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Association between aberrations in DNA methylation patterns of spermatozoa and abnormalities in semen parameters of subfertile males

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Abstract

Infertility affects 10–15% of couples and approximately 50% of cases are linked to male factor infertility. In recent decades, many studies have found that 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. Besides, 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. Likewise, a recent study detected aberrant in DNA methylation levels for genes involved in the spermatogenic program and located outside of imprinted regions in poor quality spermatozoa. The present thesis aimed (I) to determine whether sperm DNA methylation level at CpG dinucleotides is different in males suffering from a reduction in fecundity compared to proven fertile males, (II) to determine the relationship between changes in sperm DNA methylation levels in this gene and abnormalities in semen parameters, (III) to identify whether cigarette smoking alters sperm DNA methylation patterns and to determine whether this alteration in sperm DNA methylation is associated with basic semen parameters like sperm count, sperm motility, and morphology, (IV) to evaluate the association between the gene expressions level for the genes included in this study and semen parameters, addition to assessing the correlation between the gene expression and the alteration in spermatozoa DNA methylation levels. The strategy of this research was applied as the following: Infinium 450K BeadChip array was used as screening study, to evaluate the variation in sperm DNA methylation levels between cases and controls groups. Then the differentially methylated CpGs (DMC) underwent to deep bisulfite sequencing to validate on large cohort samples (as validation study). In the end, Quantitative RT-PCR assay was used to evaluate the expression of the gene included in this study. According to the results of the validation study, the bioinformatics analysis found that more than one CpG showed a difference in the methylation level addition to CpGs obtained from the screening study between subfertile males and proven fertile males. Some of this CpGs were located in gene bodies and CpG islands (*PRICKLE2, ALS2CR12, ALDH3B2, PTGIR, KCNJ5, MLPH, SMC1b, GAA, PRRC2A, MAPK8IP3, PDE11A, ANXA2, CGβ,* and *TYRO3*), while the

other CpGs were located in intergenic regions. Besides, a significant correlation was found between the methylation levels at the CpGs in genes related amplicon of subfertile males and semen parameters like sperm count, a percentage of total sperm motility, the percentage of progressive motility, a percentage of non-progressive motility, a percentage of immotile sperm and sperm vitality. On the other hand, this study showed more than one CpGs have significant differences in methylation levels between current smokers as compared to never smoked males like CpGs namely cg07869343 and cg19169023, which are located in the *MAPK8IP3* and *TKR* genes. Moreover, the results showed a significant correlation between the methylation levels at more than one CpGs in the genes related amplicon of current smoker males and semen parameters such as sperm count, the percentage of total sperm motility, a percentage of sperm immotile, a percentage of progressive motility, and a percentage of sperm normal form. In conclusion, this study identified CpGs related to different genes have an alteration in sperm DNA methylation levels in cases compared to controls groups. 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.

Zusammenfassung

Unfruchtbarkeit betrifft 10 - 15% der Paare und etwa 50 % der Fälle werden an männliche Faktorunfruchtbarkeit verbunden. In den letzten Jahrzehnten haben viele Studien haben festgestellt, dass die genetische Ursachen, abweichendes Muster der DNA-Methylierung und Histonmodifikation gezeigt haben, eine Rolle in der männlichen Unfruchtbarkeit oder Fruchtbarkeit Rückgang zu spielen, und haben in 15 identifiziert wurde - 30% der infertile Männer. Neben, mehrere Studien haben gezeigt, dass die Veränderungen in der DNA-Methylierung spezifischer Gene in Keimzellen verbunden sind mit oligozoospermie, reduzierte progressive Beweglichkeit der Spermien, die Morphologie der Spermien und abnormal. Ebenso wird eine aktuelle Studie entdeckt aberrante DNA-Methylierung von Genen, die in die spermatogene programm beteiligten und außerhalb der geprägten Regionen in schlechter Qualität der Spermien. Die vorliegende Arbeit soll (I), um zu bestimmen, ob die Spermien-DNA Methylierung bei CpG dinucleotides verschiedenen in Männer leiden unter einer Verringerung der Fruchtbarkeit ist im Vergleich zu den bewährten fruchtbare Männer, (II) die Beziehung zwischen Veränderungen der Spermien-DNA Methylierung Ebenen in diesem Gen und Missbildungen im Samen Parameter zu bestimmen, (iii) um zu ermitteln, ob das Rauchen verändert Spermien-DNA Methylierungsmuster und zu ermitteln, ob diese Änderung der Spermien-DNA Methylierung mit grundlegenden Samen Parameter wie die Zahl der Spermien, die Beweglichkeit der Spermien zugeordnet ist, und die Morphologie, (iv) die Assoziation zwischen den gen Ausdrücke für die Gene, die in dieser Studie und Samen Parameter enthalten, zusätzlich zu der Bewertung der Korrelation zwischen der Genexpression und der Veränderung der Spermien-DNA-Methylierung. Die Strategie dieser Forschung war, wie die Folgenden: Infinium 450K BeadChip Array war als screening Studie verwendet, die Veränderung der Spermien-DNA Methylierung Ebenen zwischen Fällen und Kontrollen Gruppen zu bewerten. Dann die differentiell methylierte CpGs (DMC) wurde zu tief Bisulfite Sequencing auf große Kohorte Proben (als validierungsstudie) bestätigen. Am Ende, Quantitative RT-PCR Assay wurde verwendet, um die Expression des Gens in dieser Studie zu bewerten. Nach den Ergebnissen der Validierungsstudie, die bioinformatik Analyse ergab, dass mehr als ein CpG zeigten einen Unterschied in der methylierung Ebene neben der CpGs vom screening Studie zwischen subfertile Männer und bewährte fruchtbare Männer

gewonnen. Einige dieser CpGs wurden in gen Stellen und CpG-Inseln (*KRIBBELN 2, ALS 2 CR 12, ALDH3 B2, PTGIR, Kcnj 5, MLPH, SMC 1b, GAA, PRRC2A, MAPK 8 IP3, PDE 11 A, ANXA2, CGβ* und *TYRO3*), während die anderen CpGs in intergenic Regionen entfernt wurden. Außerdem wurde eine signifikante Korrelation zwischen der Methylierung der CpGs in Genen amplikon der Subfertile Männer und Samen Parameter wie die Zahl der Spermien gefunden wurde, einen Prozentsatz der gesamten Beweglichkeit der Spermien, der Prozentsatz der Spermienbeweglichkeit, ein Anteil von Nicht-progressive Motilität, einen Prozentsatz der Immotile Spermien und Spermien Vitalität. Auf der anderen Seite ist diese Studie zeigte mehr als ein CpGs haben signifikante Unterschiede in der Methylierung Ebenen zwischen Rauchern gegenüber nie geraucht Männchen wie CpGs nämlich cg cg 07869343 und 19169023, die in der *Mapk 8 IP3* und *TKR* Gene befinden. Darüber hinaus. Die Ergebnisse zeigten einen signifikanten Zusammenhang zwischen der Methylierung Niveaus an mehr als einem CpGs in den Genen amplikon der aktuellen Raucher Männer und Samen Parameter wie die Zahl der Spermien, der Prozentsatz der gesamten Beweglichkeit der Spermien, einen Prozentsatz der Spermien Immotile, einen Prozentsatz der Spermienbeweglichkeit und ein Prozentsatz der Spermien normale Form an. Im Ergebnis dieser Studie identifizierten CpGs im Zusammenhang mit verschiedenen Genen haben eine Veränderung in der Spermien-DNA Methylierung Ebenen in Fällen verglichen mit Kontrollen Gruppen. Zusätzlich wird eine Verbindung zwischen den Veränderungen in der Methylierung für diese CpGs und verschiedenen Samen Parameter gefunden wurde. Die beobachteten Abweichungen können einen Einfluss auf die spermien Phänotyp. Weitere Studien sind notwendig, um die Mechanismen, die im Zusammenhang mit diesen Veränderungen und deren Bedeutung und funktionelle Konsequenzen für männliche Fruchtbarkeit zu entdecken, zu klären.

Dedication

I would like to express my special thanks to the following individuals

To my ***father*** who taught me how to give without limits,

To my ***mother*** who supported me by prayer (Doaa),

To my ***brothers*** and ***sisters*** who shared different feelings with me,

To my ***brother*** "***Abu Osama***" and to our heroes martyrs,

To my dear ***brothers*** whether they are under the earth or above it,

To each person in my big family and the family of my wife especially the ***mother of my wife***,

To my teachers in Islamic University-Gaza (***Prof. Dr. Maged Yassin and Prof. Dr. Fadel Sharif***) who had a major role in teaching me the basics of scientific research and how the scientific manuscripts were written

Finally, there are insufficient words to show my appreciation and respect for my wife, ***Eman*** for her continued support and prayer (Doaa) to me, as well as for her great patience. Besides my sons ‘‘***Odai and Marwan*”** how much I miss them,

Gaza Strip is a part of Palestine and it will remain as the title of steadfastness and challenge over the time. Best greetings to the beloved Gaza and his people.

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To all of these individuals, I owe many thanks for their insights and unlimited support

Scientific papers

This thesis is a cumulative thesis based on the published manuscripts as following. The papers bound in the thesis are identical to the published versions.

1. **Laqqan M**, Tierling S, Alkhaled Y, Lo Porto C, Solomayer E.F, Hammadeh M.E. Spermatozoa from males with reduced fecundity exhibit differential DNA methylation patterns. Andrology. 2017 May 23.
2. **Laqqan M**, Solomayer E.F, Hammadeh M.E. Aberrations in sperm DNA methylation patterns are associated with abnormalities in semen parameters of subfertile males. Reproductive Biology. 2017 May 26.
3. **Laqqan M**, Solomayer E.F, Hammadeh M.E. Association between alterations in DNA methylation level of spermatozoa at CpGs dinucleotide and male subfertility problems. Andrologia. 2017;00:e12832. https://doi.org/10.1111/and.12832.
4. **Laqqan M**, Tierling S, Alkhaled Y, Porto CL, Solomayer E.F, Hammadeh M.E. Aberrant DNA methylation patterns of human spermatozoa in current smoker males. Reproductive Toxicology. 2017 May 30.
5. Al Khaled Y, Tierling S, **Laqqan M**, Lo Porto C, Hammadeh M.E. Cigarette smoking induces only marginal changes in sperm DNA methylation levels of patients undergoing intracytoplasmic sperm injection treatment. Andrologia. 2017 May 14.

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List of abbreviations

3'-UTR	3-untranslated region
5meC	5-methylcytosine
5'-UTR	5-untranslated region
ADAMTS14	ADAM Metallopeptidase With Thrombospondin Type 1 Motif 14
ATP	Adenosine triphosphate
ALDH3B2	Aldehyde dehydrogenase 3 family member B2
ALS2CR12	Amyotrophic lateral sclerosis 2 chromosome region 12
ANXA2	Annexin A2
APOL6	Apolipoprotein L6
ASAP1	Arf-GAP with SH3 domain, ankyrin repeat and PH domain 1
x g	Centrifuge rotor speed in g
CGβ	Chorionic gonadotropin beta subunit 3
CHD	Chromodomain, helicase, DNA binding
COL4A1	Collagen type IV alpha 1 chain
CGIs	CpG islands
CpG	Cytosin-Phosphate-Guanin site
DNA	Deoxyribonucleic acid
DMRs	Differentially methylated regions
DNMT	DNA methyltransferase
GAA	Glucosidase Alpha, Acid
HATs	Histone acetyltransferases
HDACs	Histone deacetylases
HMTs	Histone methyltransferases
ISWI	Imitation switch

INO80	Inositol requiring 80
lncRNA	Long non-coding RNA
HLA	Major histocompatibility complex
MARs	Matrix attachment regions
MLPH	Melanophilin
MAGI1	Membrane Associated Guanylate Kinase
mRNA	Messenger RNA
µl	Microliters
miRNA	MicroRNA
MAPK8IP3	Mitogen-Activated Protein Kinase 8 Interacting Protein 3
ng	Nanogram
ncRNAs	Noncoding RNAs
PGAM5	PGAM family member 5, mitochondrial serine/threonine protein phosphatase
PDE11A	Phosphodiesterase 11A
piRNAs	Piwi-interacting RNAs
PCR	Polymerase Chain Reaction
PTMs	Post-translational modifications of histone
KCNJ5	Potassium voltage-gated channel subfamily J member 5
PRICKLE2	Prickle planar cell polarity protein 2
PGCs	Primodial germ cells
PRRC2A	Proline Rich Coiled-Coil 2A
PTGIR	Prostaglandin I2 (prostacyclin) receptor (IP)
P1	Protamines 1
P2	Protamines 2
PTPRN2	Protein tyrosine phosphatase, receptor type N2

ROS	Reactive oxygen species
RT-PCR	Real-Time Polymerase Chain Reaction
RNA	Ribonucleic acid
SAM	S-adenosylmethionine
SNP	Single Nucleotide Polymorphism
siRNAs	Small interfering RNAs
SD	Standard Deviation
SMC1B	Structural Maintenance Of Chromosomes 1B
SWI/SNF	Switching defective/sucrose nonfermenting
Tsx	Testis-specific X-linked
TF	Transcription factors
TPs	Transition proteins
TYRO3	Tyrosine-protein Kinase receptor
UBE2G2	Ubiquitin conjugating enzyme E2 G2
WHO	World Health Organization
bp	Base pairs

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1 Introduction

1.1 Overview

Infertility is a growing problem in human health and is defined as the inability to achieve pregnancy after 12 months, despite unprotected sexual intercourse (**Poongothai *et al*., 2009**). In addition, infertility is a complex disease related to several factors such as hormonal, biological, genetic, and lifestyle factors (**Dada *et al*., 2001; Krausz *et al*., 2014; Yassin *et al*., 2017**). Approximately 10–15% of couples suffering from infertility problems and about 50% of cases are correlated with male factor (**Raheem *et al*., 2012; Inhorn & Patrizio, 2015; Kumar & Singh, 2015**). Oligozoospermia is one of the most prevalent disorders in male suffered from infertility problems, which characterized by abnormally decrease in the semen parameters. Besides that, the genetic problems were considered as one of the causes lead to male infertility problems, despite the differences in their origins and their etiologies. For examples: chromosome abnormalities, alterations linked to the Y chromosome and single gene mutations (**Müslümanoğlu *et al*., 2005; Kitamura *et al*., 2015**). Also, the recent studies found that the epigenetic alterations, abnormality in the sperm DNA methylation patterns and histone modification play a role in male infertility or fecundity decline (**Esteves, 2013; Soubry, 2015; Jenkins *et al*., 2016a**). The molecular mechanisms of these defects need more explanation since male infertility has been classified as a phenotype of the deficiency in the transcription of various genes (**Matzuk & Lamb, 2002**). So, epigenetic is considered as one of the most promising approaches, which have the ability to explain some etiologies of fertility disorders.

1.2 Epigenetics

Epigenetics refers to heritable and reversible forms of gene activity and expression without any actual modification in the DNA sequences, or epigenetics defined as changes occur in gene expression by changing DNA and histone structure without changing the DNA sequence itself. The epigenetic processes include several

of activities like such as DNA methylation, post-translational modifications of histone (PTMs), chromatin remodeling, and RNA interference (**Vaissière *et al.*, 2008**) (**Figure 1.1**), and these epigenetic alterations can be inherited through both mitotic and meiotic divisions (**Ferfouri *et al.*, 2013; Urdinguio *et al.*, 2015**). The body of the human has at least 400 different kind of cells and the DNA is similar in all cells (**Razin, 1998**). The regulation of mRNA transcription is considered the main effector of cellular differentiation and diversity; epigenetic and histones modification is a key modulator of this process **(Mitchell *et al.*, 2016).** The DNA wraps around histones to form nucleosomes and the nucleosomes are grouping into a special order of structures called chromatin. The gene expression was controlled through the chromatin modifications (**Klose & Bird, 2006**). The compactness of DNA in a particular region of chromatin is regulated by epigenetics, which plays a critical role in deciding which genes will be expressed and when.

