129	0.099	0.352	≤ 0.026	≤ 0.017	0.322
CpG17	r	-0.017	0.156	-0.199	-0.259	-0.066	0.335	-0.318	0.270
	P value	0.935	0.456	0.340	0.212	0.752	0.101	0.121	0.191
CpG18	r	-0.015	0.299	-0.081	-0.114	-0.099	0.223	-0.450	0.007
	P value	0.945	0.146	0.699	0.586	0.637	0.285	≤ 0.024	0.974
CpG19	r	-0.039	0.193	-0.251	-0.314	-0.087	0.384	-0.325	0.196
	P value	0.852	0.356	0.226	0.126	0.679	0.058	0.113	0.349

CpG20	r	-0.056	0.269	-0.231	-0.280	-0.108	0.364	-0.350	0.112
	P value	0.789	0.194	0.266	0.175	0.606	0.074	0.087	0.593
CpG21	r	-0.118	0.268	-0.272	-0.375	-0.055	0.401	-0.419	0.184
	P value	0.573	0.196	0.188	0.065	0.792	≤ 0.047	≤ 0.037	0.377
CpG22	r	-0.062	0.254	-0.301	-0.311	-0.169	0.429	-0.475	0.159
	P value	0.770	0.221	0.144	0.131	0.418	≤ 0.032	≤ 0.016	0.447
CpG23	r	0.013	0.322	-0.275	-0.166	0.021	0.286	-0.367	0.135
	P value	0.949	0.116	0.183	0.427	0.919	0.166	0.071	0.520
CpG24	r	-0.103	0.236	-0.231	-0.270	-0.195	0.366	-0.437	0.290
	P value	0.625	0.256	0.266	0.192	0.350	0.072	≤ 0.029	0.160
CpG25	r	-0.071	0.152	-0.210	-0.214	-0.187	0.347	-0.475	0.228
	P value	0.736	0.469	0.313	0.304	0.372	0.090	≤ 0.016	0.274
CpG26	r	0.063	0.110	-0.142	-0.208	-0.131	0.268	-0.307	0.242
	P value	0.766	0.600	0.498	0.320	0.534	0.196	0.136	0.243

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Publications and Posters

Publications

Alkhaled, Y., Laqqan, M., Tierling, S., Porto, C. L. & Hammadeh, M. E. (2018). DNA methylation levels of spermatozoa from subfertile and proven fertile males and its relation to standard sperm parameters. Andrologia.

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Curriculum Vitae