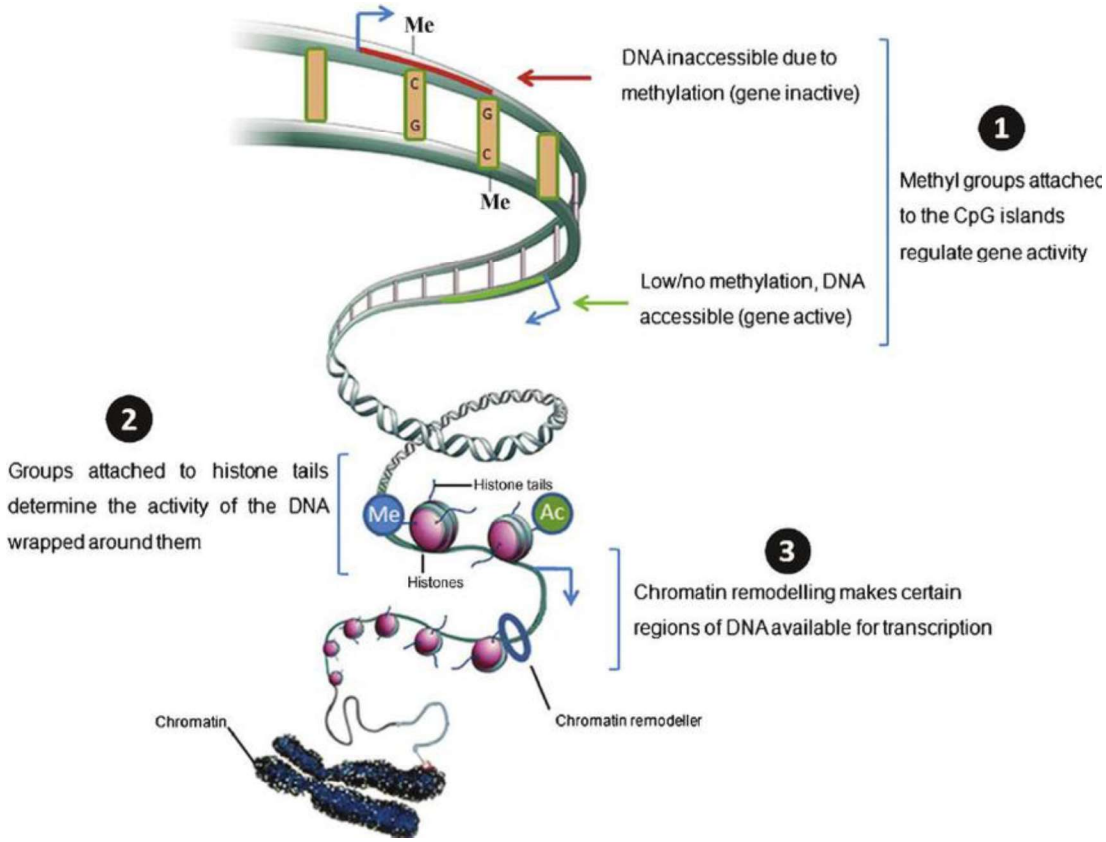

Figure 1-1 Major epigenetic mechanisms influencing gene expression (**Rajender *et al.*, 2011**).

1.2.1 DNA methylation

DNA methylation is an epigenetic mechanism, used to control the expression of the genes in all cells, and occurs by the transfer of a methyl (CH3) group from S-adenosylmethionine to a fifth carbon atom of cytosine pyrimidine ring (5meC) in the CpG islands (CGIs) clusters (**Schübeler, 2015**) (**Figure 1.2**). The methylated state of CpGs has a critical impact on genes transcription during embryonic growth, genomic imprinting, X-chromosome inactivation, and development of tumors (**Liyanage *et al.*, 2014; Heyn *et al.*, 2016**). In human, DNA methyltransferase (DNMT) is responsible for the transfer of a methyl group (CH3) from S -adenosylmethionine to 5-cytosine of CpG dinucleotides to form 5-methylcytosine (5meC) (**Celik *et al.*, 2016**). The CpG islands (CGIs) are short (~500 base pairs long) interspersed C+G rich DNA sequences and found in the promoter or in the regulatory regions of transposable elements (**Zhu *et al.*, 2008; Illingworth & Bird, 2009**).

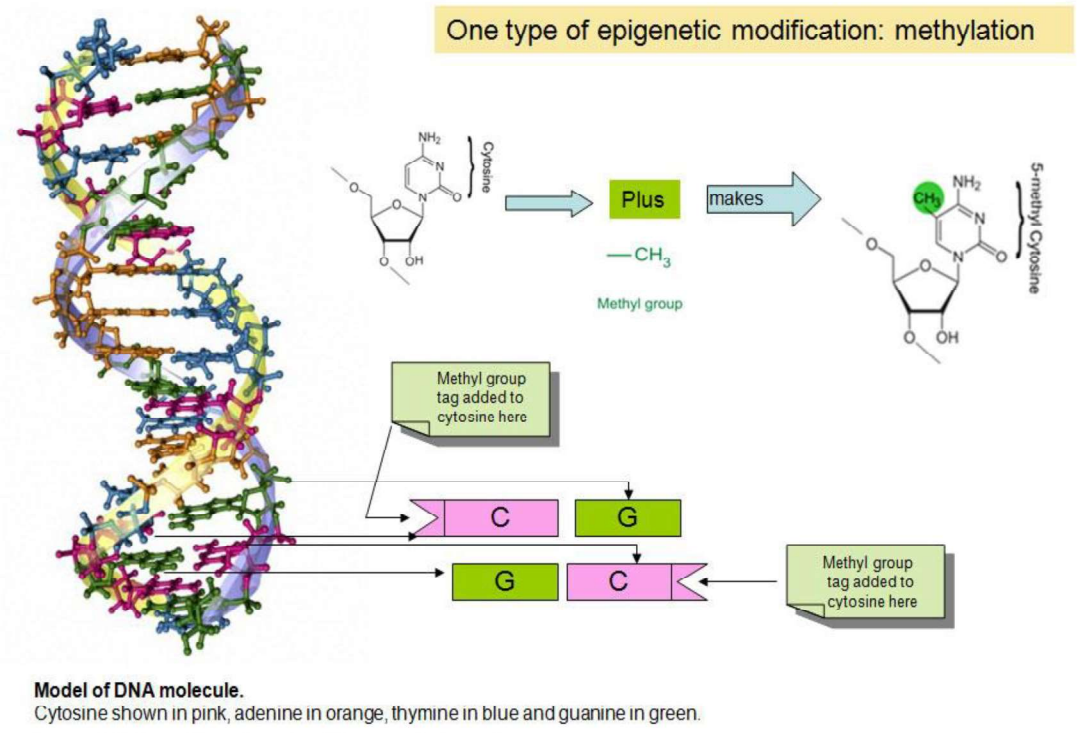

Figure 1-2 Chemical structures of cytosine and 5-methyl cytosine in DNA (Information from: http://bscb.org/learning-resources/softcell-e-learning/epigenetics-its-not-just-genes-that-make-us/).

The methylation of these CGIS is correlated with silencing or inactivation of promoters through inhibiting the attached of a transcription factor with methylated cytosines or suppression mediated by methyl-CpG-binding proteins (**Jones, 2012)**, while the hypomethylation usually correlated with gene expression activation (**Deaton & Bird, 2011**) (**Figure 1.3**). Suppression of transcription by CpG methylation can occur by different mechanisms. (I) The presence of methyl groups at specific CpG sites leads to directly blocked for the DNA recognition and binding of transcription factors (**Nomura *et al*., 2007**), (II) arrive the transcription factor to the regulatory elements might be blocked (**Handy *et al*., 2011**), (III) histone deacetylation promotes chromatin condensation, further inhibiting transcription (**Chen *et al*., 2016).**

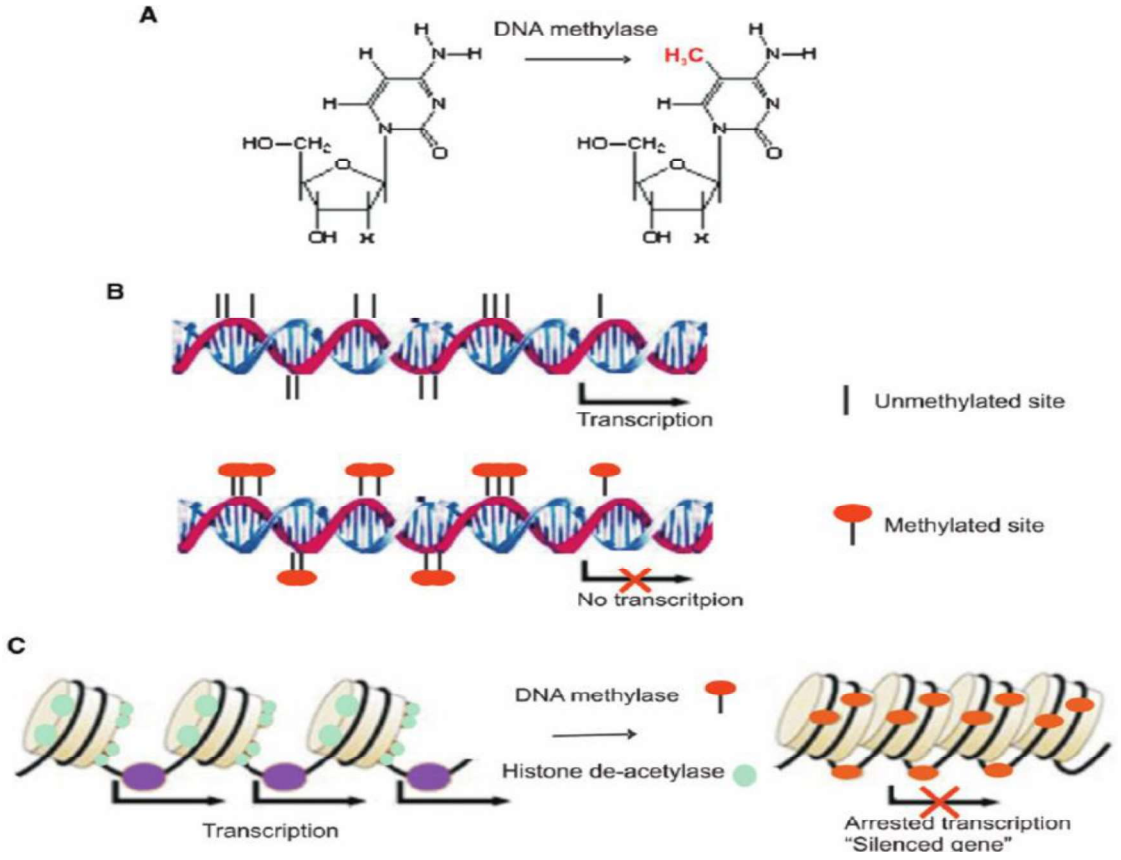

Figure 1-3 Epigenetic regulation of gene expression

As described in figure 1-3 (A) DNA methylase catalyzes the transfer of a methyl group to the cytosine residues in CpG dinucleotide sequences. (B) Inhibition the gene expression by methylation of the CpG islands in the gene promoter region. (C) Histone acetylation plays an important role in the regulation of gene expression. Hyperacetylated chromatin is transcriptionally active whereas hypoacetylated chromatin is silent. Methyl-CpG-binding proteins interact with histone deacetylase causing gene silencing (**Muhonen & Holthofer, 2009**).

In vertebrates, CpG islands are usually related with the promoters of many housekeeping genes which are necessary for general cell functions, as well as certain tissue-specific genes, and methylation of these sites attenuates or halts transcription (**Gardiner-Garden, 1987; Mitchell *et al*., 2016**). Also, the methylated CpG clusters distributed within genes, intergenic regions, and within silencers and enhancers, which plays a role in cellular physiology. These CpG sites are considered as a subject for research, especially they play an important role in controlling of genes transcription (**Mitchell *et al*., 2016**). The DNA methylation patterns within regulatory regions are characterized as stable; nevertheless, the dynamic de novo methylation occurs in the regulatory regions to avoid re-expression (**Weber *et al*., 2007**). The regulatory mechanisms depending on the DNA methyltransferase (DNMT) protein family, which includes different kinds of DNA methyltransferases (DNMTs) like DNMT1 which is responsible for maintenance of DNA methylation during DNA replication, besides facilitates the activities of other enzymes (DNMT3A and DNMT3B) (**Goll & Bestor, 2005; Jones, 2012**). While, DNMT3A, DNMT3B, and DNMT3L mediate de novo methylation of genomic DNA during the early phase of embryo growth specifically in germ cells, and they have an active role in the process of spermatozoa production (**Kaneda *et al*., 2004**). Recent findings showed that epigenetic regulation contributed in neurodevelopment, aging, and many human diseases (**Kaas *et al*., 2013; Jones *et al*., 2015**).

1.2.2 Histone modifications

The DNA of eukaryotic is packaged into a compacted chromatin structure and the nucleosome is the essential compound of this structure. Nucleosomes are formed from eight histone molecules with two copies of histones H2A, H2B, H3, and H4 (**Barnes *et al*., 2005**) (**Figure 1.4**), which is fundamental to spermatozoa production, structure, and function (**Okada *et al*., 2007**). Histone modifications are performed by acetylation of lysines, methylation of lysines and arginines, ubiquitylation of lysines, and phosphorylation of serines and threonines (**Kouzarides, 2007**). Histone modifications are known as chromatin regulatory proteins, ultimately leading to variations in DNA transcription, replication, repair, and recombination (**Dada *et al*., 2012; Tvrda *et al*., 2015**). The open and/or closed chromatin statuses determine the

gene accessibility to the transcriptional machinery and regulated through the modifications to both DNA and histone tails. Briefly, the open status is less densely packed chromatin state allows binding of transcription factors (TF) and the general transcriptional machinery to the gene regulatory region and leads to transcriptionally active. This open status is correlated with demethylated DNA and highly acetylated histone tails (H3, and H4). In contrast, the closed status is more densely packed, does not allow the binding of transcription factors and leads to transcriptionally silent. This close status is characterized by the following, DNA methylation and deacetylated; methylated histone lysine (H3K9 and H3K27) residues (**Carlberg & Molnár, 2016**; **Prakash, 2017**) (**Figure 1.3 "C"& Figure 1.5**).

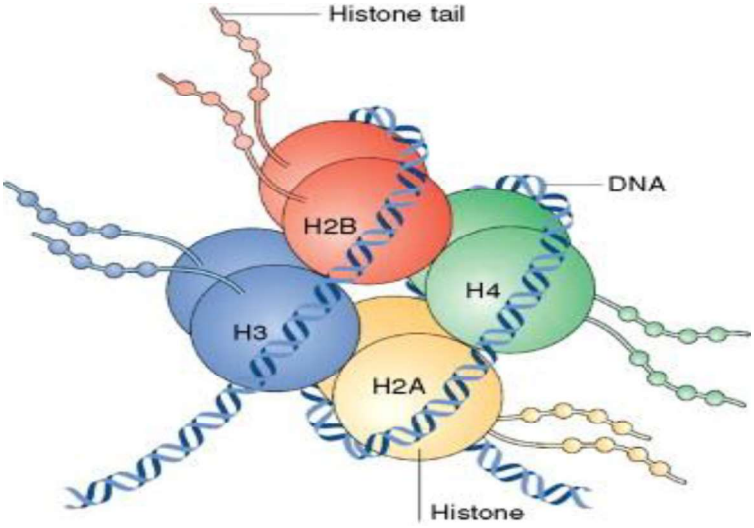

Figure 1-4 A structure of Nucleosome

As described in figure 1-4: A histone octamer composed of two copies each of the histones H2A, H2B, H3, and H4. A histone octamer surrounded by with a DNA strand wrapped (**Tsankova *et al.*, 2007**).

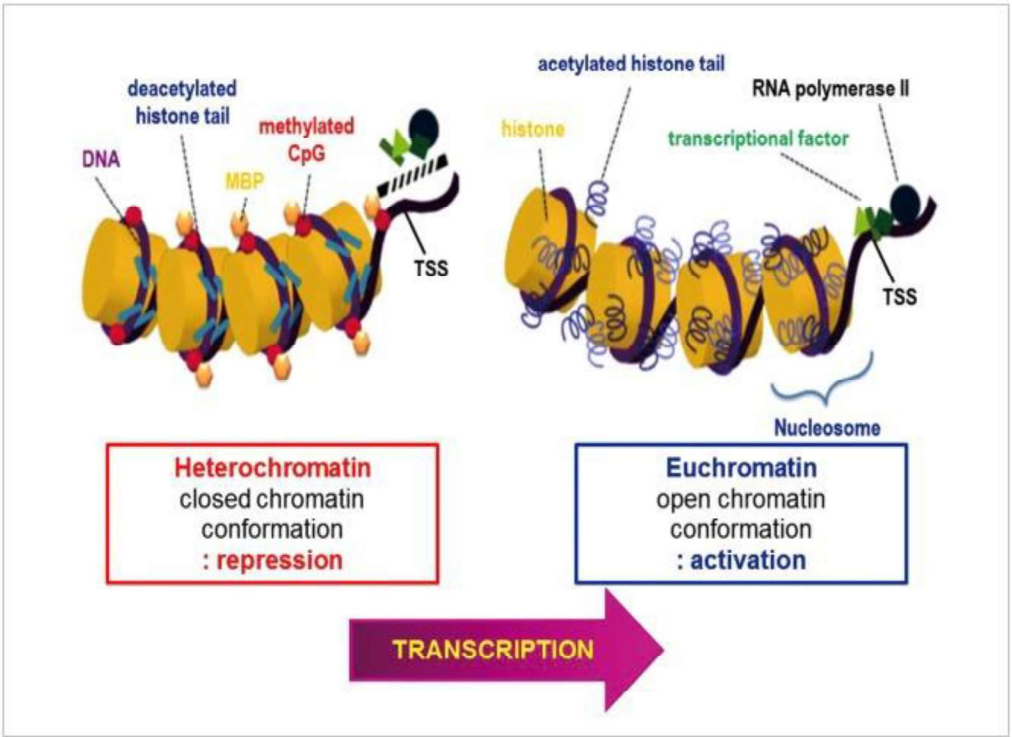

Figure 1-5 Open and closed status of chromatin
As described in figure 1-5: the close status on the left is characterized by DNA methylation and deacetylated histones, is condensed and inaccessible to transcription factors, which is repressive regulation of transcription. In contrast, open status on the right is in a loose form and transcriptionally active; DNA is unmethylated and histone tails acetylated, which is active regulation of transcription (**Hatzimichael & Crook, 2013**).

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are the major enzymes that contribute in the Histone acetylation. HATs catalyze the addition of acetyl group to the lysine residues and associated with the local chromatin expansion and activation of gene expression (**Rice *et al.*, 2001**). While HDACs removes the acetyl group from the lysine residue which and leads to chromatin condensation and inhibition of gene expression (**Tsankova *et al.*, 2010**). A complex interaction occurs between modifications of the histone tails of H3 and H4, some of which work antagonistically to regulate the change from an active to inactive chromatin state and one of them termed as histone code (**Lachner *et al.*, 2003**). Histone methyltransferases (HMTs) are enzymes responsible for the addition of methyl groups to either lysine and/or arginine residues (**Upadhyay & Cheng, 2011**). In contrast to DNA methylation, histone methylation is associated with either suppression or activation of gene expression depending on the number and the

position of methyl group that added to amino acid residues, for examples: methylation of histone H3 at Lys4 (H3K4), Lys36 (H3K36) and Lys79 (H3K79) has an activating influence on gene expression, while methylation of histone H3 at Lys9 (H3K9) and Lys27 (H3K27) is correlated with gene silencing (**Filipponi & Feil, 2009; Upadhyay *et al*., 2011; Ho *et al*., 2015**). Additionally, chromatin remodeling complexes play a vital role in remodels nucleosome structure to cover or expose regions of DNA for transcriptional regulation. Currently, four different families of chromatin remodeling complexes were identified SWI/SNF (switching defective/sucrose non-fermenting), CHD (chromo-domain, helicase, DNA binding), ISWI (imitation switch), and INO80 (inositol requiring 80). All of these types need to ATP (ATP hydrolysis) to change histone-DNA and act together with chromatin modifiers to direct nucleosome dynamics (**Clapier & Cairns, 2009; Hargreaves & Crabtree, 2011**). It is worth mentioning that, histone methylation is considerably more complex compared to histone acetylation, and it can occur on conserved lysine and arginine residues and across all four histone proteins (**Zhang & Reinberg, 2001**). Recently, the researchers believed that the histone methylation is a more permanent, stable modification (**Bannister & Kouzarides, 2005; Black *et al*., 2012**).

1.3 Epigenetic and spermatozoa development

Spermatozoa are very organized and differentiated cells, which connected to specific structural and functional integrity. In the past, the major function of the spermatozoa was to transfer the paternal genetic information encoded in the DNA sequence to the embryo, besides the appropriate epigenetic information (**Carrell & Hammoud, 2010; Rando & Daddy, 2012**). While, the current information pointed that the spermatozoa structures have active participation in the early development stage (**Krawetz, 2005; Carrell, 2008; Inbar-Feigenberg *et al*., 2013**). Each type of cell have private epigenetic signature which shows in the developmental history and environmental influences and finally reflected in the cell's functions (**Dada *et al*., 2012**). However, the epigenetic of spermatozoa has been dynamically changing during development (**Surani *et al*., 2007; Saitou *et al*., 2012**).

1.3.1 Histone-to-protamine transition in spermatozoa

The structure of spermatozoa DNA is very special compared to the structure of the somatic cell, whereas the main part of DNA in spermatozoa is coiled into very compressed, and this issue occurs due to an incorporation of protamines. A less amount of DNA is joined with histones in a very relaxed form and the residual amount of DNA is joined to the sperm nuclear matrix within matrix attachment regions (MARs) (**Agarwal & Said, 2003**). During the progress of spermatogenesis, the spermatozoa undergo a dramatic change in the genome that not only influence genetic and epigenetic information but also in the structure of nuclear such as in chromatin structure. The majority (90–95%) of histones are replaced with protamines late in the haploid stage of spermatogenesis and these proteins are vital for the spermatozoa to become effective (**Rousseaux *et al*., 2005; Oliva, 2006; Kitamura *et al*., 2015**). Protamines are small molecules created during advanced stages of spermatogenesis and rich of arginine (**Bao & Bedford, 2016**). The DNA compaction plays a very important role in the formation and development of spermatozoa and in protecting the spermatozoa genome from the environmental factors, and harmful molecules within the female reproductive system (**H Cree *et al*., 2011)** (**Figure 1.6**). Acetylation process is controlled through the interactions between the acetylase and deacetylase enzymes. The major purpose of histone hyperacetylation represent in the open of chromatin structure and which stimulates DNA strand breaks caused by topoisomerase enzyme and facilitates the replacement of histones with transition proteins (TPs) (**Nair *et al*., 2008; Rousseaux *et al*., 2011**). Hyperacetylated considered as a starting point and then several extra events lead to the final protamine replacement such as the transition proteins are eliminated and replaced by protamines (**Rousseaux *et al*., 2009; Tvrda *et al*., 2015**). Two protamines (P1 & P2) are approximately equally expressed in human **(Carrell *et al*., 2007).** The phosphorylation of protamines and the ratio of the two protamines in human are essential for optimal sperm function. The normal ratio of P1/ P2 in proven fertile males found between 0.8-1.2 (**Carrell & LIU 2001**), and any defect in this ratio, higher or lower than normal correlated with declined sperm counts, and function, increased DNA damage and decreased males fecundity (**Aoki *et al*. 2005; Simon *et al*., 2011**). In addition, change in the ratio of P1 to P2 indicates abnormal

spermatogenesis and is a probable direct cause of abnormal methylation of differentially methylated regions (DMRs) (**Hammoud *et al.*, 2010; Hammoud *et al.*, 2011**).

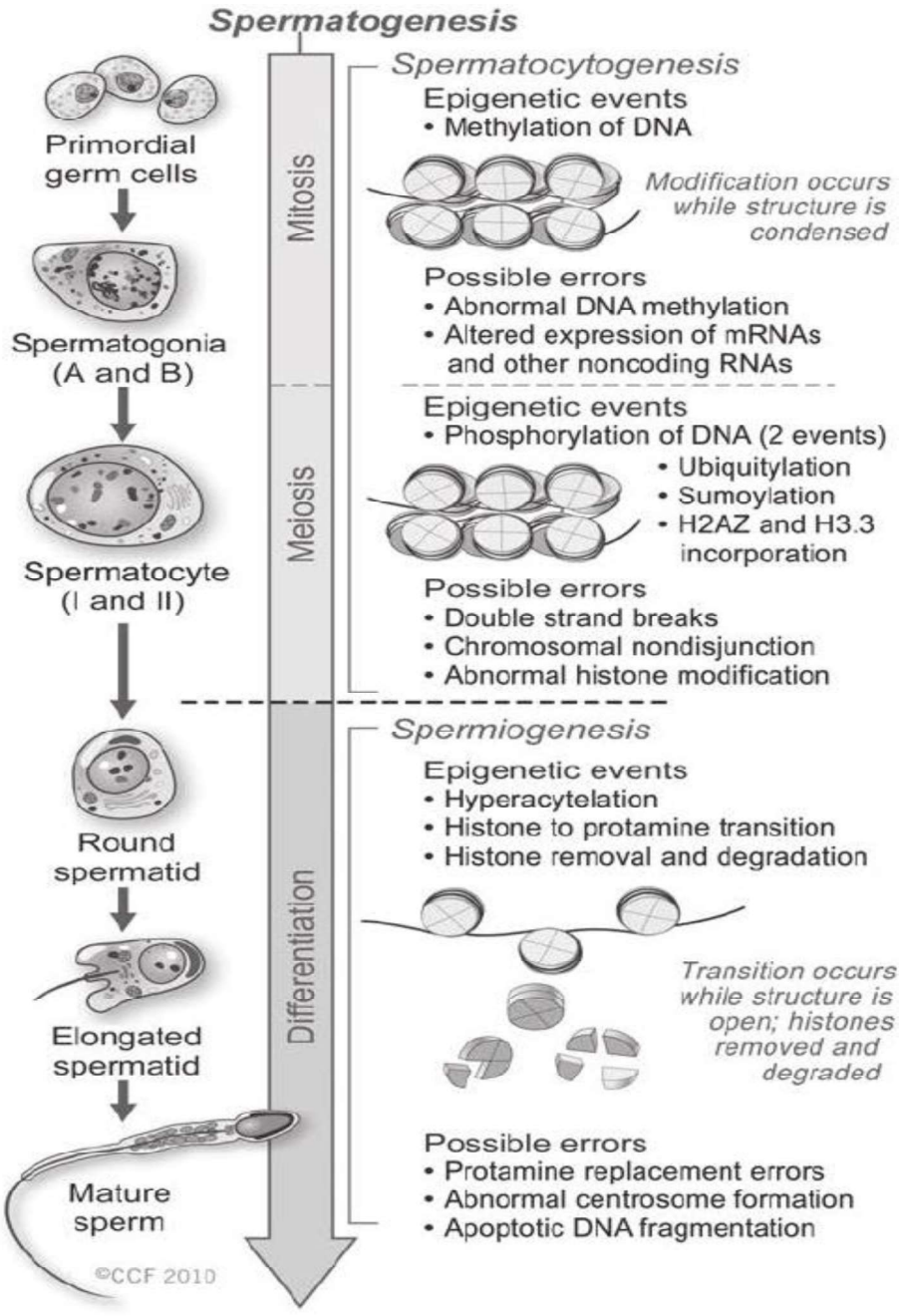

Figure 1-6 Epigenetic events during spermatogenesis

As described in figure 1-6: In primodial germ cells (PGCs), DNA methylation occurs to set up the paternal specific imprints. Hyperacetylation occurs during spermiogenesis to assist in the Histone-Protamine exchange (**Tvrda *et al.*, 2015**).

1.4 Environmental factors and epigenetic modifications

Male fecundity influenced by different factors and environmental factors are one of this factors. Several kinds of toxins like volatile organic solvents, physical agents, chemical dust, and pesticides had been associated with male infertility problems (**Hruska *et al*., 2000; Olooto, 2017**). Besides, lifestyles such as tobacco smoking and alcohol intake also contribute to the male infertility problems (**Gaur *et al*., 2007; Aitken *et al*., 2014**). Environment factors have a strong influence on the epigenetic modifications and alteration in gene expression and having a profound role on development and health as well as development and progression of the disease (**Poulsen *et al*., 2007; Miao *et al*., 2014**). Indeed, several of previous studies have assessed the effects of different environmental agents on the male fertility and showed that the environmental factors lead to aberrant in epigenetic regulation which adversely affects semen parameters (**Jenkins & Carrell, 2011; Huang *et al*., 2015**).

Nutrition: Diet consists of different types of supplements (folate, methionine, vitamin B6, and B12) which correlated with epigenetic modification and DNA methylation (**Feil & Fraga, 2012**). This compounds can influence on the carbon metabolism and consequently, the available amount of S-adenosylmethionine (SAM) can be affected which in turn affects the process of DNA methylation (**Van den Veyver, 2002**).

Environmental toxicants: The male fertility could be influenced by gonadal endocrine disruption or by direct damage on steroidogenesis (**Hammoud *et al*., 2010**). Endocrine disruptors and steroidotoxic agents are present in air pollution (**Jensen *et al*., 2002**), which contains a high level of carbon monoxide, nitrous dioxide, and lead. These toxic agents could influence on semen parameters and modification in the integrity of sperm DNA (**Mendiola *et al*., 2014; Santi *et al*., 2016**). Several of previous studies found that exposure to endocrine disrupting chemicals during the early stage of development leads to continual changes in DNA methylation. Whereas, the endocrine disruptors have the ability to change DNA methylation patterns found in the key genes and lead to significant changes in transcriptional process (**Li *et al*., 2003; Guerrero-Bosagna *et al*., 2008; Kumar *et***

***al*., 2013**). Another study showed that the used of 5-aza-20-deoxycytidine as anticancer agent causes a decrease in the global DNA methylation which leads to changed sperm morphology, reduced sperm motility, reduced fertilization capacity, and reduced the survival rate of the embryo (**Oakes *et al*., 2007**).

Cigarette smoking: Cigarette smoke is recognized as one of the environmental factors that effect on the DNA methylation (**Breitling *et al*., 2011**). 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 (**Sharpe, 2010; Breitling *et al*., 2011; Nielsen *et al*., 2016**), and sperm DNA damage (**Niu et al., 2010; Caserta *et al*., 2015**). Approximately 4000 chemical compounds produce from cigarette combustion such as nicotine, carbon monoxide, cadmium, and other mutagenic compounds which inhaled by smokers (**Figure 1.7**), all of this compounds have potentially to influences on the germ cells of males (**Wu *et al*., 2016**) and can reduce the activity of sperm mitochondrial, and damage the chromatin structure in human sperm, consequently reducing the fertilization capacity of spermatozoa (**Calogero *et al*., 2009; Sharma *et al*., 2013; Condorelli *et al*., 2017**).

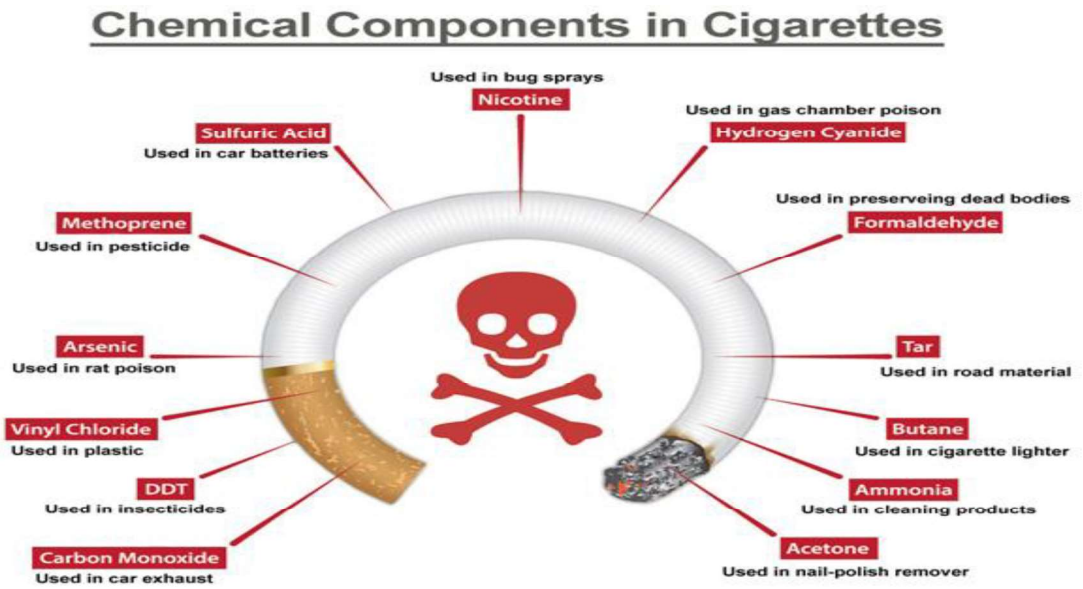

Figure 1-7 Harmful chemical compounds found in cigarettes smoke
As described in figure 1-7: Cigarette smoke contains over 4000 chemical compounds. Only a few of them are provided in this figure (information from http://hamrah.co/en/pages/nicotine/).

Recent studies found that the cigarette smoking have adversely influence in the transcriptome (**Charlesworth *et al*., 2010; Shen et al., 2016**), causes chromosomal aberration (**Kumar *et al*., 2015; Vaghela *et al*., 2016**), inverse effect on the protamination process by disrupting P2 (**Hammadeh *et al*.,2010, Hamad *et al*., 2014**), alteration in the sperm DNA methylation pattern (**Zeilinger *et al*., 2013; Thomas, 2016**), and decreased in the sperm membrane permeability and in the activity of acrosin (**Sofikitis *et al*., 2000; Sreenivasa *et al*., 2016**). Nevertheless, the influence of cigarette smoking on sperm DNA methylation remains controversial, and there are various mechanisms have been hypothesized to clarify this issue (**Sakkas *et al*., 2002; Calogero *et al*., 2009**). One of mechanisms candidate is the oxidative stress caused by increase in the generation level of reactive oxygen species (ROS) or free oxygen radicals within the testis plus decreased in the levels of antioxidant such as Vitamin E and Vitamin C, which lead to impaired spermatogenesis (**Lee *et al*., 2007; Meri et al., 2013**). Depend on the available data, which related to environmental factors and epigenetic alteration, it may be concluded that we should be focused on the environmental toxins as one of strong etiology that related with epigenetic changes in spermatozoa, in order to assist in improving diagnostic and treatment options in males who suffer from infertility problems (**Skinner *et al*., 2010; Jenkins *et al*., 2011**).

1.5 Sperm DNA methylation and male infertility

The spermatozoa are specialized cells with a flagellum, produced through the spermatogenesis process. This process includes a complex of events such as extensive cellular, epigenetic and chromatin changes. The mature spermatozoa will be shown at the end of this process, which is required for reproduction, fertilization, and normal embryo development (**Ioannou *et al*., 2016**). Spermatozoa characterized by unique DNA methylation patterns which generated in the early stages of spermatogenesis and needed for normal spermatogenesis and sperm development (**Santos & Dean, 2004; Ankolkar *et al*., 2012; Hackett & Surani, 2013**). Unlike somatic cells, germ cells have hypomethylated DNA (**Oakes et al., 2007**). The increase in the methylation levels of human spermatozoa has been shown to

influence human male fertility. For example, hypermethylation of the promoter region of CREM gene in spermatids leads to an abnormality in the P1/P2 ratios and is correlated with incomplete sperm chromatin compaction, reduced sperm motility, and male subfertility (**Nanassy & Carrell, 2011**). Several studies have illustrated that changes in the DNA methylation levels 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; Nanassy & Carrell, 2011; Li *et al*., 2013; Montjean *et al*., 2015**). Besides, other studies found a correlation between the alterations in sperm DNA methylation pattern and male infertility problems (**Navarro-Costa *et al*., 2010; Bahreinian *et al*., 2015; Tavalaee *et al*., 2015; Jenkins *et al*., 2016a**). Aberrant in 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**). Cheng *et al* revealed a significant relationship between DNMT1 polymorphisms and oligozoospermia compared to proven fertile, and this result suggests that these polymorphisms may influence in the DNMT1 expression levels and a defect in the spermatogenesis (**Cheng *et al*., 2014**). All of the previous studies support the hypothesis that spermatozoa DNA methylation patterns of printed as well as non-imprinted genes are necessary for normal sperm function, embryo development, and male fecundity. 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 study of the DNA methylation of CpG dinucleotides at differentially methylated regions (DMRs) is the best epigenetic mark to understand some infertility problems in males.

1.6 Human spermatozoa RNAs and male infertility

The nucleus of spermatozoa considered inert because of it is extreme compaction. However, spermatozoa RNA can act as an epigenetic regulator (**Grunewald *et al*., 2005**). Indeed, spermatozoa contain many specific kinds of RNAs

like mRNAs, miRNAs, noncoding RNAs (ncRNAs), small interfering RNAs (siRNAs), and Piwi-interacting RNAs (piRNAs) (**Yan *et al*., 2008; Frost *et al*., 2010; Song *et al*., 2011; Hamatani, 2012**). Both of miRNAs and noncoding RNAs have the ability to regulate the different genes expression by various processes and it is known that the differential susceptibility to microRNAs contributes to tissue-specific gene expression (**Ostermeier *et al*., 2002; Tani *et al*., 2010; Liu *et al*., 2012**). Additionally, the small non-coding RNAs and long noncoding RNAs (lncRNAs) have a significant function in germline development (**Yadav & Kotaja, 2014**). On the other hand, at transcriptional and post-transcriptional of miRNAs and siRNAs regulate the mRNA translation and degradation either by triggering endonuclease cleavage, promoting translation repression, or accelerating mRNA decapping (**Yadav & Kotaja, 2014**). Moreover, it has been found that spermatozoa RNA is capable of saving nucleosome-bound DNA and blocks the protamination in specific regions of chromatin (**Hamatani, 2012**) (**Figure 1.8**). In human, the mature spermatozoa have a specific mRNAs, and some of them encoding protamines and hormonal receptors (**Dadoune, 2009**). Furthermore, it has been found that the sperm RNAs could help in the stabilization of nuclear envelope, an interaction between DNA and histones during the protamine transition, and they have a significant function in marking the DNA sequences that stay bound to histones (**Hamatani, 2012**). A high amount of messenger RNAs (mRNAs) stored during spermatogenesis and which considers as an important feature, whereas the mRNAs are playing an important role in recognizing genes associated with the spermatogenesis and, consequently, genes correlated to infertility (**Wykes *et al*., 2000; Zhao *et al*., 2006**). Besides that, certain mRNAs remnants in the adult spermatozoa are important in fertility and early embryo development in humans (**García-Herrero *et al*., 2011**). In addition, several of previous studies illustrated that the spermatozoa RNAs have an important role in the epigenetic regulation of genes expression especially in the early stage of embryo development (**Grandjean *et al*., 2015; Chen *et al*., 2016a**). Moreover, other studies found an alteration in the expression of some genes in males who suffered from infertility problems compared to proven fertility males (**Gu *et al*., 2010; Montjean *et al*., 2012**).

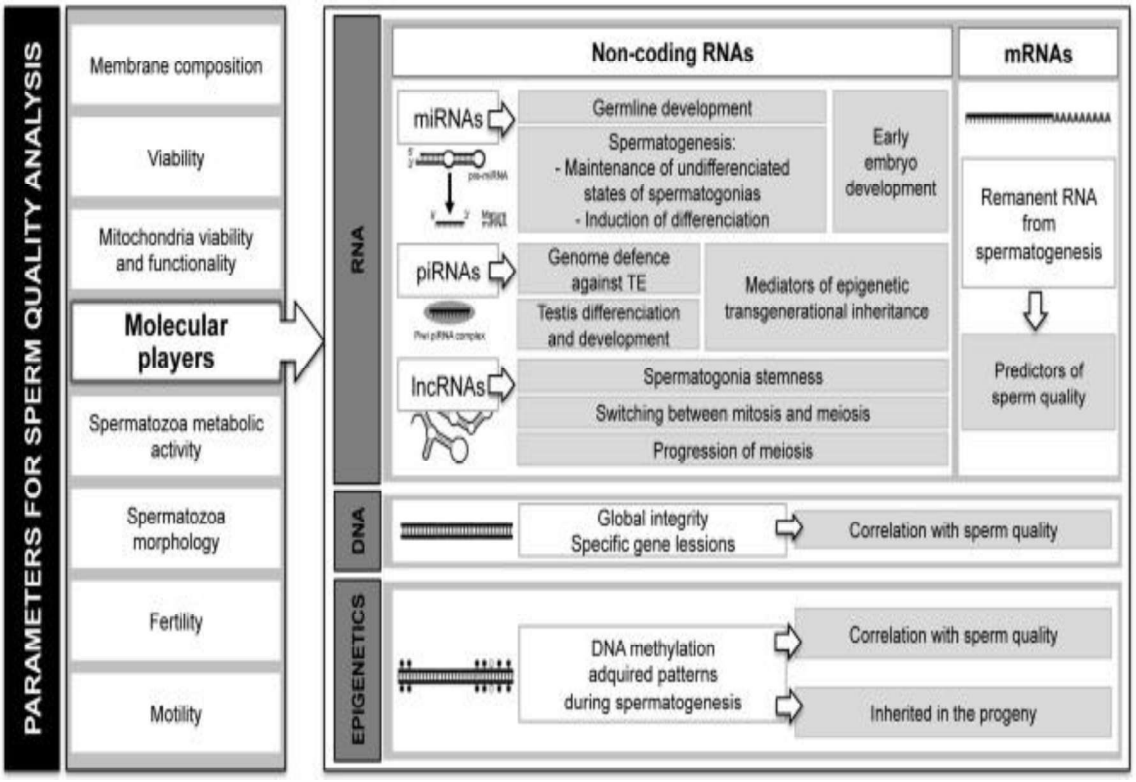

Figure 1-8 Parameters for sperm quality analysis

As described in figure 1-8: The molecular indicators that use to evaluate the sperm quality analysis and their roles on a male fertility and reproductive key process. RNA: non-coding RNAs (miRNAs, piRNAs, and lncRNAs) and mRNAs; DNA and epigenetic status (**Robles *et al*., 2017**).

This different kind of RNA could act at different levels and have different roles in regulation phase: (I) inhibit miRNA function, (II) avoid translation, (III) influence in chromatin remodeling (**Kung *et al*., 2013**). Interestingly, some lncRNAs have a specific function in the development of male germ cell such as Testis-specific X-linked (Tsx) is associated with meiosis progression and the Dmrt1-related gene (Dmr) plays a role in the switching between mitosis and meiosis of the germ cell development (**Lee & Xiao, 2012**), and depending on the information reviewed, it seems clear that the small and long non- coding RNAs have a critical role in post-transcriptional regulation, which considers a very necessary for successful spermatogenesis (**Robles *et al*., 2017**).

2 Aims of the PhD thesis

As summarized in the introduction, Epigenetic plays a critical role in the regulation of genes expression. Besides that, Spermatozoa have unique DNA methylation patterns which are created during early stages of spermatogenesis and are necessary for normal spermatozoa production and development. In patients with infertility problems, epigenetic alterations, abnormality in the sperm DNA methylation patterns and histone modification are considered as one of the causes leading to fecundity decline. So, epigenetic is considered as one of the most promising approaches, which have the ability to explain the etiologies of some infertility disorders.

The purposes of this study were summarized as the following

I. To determine whether sperm DNA methylation at CpG dinucleotides is different in males suffering from a reduction in fecundity compared to proven fertile males.
II. To determine the relationship between changes in sperm DNA methylation levels in the genes of this study, and abnormalities in semen parameters.
III. 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.
IV. To evaluate the association between the gene expressions level for the genes included in this study and semen parameters, addition to assessing the correlation between the gene expression and the alteration in spermatozoa DNA methylation levels.

3 Results

This thesis is a cumulative thesis based on five of published scientific manuscript as follows: In this thesis, we determine whether sperm DNA methylation at CpG dinucleotides is different in males suffering from subfertility problems compared to proven fertile males. Also, this study evaluated the relationship between changes in sperm DNA methylation patterns and semen parameters in subfertile males. On the other hand, two from the attached manuscript has been designed 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.

- In the first manuscript, nine regions were identified have a variation in the methylation levels between the males with reduced fecundity (as cases) and the proven fertile males (as controls) without overlap common annotation SNPs. Six out of nine differentially methylated regions (cg05799088, cg07227024, cg16338278, cg08408433, cg23081194, and cg19779893) is located in the CpG island and the bodies of the following genes (*PRICKLE2, ALS2CR12, ALDH3B2, PTGIR, UBE2G2, ADAMTS14*, respectively), while three were found in intergenic regions (cg04807108, cg25750688, and cg19406113). In addition, an association between changes in the methylation level for these CpGs and different semen parameters was found.
- In the second manuscript, eight CpG were identified have a variation in the methylation levels between subfertile males, who have been unable to have children after ten years of attempting childbearing (cases) and proven fertile males (controls) without overlap common annotation SNPs. Six out of eight differentially methylated regions (cg09737095, cg14271023, cg17662493, cg26038514, cg20311754, and cg12403190) were located in CpG island and the bodies of the following genes (*KCNJ5, MLPH, SMC1B, ASAP1, COL4A1, MAGI1*, respectively), and two were found in intergenic regions (cg13496755 and cg04733911). In addition, an association was shown between the

alterations in the methylation levels at these CpGs and sperm count, a percentage of total sperm motility.

- In the third manuscript, only seven CpG regions showed a significant difference in the mean methylation level in proven fertile males as “control group” compared to subfertile males, who have been unable to have children as the “case group” without overlap common annotation SNPs. Each one of this CpGs (cg05813498, cg06833981, cg07869343, cg02745784, cg09785377, cg22963027 and cg19169023) was located in gene bodies and CpG islands (*GAA, PRRC2A, MAPK8IP3, PDE11A, ANXA2, CGβ, TYRO3,* respectively), and these genes were found to be related to male fertility, sperm count and sperm function.
- The fourth manuscript showed ninety-five CpG have significant differences in current smokers (cases) as compared to never smoked males (controls). However, only 11 of those have significant differences in cases as compared to controls without overlap common annotation SNPs. Six out of eleven CpGs (cg20978247, cg07869343, cg19169023, cg09432376, cg00648582, cg23841288) were located in CpG the bodies of the following genes (*HLA, MAPK8IP3, TYRO3, APOL6, PGAM5, PTPRN2*, respectively), and two were found in intergenic regions (cg01584086, cg23109721, cg27391564, cg15412446, cg08108333). Besides that, a significant correlation has been found between the percentage of total sperm motility, the percentage of sperm immotile and the methylation level at CpGs in gene related amplicon included in this study.

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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 ($p \leq 0.005$, $p \leq 0.004$, $p \leq 0.012$, $p \leq 0.028$, $p \leq 0.012$, $p < 0.009$, 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 ($p \leq 0.017$, $p \leq 0.050$, $p \leq 0.040$, $p < 0.029$, $p < 0.005$, $p < 0.050$, $p < 0.013$, $p < 0.016$, $p < 0.017$, $p \leq 0.024$, $p \leq 0.037$, $p \leq 0.016$, $p \leq 0.029$, and $p \leq 0.016$, respectively).

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

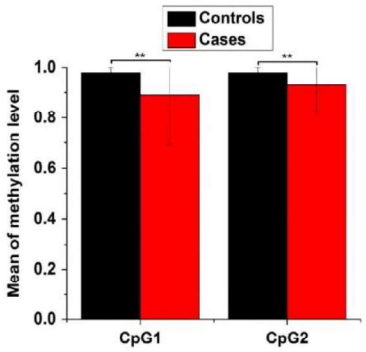


Figure 2 Methylation level at CpGs in *ALS2CR12* gene-related amplicon. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

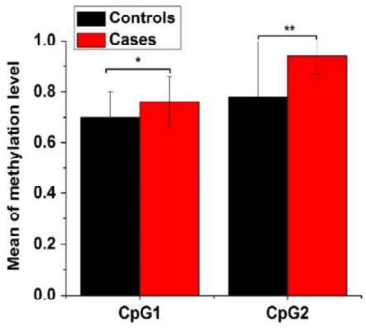


Table 2 Descriptive characteristics for the study population and for cases compared to controls ($n = 111$)

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. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

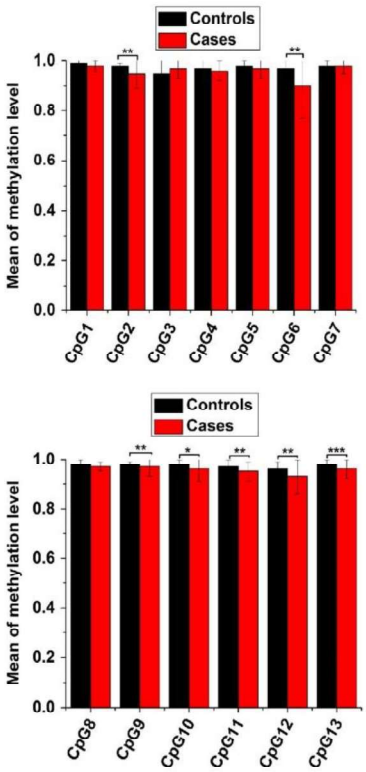
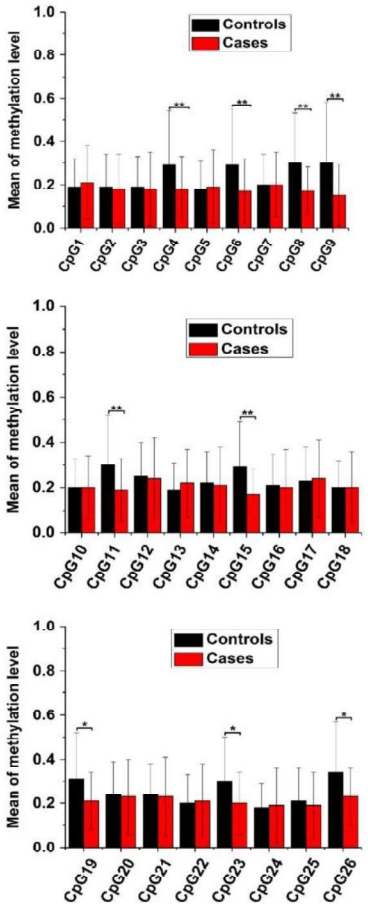


Figure 4 Methylation level at CpGs in *PTGIR* gene-related amplicon. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.



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 ($p \leq 0.001$, $p \leq 0.0001$, $p \leq 0.0001$, $p \leq 0.0001$, 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).

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Original article

Aberrations in sperm DNA methylation patterns are associated with abnormalities in semen parameters of subfertile males

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ABSTRACT

Infertility affects about 15% of couples worldwide, with approximately 7% of males suffering from infertility problems. This study was designed to assess the relationship between alterations in sperm DNA methylation patterns and semen parameters in subfertile males. Of a total of 108 males, 30 samples were subjected to 450 K BeadChip arrays to evaluate the variation in DNA methylation level between cases and controls. Three CpG sites showed the highest difference in methylation levels (cg09737095, cg14271023, and cg17662493), which are located in the *KCNJ5*, *MLPH*, and *SMC1β* genes, respectively; these were selected for further analysis using deep bisulfite sequencing in 78 independent samples (21 proven fertile "controls", and 57 subfertile "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 decrease in methylation levels at six CpGs (CpG1. CpG3. CpG4. CpG6. CpG7. and CpG8) in the *KCNJ5* gene-related amplicon ($p \leq 0.001$, $p \leq 0.009$, $p \leq 0.007$, $p \leq 0.007$, $p \leq 0.020$, and $p \leq 0.016$, respectively), and at (CpG1, CpG2, and CpG4) in the *MLPH* gene-related amplicon ($p \leq 0.003$, $p \leq 0.005$, and $p \leq 0.0001$, respectively), while there was a significant increase in the methylation level at six out of eight CpGs in the *SMC1β* gene-related amplicon in cases compared to controls. Our results show that three CpGs have a significant difference in sperm DNA methylation levels in subfertile males compared to proven fertile males.

1. Introduction

Infertility is a complex disease related to several factors such as hormonal, biological, genetic, and lifestyle factors [1–3]. Infertility affects about 15% of couples worldwide [4]. Besides, approximately 7% of males suffer from infertility problems [5]. A common reason for infertility in human couples is male subfertility [6], and approximately 15–30% of couples are diagnosed with unexplained infertility after a routine analysis [7]. Several known causes lead to infertility like genetic causes, which constitute nearly 30% of male infertility cases [8], and there are other causes; however, about 50% of cases of male infertility are still diagnosed as unexplained [9]. Interestingly, several recent studies have shown a relationship between epigenetic alterations and male subfertility [10–13]. Therefore, epigenetics appears to be a promising research field for studying and explaining some of the causes of male infertility.

Epigenetics is defined as alterations to DNA that turn genes "on" or "off" without changes to the DNA sequence [14,15]. Epigenetics includes modifications to histone proteins and DNA methylation; here, we focused on DNA methylation and subfertility in males. DNA methylation is a major epigenetic modification involving the addition of a methyl group to the 5 position of cytosine by DNA methyltransferase to form 5-methylcytosine (5-mC) [16,17]; this addition leads to transcriptional silencing of mammalian genes [18]. The CpG dinucleotides can be found in clusters that have been termed CpG islands and characterised by less methylation than non-CpG islands [17]. It is worth mentioning that CpG islands are found in about 60–70% of gene promoters, and these promoters can be divided according to their CpG density [19,20]. The anomalies of sperm DNA methylation patterns are still under experimental investigation. In contrast, several previous studies have reported that these abnormalities can be transmitted to offspring and can influence the offspring's susceptibility to disease [21,22]. Therefore, alterations in sperm DNA methylation patterns of subfertile males may potentially be responsible for the increased risk of imprinting disorders, perinatal mortality, and several other pregnancy-related complications that are seen in assisted reproductive technology (ART) babies [23]. This study is designed to further evaluate the association between anomalies in sperm DNA methylation patterns and semen parameters of subfertile males.

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For this purpose, the present study tested the differences in sperm DNA methylation patterns between proven fertile males and subfertile males, who have been unable to have children after ten years of attempting childbearing. Also, this study will evaluate the relationship between changes in sperm DNA patterns and semen parameters in subfertile males.

2. Material and methods

2.1. Sample collection and semen analysis

Institutional review board approval (No. PHRC/HC/13/14) was obtained from the Ethics Committee of Helsinki before the initiation of this study. Besides, all participants gave written consent before participation in this study. All samples were analysed in the laboratory of the Molecular Biology, Genetics & Epigenetics Department at the University of Saarland, and the samples were analysed according to standard operating procedures. Briefly, one hundred and eight samples with a mean age of 38.5 ± 4.7 were collected (36 samples from proven fertile males as a "control group" and 72 samples from subfertile males, who have been unable to have children after ten years of attempting childbearing as the "case group"). 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, 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. carbon monoxide, 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 sperm count was assessed immediately using a Meckler counting chamber (Sefi-Medica, Haifa, Israel). The sperm parameters were analysed (sperm counts, a percentage of total sperm motile, and percentage of progressive motility) according to World Health Organisation guidelines [24]. Before DNA extraction from sperm, somatic cells were removed from all semen samples through the use of Somatic Cell Lysis Buffer (SCLB) which is widely used for sperm cell purification [25,26]. First, pure populations of spermatozoa were obtained through a 50% gradient (20 min at 300 × g), and then pure spermatozoa were incubated with Somatic Cell Lysis Buffer (SCLB) on ice for 30 min, and washed twice with phosphate-buffered saline (10 min at 500 × g). The absence of somatic cell contamination was confirmed by microscopic examination.

2.2. 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). The concentration and purity of extracted DNA were determined with the use of a Nanodrop spectrophotometer ND-2000c (Thermo Scientific). Five hundred nanograms of extracted 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 [27]. To confirm the effectiveness of the protocol used in removing somatic cells from the semen samples that was 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 round cell contamination.

2.3. Screening study by infinium 450 K BeadChip array

Thirty semen samples were used in the array screening study with a mean age of 39.4 ± 3.5; this included 15 samples from proven fertile males "controls" and 15 from subfertile males who have been unable to have children after ten years of attempting childbearing "cases". 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 [28], and the arrays were scanned using the Illumina iScan. β-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 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 [29]. 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 hybridisation, 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. Validation study by bisulfite profiling (Bi-PROF)

In this study, and according to the results of screening study, three CpG sites that have the greatest difference in methylation level between the case and control groups were subjected to further analysis using local deep bisulfite sequencing (**Bi-PROF**) [30], according to the manufacturer's instructions. In the validation study, 78 samples (independent samples) were used and distributed as follows: 57 samples as cases and 21 samples as controls. 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 "MyTaq™ 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 microlitres 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 Agencourt® AMPure XP

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

cgID	Annotation	Chr-	nt (hg19)	At (°C)	Product size (bp)	DMCs	Primer sequence (5′–3′)	
cg09737095	*KCNJ5*	chr11	128787688	54.7	361pb	8	**F**	GTAAAGAGAAAGGAGAGTTA
							R	CACAACTAAAACAAATACTAA
cg14271023	*MLPH*	Chr2	238410067	52.4	244pb	4	**F**	AGATATTTTTGTGATGTGGT
							R	TACCTAACCTAAAATAAATT
cg17662493	*SMC1β*	Chr22	45806309	54.7	357pb	8	**F**	GTTAATGTAGTTGTTATTATTATTA
							R	AAAATCTTACTCTCTTACCCA

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

beads (Beckman Coulter, USA), measured using Quant-iT™ DNA Assay Kit (Fisher Scientific, USA) according to the manufacturer's 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 [31], excluding all reads containing ≥ 10% of missing CpG sites (maximal fraction of unrecognized sites ≥ 0.1). The obtained alignment sequence showed an absence of alterations at CpG positions.

2.5. Statistical analysis

Data obtained from the validation study were analysed using IBM SPSS for Windows software package version 24.0 (SPSS Inc., USA). Samples included in this study were non-normally distributed (non-parametric) according to the values of the skewedness 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, the Spearman's test was applied to assess the correlation coefficient between methylation level and other sperm parameters. The computed P values were adjusted with the use of Bonferroni correction. P values ≤ 0.05 were considered to be statistically significant.

3. Results

In a screening study, thirty DNA samples from spermatozoa (15 cases, and 15 controls) were subjected to an Infinium 450 K BeadChip analysis, to evaluate the differences in methylation patterns between study groups. Supplementary Table S1 summarises the location of the most differentially methylated CpGs (DMC), based on the mean DNA methylation difference of ≥20% between the case and control groups. We found only eight CpG positions following the applied criteria. Six out of eight differentially methylated regions (cg09737095, cg14271023, cg17662493, cg26038514, cg20311754, and cg12403190) were located in gene bodies and CpG islands, and two were found in intergenic regions (cg13496755 and cg04733911). From the results of the screening study, three CpG sites have the highest difference in methylation level were subject to validation study in 78 samples: I) cg09737095 was located in exon 3 of the *KCNJ5* gene, II) cg14271023 was located in intron 3 of the *MLPH* gene, and III) cg17662493 was located in intron 1 of the *SMC1β* gene. Table 2 illustrates that the age of the cohort study population was between 20.0-45.0 years, with a mean age of 37.7 ± 5.9 years, besides 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 21 males with a mean age of 38.5 ± 5.2 years as the "controls", and the subfertile group was composed of 57 males who have been unable to have children after ten years of attempting childbearing, with a mean age 37.4 ± 6.1 years as the "cases". The semen parameters showed a significant decrease in semen volume, sperm count, the percentage of total sperm motility, the percentage of progressive motility, the percentage of normal form sperm, and sperm vitality in the case group compared to the control group ($p \leq 0.000001$). Conversely, a significant increase in the percentage of immotile sperm ($p \leq 0.000001$) 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 neighbouring CpGs obtained from the screening study. The mean DNA methylation levels at six out of eight CpGs related to the *KCNJ5* gene amplicon (CpG1, CpG3, CpG4, CpG6, CpG7, and CpG8) showed a significant difference in the case group compared to the control group ($p \leq 0.001$, $p \leq 0.009$, $p \leq 0.007$, $p \leq 0.007$, $p \leq 0.020$, and $p \leq 0.016$, respectively) (Fig. 1). Moreover, the results showed that three of the four CpGs related to the *MLPH* gene amplicon (CpG1, CpG2, and CpG4) had a significant difference in the mean methylation level ($p \leq 0.003$, $p \leq 0.005$, and $p \leq 0.0001$, respectively) in cases compared to controls (Fig. 2). Besides that, six of the eight CpGs related to the *SMC1β* gene amplicon (CpG1, CpG3, CpG4, CpG6, CpG7, and CpG8) showed a significant difference in the case group compared to the control group ($p \leq 0.013$, $p \leq 0.013$, $p \leq 0.030$, $p \leq 0.017$, $p \leq 0.033$, and $p \leq 0.050$, respectively) (Fig. 3).

Finally, this study evaluated 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 negative correlation was found between the percentage of non-progressive motility and the methylation levels at CpG2 ($p \leq 0.016$) in the *KCNJ5* gene-related amplicon. Besides, a significant negative correlation was observed between the percentage of total sperm motility, the percentage of progressive motility and the methylation level at CpG1 in the *MLPH* gene amplicon ($p \leq 0.0001$, $p \leq 0.00002$, respectively). Furthermore, a significant negative correlation has been shown between the methylation level at CpG3 and the percentage of total sperm motility ($p \leq 0.008$), in addition to the methylation level at CpG8 and a percentage of non-progressive motility ($p \leq 0.003$)

Table 2
Descriptive characteristics for the study population (n = 78).

Variables	Mean ± SD	Minimum	Maximum
Age (Year)	37.7 ± 5.9	20.00	45.00
Semen Volume (mL)	1.60 ± 1.16	0.50	5.20
Sperm Count (mill/ml)	30.22 ± 32.85	4.00	123.00
Percentage of total sperm motility	38.64 ± 19.35	10.00	95.00
Percentage of progressive motility	27.85 ± 16.37	3.00	79.80
Percentage of non-progressive motility	10.79 ± 5.47	4.00	25.00
Percentage of immotile sperm	61.36 ± 19.35	5.00	90.00
Percentage of sperm normal form	20.22 ± 9.54	8.00	46.00
Sperm vitality	31.26 ± 16.25	9.00	69.00

All values are expressed as mean ± SD, SD: Standard deviation.

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

Variables	Controls (n = 21) Mean ± SD	Cases (n = 57) Mean ± SD	p value	Corrected P value
Age (year)	38.5 ± 5.2	37.4 ± 6.1	0.623[a]	1.00
Semen Volume (mL)	3.17 ± 1.19	1.02 ± 0.29	≤ 0.0001[a]	≤ 0.000001
Sperm Count (mill/ml)	69.79 ± 34.25	15.65 ± 16.17	≤ 0.0001[a]	≤ 0.000001
Percentage of total sperm motility	60.10 ± 15.84	30.74 ± 13.75	≤ 0.0001[a]	≤ 0.000001
Percentage of progressive motility	47.72 ± 11.58	20.53 ± 10.86	≤ 0.0001[a]	≤ 0.000001
Percentage of non-progressive motility	12.37 ± 6.42	10.21 ± 5.01	0.242[a]	1.00
Percentage of immotile sperm	39.90 ± 15.84	69.26 ± 13.75	≤ 0.0001[a]	≤ 0.000001
Percentage of sperm normal form	30.81 ± 8.46	16.32 ± 6.47	≤ 0.0001[a]	≤ 0.000001
Sperm vitality	46.95 ± 13.82	25.47 ± 12.98	≤ 0.0001[a]	≤ 0.000001

All values are expressed as mean ±SD, SD: Standard deviation.
P ≤ 0.05: Signifiant, P > 0.05: Not signifiant.
[a] Mann–Whitney test.

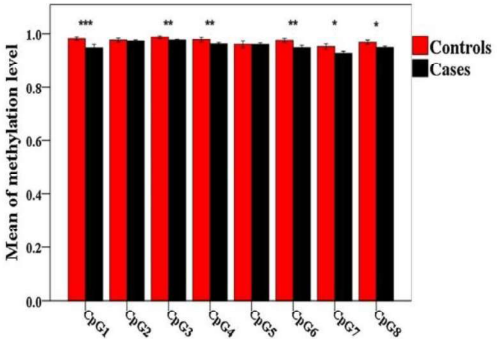

Fig. 1. Methylation level at DMC in *KCNJ5* gene-related amplicon in cases compared to controls.
*: P ≤ 0.05
**: P ≤ 0.01
***: P ≤ 0.001

related to the *SMC1β* gene amplicon. On the other hand, a significant positive correlation was observed between the methylation level at CpG7 and sperm count ($p \leq 0.007$) and between the methylation level at CpG2 and the percentage of normal sperm ($p \leq 0.001$) related to the *SMC1β* gene amplicon.

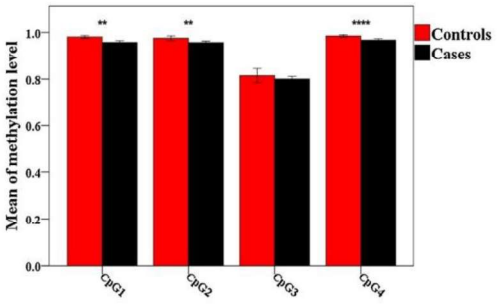

Fig. 2. Methylation level at DMC in *MLPH* gene-related amplicon in cases compared to controls.
*: P ≤ 0.05
**: P < 0.01
***: P ≤ 0.001
****: P ≤ 0.0001

4. Discussion

This study evaluated the methylation levels at DMC in the *KCNJ5, MLPH,* and *SMC1β* gene-related amplicons in spermatozoa obtained from subfertile males who have been unable to have children after ten years of attempting childbearing compared to proven fertile males. The DNA methylation in germline-related genes plays a vital role in the proper spermatogenesis process [32], and various studies have found that the defects in spermatogenesis and reduction in the reproductive potential of males could be caused by abnormal methylation in genes expressed in the testes [33–36]. Moreover, it has been shown that alterations in the sperm DNA methylation levels may affect male fecundity and semen parameters [12,23,37]. According to the results of the screening study, three CpGs (cg09737095, cg14271023, and cg17662493) have the highest difference in methylation level were validated on 78 samples, and these CpGs are located in the gene body and islands and were found to be related to the fertility of males, sperm count, and sperm function [38–41].

- cg09737095: located in exon 3 of the *KCNJ5* gene, without any overlap between a DNase I hypersensitivity cluster and transcription factor binding site [42].
- cg14271023: located in intron 3 of the *MLPH* gene, within a strong DNase I hypersensitivity cluster and the transcription factor binding site like EP300, TCF7L2, E2F1, and GATA3 [42].
- cg17662493: located in intron 1 of the *SMC1β* gene, only mapped to a weak DNase I hypersensitivity cluster without any linkage to active chromatin marks or transcription factors [42].

The results obtained from validation study showed a significant decrease in the methylation levels at six CpGs (CpG1, CpG3, CpG4, CpG6, CpG7, and CpG8) in *KCNJ5* gene-related amplicon and at three CpG (CpG1, CpG2, and CpG4) in *MLPH* gene-related amplicon, versus a significant increase in methylation level at six CpGs (CpG1, CpG3, CpG4, CpG6, CpG7, and CpG8) out of eight in *SMC1β* gene-related amplicon in cases compared to controls group. These results indicate that variations in sperm DNA methylation at DMC in *KCNJ5, MLPH,* and *SMC1β* genes might be among the mechanisms that lead to a defect in spermatogenesis, or reduced in semen parameters. These alterations in sperm DNA methylation level are in line with that which has been previously highlighted by researchers [43,44], who found alterations in the methylation levels of sperm DNA from males who suffer from a reduction in reproductive potential compared to the control group. In addition, other studies found a strong relationship between a change in the level of sperm DNA methylation and infertility problems in males [37,45–47]. With regard to the genes of this study, the

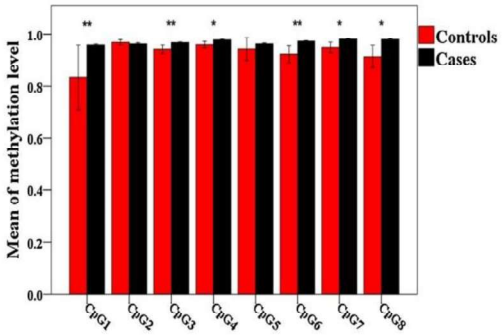

Fig. 3. Methylation level at DMC in *SMC1β* gene-related amplicon in cases compared to controls.
*: $P \leq 0.05$
**: $P \leq 0.01$

melanophilin (*MLPH*) or *SLAC2-A* gene encodes a member of the exophilin subfamily of Rab effector proteins and expressed with high levels in the brain, lung, and testis [48], and acts as linker protein between the GTP-bound form of Rab27A and myosin Va, an actin-based motor protein, and that the tripartite protein complex (Rab27A_Slac2-a_myosin Va) regulates melanosome transport to the actin-enriched cell periphery [38,49]. This complex is very important for sperm, especially in acrosome biogenesis [50,51]. Also, we should mention that the *MLPH* gene contributes to the development of spermatid tail, through the intramanchette transport (IMT) mechanism [52,53]. Furthermore, expression of the structural maintenance of chromosomes 1 Beta (*SMC1β*) gene is shown in the M phase of spermatogonia as a checkpoint gene [54], and forms the core of several protein complexes, the most important of which are cohesin and condensin. Takabayashi and his colleagues reported that the cohesin and condensin proteins play important roles in the maintenance of sister chromatid cohesion during both mitosis and meiosis, and the abnormality in the meiotic chromosome dynamics due to a lack of these protein components is thought to lead to incorrect chromosome segregation, chromosomal aberrations, and aneuploidy [40]; this, in turn, can result in spermatogonial apoptosis, meiotic arrest, or gametes with genomic instability, leading to either infertility or congenital malformations of the offspring [55]. Also, the expression of *SMC1β* has diverse functions within cells [56,57], stabilising cells' genetic information, DNA repair and recombination [58,59]. In the present study, a highly significant decrease was observed in cases compared to control groups in each of the following parameters: semen volume, sperm count, percentage of total sperm motility, percentage of progressive motility, percentage of normal form sperm, and these agrees with the previous studies results [60,61].

Finally, the results revealed a significant negative correlation between a percentage of total sperm motility, a percentage of progressive motility and the methylation level at CpG1 in the *MLPH* gene-related amplicon. In addition, a significant negative correlation was shown between the methylation level at CpG2 in the *KCNJ5* gene-related amplicon, CpG8 in the *SMC1β* gene-related amplicon and percentages of non-progressive motility. Moreover, a similar correlation has been found between the methylation level at CpG3 in the *SMC1β* gene-related amplicon and the percentage of total sperm motility. Conversely, a significant positive correlation was detected between the percentage of sperm normal form and methylation level at CpG2 in the *SMC1β* gene-related amplicon. In addition, a similar correlation between the sperm count and the methylation level at CpG7 in the *SMC1β* gene-related amplicon was found. These correlations are consistent with the results of other authors who found a high correlation between sperm count, sperm motility, and sperm DNA methylation levels [62]. Also, the results of other previous studies found an association between alterations in the methylation of imprinted genes and oligospermia in males with infertility problems [63]. On the other hand, these correlations disagree with the results of the previous study, which reported no correlation between alteration in the sperm DNA methylation levels and abnormal semen parameters [64,65].

5. Conclusions

In conclusion, the methylation levels at these CpGs were significantly different in subfertile males, who have been unable to have children after ten years of attempting childbearing, compared to proven fertile males. In addition, an association was found between the alterations in methylation levels at these CpGs and sperm count and sperm motility. Therefore, more studies are needed to elucidate the mechanisms relating to these alterations and to identify their significance and consequences on male infertility.

Conflict of interest

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

Acknowledgments

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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.repbio.2017.05.010.

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ORIGINAL ARTICLE

WILEY ANDROLOGIA

Association between alterations in DNA methylation level of spermatozoa at CpGs dinucleotide and male subfertility problems

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Summary

The purpose of this study was to assess the relationship between alterations in sperm DNA methylation levels and sperm count and sperm motility. Five CpG sites underwent deep bisulphite sequencing to validate the observed methylation difference in 78 samples (28 proven fertile males "controls," and 50 subfertile males "cases"). The results showed that variation in methylation levels was found in more than one CpG: the DNA methylation levels in CpG1, CpG2 and CpG3 of the *PRRC2A* gene-related amplicon showed high significant differences in the case group compared to the control group ($p \leq .0001$, $p \leq .003$, and $p \leq .0001$ respectively). Moreover, three CpGs of the four CpGs tested within the *ANXA2* gene-related amplicon (CpG1, CpG3 and CpG4) were significantly different ($p \leq .002$, $p \leq .001$, and $p \leq .0001$, respectively) in the case group compared to the control group. In addition, a significant difference was found in seven CpGs of the twenty-two CpGs tested within the *MAPK8Ip3* gene-related amplicon, besides six CpGs of the ten CpGs tested within the *GAA* gene-related amplicon between case and control groups. In conclusion, this study identifies that CpGs have a significantly different in methylation levels of sperm DNA for subfertile males.

KEYWORDS
CpGs, DNA methylation, spermatozoa, subfertility

1 | INTRODUCTION

Decreased fecundity is a complex pathology with many subtle associations with genetic, hormonal, biological and lifestyle factors that contribute to the disease (Barazani, Katz, Nagler, & Stember, 2014; Ferlin & Foresta, 2014; Yassin, Lubbad, Taha, Laqqan, & Jamiea, 2017). Male subfertility is the most common causes of infertility problems in couples. It is believed that male reproductive dysfunction is an independent reason of reduced fecundity in nearly 30% of subfertile couples (Anawalt, 2013). In another 20% of couples, abnormal male reproductive function contributes to a couple's inability to conceive (Anawalt, 2013; Poongothai, Gopenath, & Manonayaki, 2009). Infertility in male linked with a decrease in semen quality, quantity and reduced male fertility may also have unexplained reasons (Gelbaya, Potdar, Jeve, & Nardo, 2014). Moreover, the genetic anomalies (such as karyotype anomalies and Y chromosome microdeletions) are considered an important reason for male infertility. However, some cases have normal semen parameters after semen evaluation, but these cases are still diagnosed as idiopathic male infertility (Hamada, Esteves, & Agarwal, 2011). These idiopathic cases represented approximately 60%–75% of male infertility (Erenpreiss, Spano, Erenpreisa, Bungum, & Giwercman, 2006; Filipponi & Feil, 2009; McLachlan & O'Bryan, 2010). Many research studies have concentrated on the role of the DNA methylation and genetic basis of male infertility, but thus far, they have been able to explain not more than 15% of infertile cases (Gianotten, Lombardi, Zwinderman, Lilford, & Van Der Veen, 2004; Krausz, Escamilla, & Chianese, 2015).

In mammals, DNA methylation occurs at the 5C position of the cytosine pyrimidine ring that is mainly found in cytosine-guanine (CpG) dinucleotides and this is the most important epigenetic alteration in eukaryotes (Rajender, Avery, & Agarwal, 2011). The DNA methylation process is regulated by DNA methyltransferases (DNMTs), which add methyl groups from S-adenosyl-methionine to the 5C position of cytosine residues of CpG dinucleotides (Biermann & Steger, 2007; Nimura et al., 2006). Methylation patterns of cytosine residues within CpG dinucleotides convey essential epigenetic information about gene expression. CpG dinucleotides can be found in clusters that have been termed CpG islands (Takai & Jones, 2002). Indeed, hypermethylation of DNA in CpG islands is linked with the maintenance of gene suppression, while hypomethylation in these regions is associated with gene expression (Biermann & Steger, 2007). In fact, DNA methylation is essential in order to enable the correct compaction of chromatin in the sperm head and to permanently silence the promoters of genes involved in genetic imprinting (Seisenberger et al., 2013). In germ cells, the main targets of methylation are a non-CpG island in both repetitive sequences and distinct loci. Nevertheless, CpG islands (CGIs) can also be methylated (Oakes, La Salle, Smiraglia, Robaire, & Trasler, 2007). In human, correct sperm DNA methylation is suggested to be essential for both fertilisation and early foetus viability (Dada et al., 2012; Jenkins & Carrell, 2012; Romero et al., 2011). In the spermatozoa of oligozoospermic men, it was found that DNA methylation errors frequently occur and can be transmitted to the embryo (Anway, Cupp, Uzumcu, & Skinner, 2005; Hiura et al., 2014; Kobayashi et al., 2009; Nevin & Carroll, 2015). Thus, alterations in DNA methylation patterns in the spermatozoa of subfertile men may potentially account for the increased risk of imprinting diseases, and several other pregnancy-related complications seen at a higher frequency in babies conceived by ART (Calicchio, Doridot, Miralles, Mehats, & Vaiman, 2014; Kalra & Molinaro, 2008; Leung, Schones, & Natarajan, 2012). So, much remains to be understood, and we need to involve several methods and techniques to clarify the aetiology of male infertility or subfertility. The epigenetic promising approach has the potential to explain the aetiology of varied disorders, at least partially. Therefore, the purpose of this study was (i) to evaluate the variation in sperm DNA methylation level between subfertile male, and fertile male, who have had children, (ii) and to assess the relationship between alterations in sperm DNA methylation levels and phenotypic semen parameters.

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 initiation of this study. All participants were given written consent forms before participation in this study. All samples were analysed in the laboratory of the Molecular Biology, Genetics & Epigenetics Department at the University of Saarland. Samples were analysed according to standard operating procedures.

2.2 | Samples collection and semen analysis

One hundred and eight samples with mean age 38.8 ± 4.7 were collected (43 samples from fertile males, who have had children as a "control group," and 65 samples from subfertile males, and have been unable to have children as the "case group"). The exclusion criteria for participation in the study were as follows: diabetes mellitus, alcohol drinkers, the presence of antisperm antibodies, varicocele, Y chromosome microdeletions, smokers, abnormal hormonal parameters, abnormal body mass index and infertility related to the woman. In contrast, the included criteria for all study populations were as follows: (i) males were not exposed directly to environmental pollutants and (ii) males had the 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 and processed immediately using a Meckler counting chamber (Sefi-Medica, Haifa, Israel). The sperm parameters were analysed (sperm counts, percentage of total sperm motile and percentage of progressive motility) and classified into two groups (fertile & subfertile) according to World Health Organization (2010) guidelines (World Health Organization 2010). The somatic cells were removed from all semen samples through the use of a stringent somatic cell lysis protocol to ensure the absence of potential contamination from a presence of white blood cells or other somatic cells (Jenkins et al., 2016).

2.3 | Sperm DNA isolation and sodium bisulphite treatment

Sperm DNA was extracted using the Isolate II genomic DNA kit according to the standard protocol provided by the manufacturer (Bioline, UK). The concentration and purity of extracted DNA was 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 was 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 round cell contamination (Figure 1). Five hundred nanograms of extracted sperm DNA was treated with sodium bisulphite using the Epitect bisulphite conversion kit (Qiagen, Germany) that converts unmethylated cytosines to uracil, while 5-methylcytosine (5MeC) remains unaltered, as previously described (Wu, Kang, Zheng, Liu, & Liu, 2015).

2.4 | DNA methylation analysis by Infinium 450K BeadChip array

Thirty semen samples were used in the array screening phase with mean age of 39.0 ± 3.0, 15 samples from fertile males, who have had children "controls" and 15 from subfertile male "cases." After bisulphite

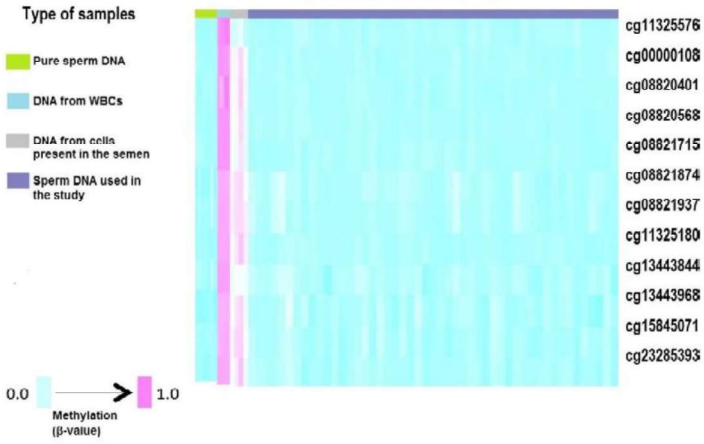

FIGURE 1 Heatmap showed the purity of sperm DNA according to the differentially methylated regions (DMR) between somatic cells (White blood cell) and spermatozoa

treatment, the DNA of these samples was subjected to Infinium 450K BeadChip arrays (Illumina, San Diego, CA, USA) following the manufacturer's recommendations (Bibikova et al., 2011), and the arrays were scanned using the Illumina iScan. β-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 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 (Assenov et al., 2014). 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 $p \leq .01$. 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.

2.5 | Bisulphite profiling (Bi-PROF)

In this work, and according to the results of the 450K analysis, we took five CpG sites and validated them using 78 samples, not including the screening phase samples, using local deep bisulphite sequencing (Bi-PROF; Gries et al., 2013). For bisulphite treatment using the Epitect bisulphite conversion kit (Qiagen, Germany), 500 ng genomic DNA was used. PCRs encompassing the differentially methylated CpGs identified by the 450K BeadChip array were performed in a 50 µl of total volume reaction using the "MyTaq™ HS Red Mix" with 2× 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

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

cgID	Chr-	nt (hg19)	AT (°C)	Product size (bp)	DMC	Primer sequence (5′–3′)
cg05813498	chr17	78,093,353	55	268	10	F: AGTTGGGTATTAATTATTTTAA
						R: AACTCAAATACATACCCCAC
cg06833981	chr6	31,597,708	54	206	3	F: TGATGATTTTTTTTTATGTGGATTT
						R: ACCCTTCCCTCATCACTATAA
cg07869343	chr16	1,797,050	52	334	22	F: GAGGTAAAGTGTAAAGTATT
						R: CTAAATAACCTATACTTCCA
cg02745784	chr2	178,970,365	52	398	1	F: AAAATGATGTAAAAATTAAAA
						R: TTTATATTCAAATCCATCCC
cg09785377	chr15	60,644,157	52	210	4	F: TGAGGAAAAATAATAAAGAGT
						R: CTAAACAATACCATTCAAAAC

DMC, Differentially methylated CpGs; Chr-, Chromosome; At, annealing temperature.

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) 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. Using BiSearch, placing primers onto common SNPs could be eliminated. PCR products (5 μl) were loaded on a 2% agarose gel stained with ethidium bromide, including the DNA ladder (Biolabs, NE). PCR products were purified using Agencourt® AMPure XP beads (Beckman Coulter, USA) and measured 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), excluding all reads containing ≥10% of missing CpG sites (maximal fraction of unrecognized sites ≥0.1). The obtained alignment sequence showed an absence of alterations at CpG positions.

2.6 | Statistical analysis

Data obtained from Bi-PROF was analysed using IBM SPSS for Windows software package version 23.0 (SPSS Inc., USA). Samples included in this study were non-normally distributed (nonparametric) according to the values of the skewness test, Kurtosis test, *Z*-value and Shapiro test. The independent-sample *t* test (Mann-Whitney test) was used to compare means of quantitative variables. In addition, the Spearman's test to assess the correlation coefficient between methylation level and other sperm parameters was used. The results in the above-mentioned procedures were accepted as statistically significant when $p \leq .05$.

3 | RESULTS

In the present study, a total of 108 samples were analysed. Of 108 samples, 30 samples were analysed by Infinium 450K BeadChip screening phase and 78 samples by local deep bisulphite sequencing. Table 2 summarises the location of the most differentially methylated CpGs (DMC), based on the mean DNA methylation difference of ≥20% between subfertile males (cases) and the fertile males (controls) group. We found only seven CpG positions following the applied criteria. From the results of the Infinium 450K BeadChip, the first five CpG sites were selected to validate the observed methylation difference in 78 samples (Table 4). Each one of these CpGs was located in gene bodies and CpG islands, and these genes were found to be related to male fertility, sperm count and sperm function. The age of the cohort study population was between 23.0 and 45.0 years, with a mean age of 38.6 ± 6.4 years, and the descriptive characteristics of the study population are illustrated in Table 3. The study population divided

TABLE 2 CpG dinucleotides that differ in their methylation levels for sperm DNA between cases (subfertile males) and controls (proven fertile) (n = 30)

cgID	Chro-	Start	Strand	Mean controls	Mean cases	Mean diff	Diff. meth. *p* value	Annotation
cg05813498	chr17	78,093,353	+	0.82977585	0.144405208	0.6853706	4.15861E−08	*GAA*
cg06833981	chr6	31,597,708	+	0.92413393	0.52358587	0.4005481	3.17235E−07	*PRRC2A*
cg07869343	chr16	1,797,050	+	0.93119657	0.491337679	0.4398589	6.70002E−07	*MAPK8IP3*
cg02745784	chr2	178,970,365	−	0.87580085	0.539144131	0.3366567	5.26616E−06	*PDE11A*
cg09785377	chr15	60,644,157	+	0.90725785	0.573759696	0.3334982	1.19285E−05	*ANXA2*
cg22963027	chr19	49,527,060	+	0.39539823	0.602667125	−0.20727	4.03738E−07	*CGβ*
cg19169023	chr15	41,853,346	+	0.9258933	0.552754264	0.373139	8.3467E−07	*TYRO3*

CpGs selected for Bi-PROF are highlighted.

TABLE 3 Descriptive characteristics for the study population (n = 78)

Variables	Mean	*SD*	Minimum	Maximum	Range
Age (years)	38.6	6.4	23.0	45.0	22.0
Sperm counts (mill/ml)	41.7	58.0	2.4	283.0	280.6
Percentage of total sperm motile	40.1	19.5	2.0	75.0	73.0
Percentage of progressive motility	24.2	14.7	0.0	55.0	55.0
Percentage of nonprogressive motility	15.9	9.9	0.0	45.0	45.0
Percentage of immotile spermatozoa	59.9	19.5	25.0	98.0	73.0

SD, Standard deviation.

into two groups (fertile and subfertile). The fertile group composed of 28 males with mean age 38.5 ± 5.6 years as "control," and the group composed of 50 subfertile males, with mean age 38.7 ± 6.9 years as "case." The sperm parameters that were investigated showed a significant decrease in the mean sperm count, the percentage of total sperm motility and the percentage of progressive motility in the case group compared to the control group ($p \leq .0001$, $p \leq .0001$, and $p \leq .0001$ respectively). Conversely, a significant increase in the percentage of immotile spermatozoa ($p \leq .0001$) in cases compared to controls (Table 4) was found. According to the results of local deep bisulphite sequencing, the study found that not only the target CpG obtained from the 450K Bead array experiments had a difference in the methylation level but also neighbouring CpGs (Table 5). The mean DNA methylation levels in all CpGs of the *PRRC2A* gene-related amplicon (CpG1, CpG2 and CpG3) showed high significant differences in the case group compared to the control group ($p \leq .0001$, $p \leq .003$, and $p \leq .0001$, respectively), and CpG1 related to *PDE11A* gene amplicon ($p \leq .0001$). Moreover, the results showed six CpGs of the ten CpGs tested related to the *GAA* amplicon (CpG2, CpG3, CpG6, CpG7, CpG9 and CpG10) had a significant difference in the mean methylation level ($p \leq .0001$, $p \leq .002$, $p \leq .001$, $p \leq .002$, $p \leq .0001$, and $p \leq .0001$, respectively) in cases compared to controls. Besides that three CpG from four CpGs related to *ANXA2* amplicon (CpG1, CpG3 and CpG4) showed a significant difference in the case group compared to the control group ($p \leq .002$, $p \leq .001$ and $p \leq .0001$ respectively). Furthermore, seven CpG from 22 CpGs related to the *MAPK8Ip3* amplicon (CpG5, CpG6, CpG7, CpG8, CpG12, CpG16 and CpG21) had a significant variance in the mean methylation level ($p \leq .004$, $p \leq .015$, $p \leq .002$, $p \leq .001$, $p \leq .005$, $p \leq .0001$ and $p \leq .008$, respectively) in cases compared to controls (Table 5).

Next, this study assessed the association between sperm DNA methylation levels obtained by Bi-PROF in different CpGs and other sperm parameters for the case group (Table 6). A significant correlation was found between the mean methylation levels in CpG1, CpG3 and CpG5 of the *GAA* gene-related amplicon and percentage of total sperm motile ($p \leq .013$, $p \leq .021$ and $p \leq .007$ respectively). Moreover, a significant correlation was found between the mean methylation levels of four CpGs related to *GAA* gene amplicon (CpG1, CpG2, CpG6 and CpG9) and the sperm count ($p \leq .049$, $p \leq .035$, $p \leq .002$ and $p \leq .018$ respectively). Likewise, a significant correlation was found between the methylation of three CpGs from twenty-two CpGs related to the *MAPK8Ip3* gene amplicon (CpG9, CpG14 and CpG22) and the percentage of progressive motility ($p \leq .022$, $p \leq .041$ and $p \leq .009$ respectively). The significant correlation was also found between the sperm count and methylation levels in CpG20 and CpG22 of the *MAPK8Ip3* gene-related amplicon ($p \leq .001$ and $p \leq .005$ respectively).

TABLE 4 Descriptive characteristics in cases compared to controls ($n = 78$)

Variables	Controls ($n = 28$)		Cases ($n = 50$)		
	Mean	*SD*	Mean	*SD*	*p* value
Age (years)	38.5	5.6	38.7	6.9	.60[a]
Sperm counts (mill/ml)	87.6	76.1	16.1	15.2	≤.0001[a]
Percentage of total sperm motile	54.9	12.3	31.8	17.8	≤.0001[a]
Percentage of progressive motility	40.3	7.2	15.2	9.1	≤.0001[a]
Percentage of nonprogressive motility	14.7	9.5	16.5	10.2	.38[a]
Percentage of immotile spermatozoa	45.0	12.3	68.3	17.8	≤.0001[a]

SD, standard deviation.
$p > .05$: not significant.
$p \leq .05$: significant.
[a]Mann-Whitney test.

4 | DISCUSSION

In several studies to date, the sperm DNA methylation profiles of specific genes from fertile and infertile human males have been compared (Heyn et al., 2012; Pacheco et al., 2011; Poplinski, Tüttelmann, Kanber, Horsthemke, & Gromoll, 2010). However, these studies limited to imprinted regions, or estimated a larger number of genes but focused only on promoter regions. What is more, only infertile patients presenting alterations connected with the process of spermatogenesis were studied. In contrast, in this study, we conducted a genomewide DNA methylation analysis to identify alterations in sperm DNA methylation between fertile male, who have had children and subfertile male, and according to the results of a genomewide DNA methylation analysis, we validated the following CpGs (cg05813498, cg06833981, cg07869343, cg02745784 and cg09785377) on 78 samples with mean age 38.6 ± 6.4 years. It is worth mentioning that the results of this study showed previously unidentified changes in DNA methylation of CpG sites located at specific genes and genomic regions.

1. cg05813498: located in the exon 21 of the *GAA* gene, located within the DNase I hypersensitivity cluster and the transcription factor binding site motif of CTCF (ENCODE Project Consortium, 2012).
2. cg06833981: located in the intron 12 of the *PRRC2A* gene, located within the transcription factor binding sites of POLR2A that plays a critical role in the synthesising messenger RNA (ENCODE Project Consortium, 2012).
3. cg07869343: located in the exon 5 of the *MAPK8IP3* gene, located within the transcription factor binding sites of (i) TEAP2C that plays a role in the early development specifically morphogenesis, (ii) TEAP2A which may activate the transcription of some genes while inhibiting the transcription of others, (iii) POLR2A that plays a role in the synthesis messenger RNA and (iv) MYC which plays a role in cell cycle progression, apoptosis and cellular transformation (ENCODE Project Consortium, 2012).
4. cg02745784: located in the intron 1 of the *PDE11A* gene, located within the DNase I hypersensitivity cluster and the transcription

TABLE 5 Methylation levels in the DMCs obtained from local deep bisulphite sequencing results in cases compared to controls (n = 78)

	Controls (n = 28)		Cases (n = 50)		
DMC	Mean	*SD*	Mean	*SD*	*p* value
Methylation level at CpGs in *GAA* gene-related amplicon					
CpG1	0.97	0.02	0.97	0.02	.892[a]
CpG2	0.97	0.03	0.91	0.10	≤.0001[a]
CpG3	0.97	0.02	0.88	0.13	≤.002[a]
CpG4	0.96	0.04	0.96	0.03	.909[a]
CpG5	0.91	0.05	0.90	0.04	.370[a]
CpG6	0.97	0.02	0.87	0.15	≤.001[a]
CpG7	0.86	0.17	0.67	0.25	≤.002[a]
CpG8	0.96	0.03	0.96	0.02	.700[a]
CpG9	0.97	0.02	0.88	0.14	≤.0001[a]
CpG10	0.94	0.04	0.87	0.10	≤.0001[a]
Methylation level at CpGs in *PRRC2A* gene-related amplicon					
CpG1	0.98	0.01	0.90	0.13	≤.0001[a]
CpG2	0.97	0.02	0.88	0.14	≤.003[a]
CpG3	0.97	0.03	0.87	0.16	≤.0001[a]
Methylation level at CpGs in *PDE11A* gene-related amplicon					
CpG1	0.98	0.03	0.89	0.15	≤.0001[a]
Methylation level at CpGs in *ANXA2* gene-related amplicon					
CpG1	0.98	0.01	0.88	0.14	≤.002[a]
CpG2	0.70	0.25	0.63	0.34	.662[a]
CpG3	0.81	0.17	0.58	0.31	≤.001[a]
CpG4	0.97	0.01	0.88	0.14	≤.0001[a]
Methylation level at CpGs in *MAPK8Ip3* gene-related amplicon					
CpG1	0.98	0.03	0.99	0.01	.925[a]
CpG2	0.98	0.02	0.98	0.02	.770[a]
CpG3	0.98	0.02	0.98	0.02	.653[a]
CpG4	0.98	0.03	0.99	0.01	.933[a]
CpG5	0.98	0.02	0.89	0.14	≤.004[a]
CpG6	0.98	0.02	0.86	0.16	≤.015[a]
CpG7	0.96	0.03	0.83	0.19	≤.002[a]
CpG8	0.96	0.02	0.87	0.14	≤.001[a]
CpG9	0.98	0.02	0.98	0.01	.478[a]
CpG10	0.97	0.03	0.97	0.02	.934[a]
CpG11	0.98	0.02	0.99	0.01	.810[a]
CpG12	0.98	0.02	0.92	0.10	≤.005[a]
CpG13	0.97	0.03	0.97	0.02	.933[a]
CpG14	0.98	0.02	0.98	0.03	.958[a]
CpG15	0.97	0.03	0.98	0.02	.835[a]
CpG16	0.97	0.03	0.93	0.06	≤.0001[a]
CpG17	0.97	0.04	0.98	0.02	.661[a]
CpG18	0.99	0.01	0.99	0.01	.543[a]
CpG19	0.98	0.02	0.98	0.02	.917[a]
CpG20	0.98	0.03	0.92	0.12	.311[a]
CpG21	0.99	0.01	0.91	0.13	≤.008[a]
CpG22	0.98	0.02	0.98	0.02	.502[a]

DMC, differentially methylated CpGs; *SD*, standard deviation.
$p > 0.05$: not significant.
$p \leq .05$: significant.
[a]Mann-Whitney test.

factor binding site TAL1 that plays a role in the regulation of transcription from RNA polymerase II promoter (ENCODE Project Consortium, 2012).

5. cg09785377: located in intron 10 of the *ANXA2* gene and located within the transcription factor binding sites of POLR2A (ENCODE Project Consortium, 2012).

The results obtained from local deep sequencing showed a significant difference between the case and control groups in the methylation levels of all CpGs in the *PRRC2A* gene-related amplicon (CpG1, CpG2 and CpG3), and CpG1 in the *PDE11A* gene-related amplicon, besides a significant variation in more than CpGs in the *GAA*, *ANXA2* and *MAPK8Ip3* gene-related amplicon (Table 5). These changes in the sperm DNA methylation levels are in agreement with previous results that showed that the methylation levels in sperm DNA tend to be altered in subfertile males compared with control males (Boissonnas et al., 2010; Montjean et al., 2015; Sato et al., 2011), as the change in the DNA methylation levels was observed mostly in sperm DNA obtained from a subfertile male, which pointed out that low methylation levels would be indicative for effective spermatogenesis (El Hajj et al., 2011; Rajender et al., 2011). Therefore, it is reasonable to assume that alteration in the methylation levels of sperm DNA in these CpGs, may have influence on the semen parameters, and may be associated with defective spermatogenesis (Kalra & Molinaro, 2008); Tunc and Tremellen (2009) suggested that alterations in global DNA methylation were associated with spermatogenic efficiency and the semen quality of infertile patients. It is worth pointing that the glucosidase alpha acid, encoded by the glucosidase alpha acid gene (*GAA*), is essential for the degradation of glycogen to glucose in lysosomes and that any defects in this gene lead to glycogen storage disease II (Pompe's disease) (Raben, Nagaraju, Lee, & Plotz, 2000), whereas the lysosomes found in the spermatozoa, especially in the acrosome, contain protease and hyaluronidase along with acid phosphatase, which plays a role in the maturation of germ cells and fertilisation (provided by RefSeq, Jul 2008, NCBI website). Besides, it should also be noted that members of the PDE protein superfamily, that is, phosphodiesterase 11A (*PDE11A*), play important roles in the control of cyclic nucleotide signalling (Jäger et al., 2012). Other studies by Fawcett et al. (2000), Yuasa, Ohgaru, Asahina, and Omori (2001), Loughney, Taylor, and Florio (2005) and Makhlouf, Kshirsagar, and Niederberger (2006) reported that the expression of this gene was prominent in prostate and testes. Baxendale and Fraser (2005) founded that strong immunosignals

TABLE 6 Correlation between the methylation level in the DMCs obtained by Bi-PROF and the different parameters investigated for the case group (n = 50)

DMC	Sperm counts (mill/ml)	% of total sperm motile	% of immotile sperm	% of progressive motility	% of nonprogressive motility
Methylation level at CpGs in *GAA* gene-related amplicon					
CpG1					
r	−.280	.351	−.351	.264	.300
p value	≤.049	≤.013	≤.013	.064	≤.034
CpG2					
r	.299	−.019	.019	.040	−.080
p value	≤.035	.898	.898	.782	.581
CpG3					
r	.014	−.326	.326	−.224	−.424
p value	.922	≤.021	≤.021	.119	≤.002
CpG4					
r	.083	.210	−.210	.190	.251
p value	.566	.143	.143	.186	.079
CpG5					
r	−.138	.374	−.374	.365	.337
p value	.338	≤.007	≤.007	≤.009	≤.017
CpG6					
r	−.419	−.071	.071	−.127	−.056
p value	≤.002	.625	.625	.378	.698
CpG7					
r	.255	.026	−.026	.071	.098
p value	.074	.858	.858	.622	.500
CpG8					
r	.046	−.240	.240	−.286	−.173
p value	.750	.093	.093	≤.044	.230
CpG9					
r	.334	−.017	.017	.046	−.086
p value	≤.018	.909	.909	.750	.554
CpG10					
r	.013	.047	−.047	.003	.171
p value	.926	.745	.745	.983	.234
Methylation level at CpGs in *PRRC2A* gene-related amplicon					
CpG1					
r	.116	−.262	.262	−.183	−.231
p value	.422	.066	.066	.202	.106
CpG2					
r	.218	−.120	.120	.054	−.203
p value	.128	.406	.406	.711	.157
CpG3					
r	−.139	−.175	.175	−.179	−.190
p value	.334	.224	.224	.214	.186
Methylation level at CpGs in *PDE11A* gene-related amplicon					
CpG1					
r	.040	−.033	.033	−.002	−.122

(Continues)

TABLE 6 (Continued)

DMC	Sperm counts (mill/ml)	% of total sperm motile	% of immotile sperm	% of progressive motility	% of nonprogressive motility
p value	.782	.822	.822	.989	.397
Methylation level at CpGs in *ANXA2* gene-related amplicon					
CpG1					
r	.287	.078	−.078	.054	.110
p value	≤.043	.593	.593	.708	.448
CpG2					
r	−.012	−.098	.098	−.173	−.015
p value	.932	.497	.497	.228	.918
CpG3					
r	.001	−.239	.239	−.272	−.157
p value	.994	.095	.095	.056	.275
CpG4					
r	−.032	.065	−.065	.218	−.126
p value	.828	.653	.653	.128	.384
Methylation level at CpGs in *MAPK8Ip3* gene-related amplicon					
CpG1					
p value	−.015	−.129	.129	−.138	−.135
r	.919	.371	.371	.339	.350
CpG2					
p value	−.129	.073	−.073	.075	.142
r	.372	.614	.614	.603	.324
CpG3					
p value	−.021	−.003	.003	−.045	.075
r	.886	.981	.981	.758	.604
CpG4					
p value	−.146	−.157	.157	−.230	−.023
r	.310	.277	.277	.108	.876
CpG5					
p value	−.097	.174	−.174	.079	.314
r	.502	.226	.226	.583	≤.027
CpG6					
p value	−.136	−.230	.230	−.278	−.170
r	.345	.109	.109	.050	.238
CpG7					
p value	.124	.198	−.198	.264	.173
r	.393	.169	.169	.064	.229
CpG8					
p value	−.243	.015	−.015	.100	−.058
r	.089	.919	.919	.489	.689
CpG9					
p value	−.082	.303	−.303	.323	.273
r	.573	≤.032	≤.032	≤.022	.055
CpG10					
p value	.093	−.141	.141	−.115	−.129

(Continues)

TABLE 6 (Continued)

DMC	Sperm counts (mill/ml)	% of total sperm motile	% of immotile sperm	% of progressive motility	% of nonprogressive motility
r	.523	.330	.330	.427	.372
CpG11					
p value	−.102	.152	−.152	.042	.182
r	.480	.292	.292	.769	.205
CpG12					
p value	−.157	−.071	.071	−.120	−.020
r	.276	.623	.623	.408	.889
CpG13					
p value	−.199	−.195	.195	−.241	−.131
r	.166	.175	.175	.091	.364
CpG14					
p value	.206	−.214	.214	−.290	−.213
r	.152	.137	.137	≤.041	.138
CpG15					
p value	−.165	−.203	.203	−.270	−.095
r	.251	.157	.157	.057	.513
CpG16					
p value	.008	−.300	.300	−.179	−.434
r	.958	≤.034	≤.034	.214	≤.002
CpG17					
p value	.040	.118	−.118	.037	.206
r	.784	.414	.414	.798	.152
CpG18					
p value	−.123	−.085	.085	−.174	.051
r	.397	.556	.556	.227	.726
CpG19					
p value	.002	.195	−.195	.128	.134
r	.987	.175	.175	.376	.355
CpG20					
p value	−.471	.022	−.022	−.042	.083
r	≤.001	.880	.880	.770	.567
CpG21					
p value	−.027	−.169	.169	−.111	−.194
r	.852	.241	.241	.441	.177
CpG22					
p value	.389	−.256	.256	−.368	−.131
r	.005	.073	.073	≤.009	.363

DMC, differentially methylated CpGs; Spearman's test, *r*, Correlation Coefficient.
$p > .05$: not significant.
$p \leq .05$: significant.

were obtained for *PDE11A* in the acrosomal cap and flagellum of spermatozoa. In addition, the annexin A2 (*ANXA2*) gene encodes a member of the annexin family, a family of Ca^{2+}-regulated phospholipid-binding and membrane-binding proteins, that is found in the acrosome and flagellum of the sperm cells and could be implicated in different events that are known to be calcium dependent, such as flagellar motility, acrosome reaction and fertilisation (Skrahina, Piljić, & Schultz, 2008). Also the protein encoded by the mitogen-activated protein kinase 8 interacting protein 3 gene (*MAPK8IP3*) is one of *MAPK* family, which observed in the spermatozoa and was observed to be correlated with a direct or indirect function in human sperm capacitation (De Lamirande & Gagnon, 2002; Thompson et al., 2001). A highly significant difference was observed in the present

study between the case and control groups in the mean sperm count, percentage of total sperm motility, percentage of progressive motility and percentage of immotile sperm, and this is in line and supports the results found by Poplinski et al. (2010) and Nanassy and Carrell (2011).

On the other hand, the results exhibited a significant correlation between methylation level at more than one CpG in *GAA*, *MAPK8Ip3* gene-related amplicon and sperm count, and percentage of total sperm motility. In addition, a significant correlation was shown between the methylation levels at different CpGs in *GAA*, *MAPK8Ip3* gene-related amplicon and percentage of progressive motility (Table 6). These correlations agree and support the studies that showed a high correlation between sperm DNA methylation levels and sperm motility (Montjean et al., 2013), besides the supporting of study that found a correlation between changes in the methylation level of imprinted genes and oligospermia in subfertile males (Marques, Carvalho, Sousa, & Barros, 2004).

5 | CONCLUSIONS

In conclusion, the methylation levels at these CpGs were significantly different in subfertile males compared to fertile males, who have had children, in addition, an association was found between the alterations in methylation levels at these CpGs and sperm parameters. Therefore, more studies are needed to clarify the mechanisms relating to these alterations and to discover their significance and functional consequences for male infertility.

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CONFLICT OF INTEREST

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

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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 500g 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 500g). 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 manufacturers 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) Mean	SD	Current smokers (n = 36) Mean	SD	p value
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 ($P \leq 0.003$, $P \leq 0.029$, and $P \leq 0.018$, respectively). Besides, two out of four CpGs in TKR gene-related amplicon showed a significant difference in the methylation level (CpG2, $P \leq 0.033$, and CpG4, $P \leq 0.0001$) 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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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**

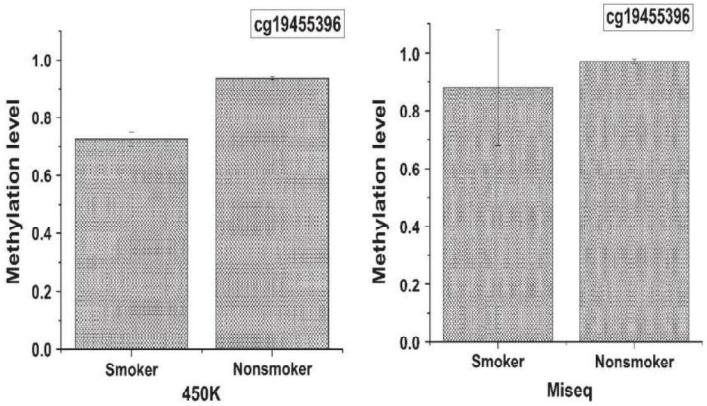

FIGURE 1 DNA methylation level at cg19455396 in smoker versus nonsmoker groups. Left: 450K BeadChip results, right: local deep bisulphite sequencing results

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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4 Discussion

In this thesis, we conducted a genome-wide DNA methylation analysis to identify alterations in sperm DNA methylation patterns between proven fertile males and males suffering from subfertile problems, and then we determined the association between changes in sperm DNA methylation levels and semen parameters. The DNA methylation in germline-related genes plays a vital role in the proper spermatogenesis process (**Carrell, 2012; Bao & Bedford, 2016**), and various studies have found that the defects in spermatogenesis and reduction in the reproductive potential of males could be caused by abnormal methylation in genes expressed in the testes (**Poplinski *et al*., 2010; Grégoire *et al*., 2013; Niederberger, 2013; Jenkins *et al*., 2016a**). Furthermore, different studies have found a strong relationship between a change in the level of DNA methylation in spermatozoa and infertility problems in males (**Hammoud *et al*., 2010; Sato *et al*., 2011; Ramasamy *et al*., 2014; Urdinguio *et al*., 2015**). The results obtained from the first three articles which related to male subfertility problems was line with the study of Aston *et al* whose 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 and 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, these changes in the sperm DNA methylation levels are in agreement with previous results that showed that the methylation levels in sperm DNA tend to be altered in subfertile males compared with control males (**El Hajj *et al*., 2011; Montjean *et al*., 2013; Montjean *et al*.,2015**). Other previous studies reported that males suffering from a reduction in fecundity have a low level of sperm DNA methylation compared with control males (**Boissonnas *et al*., 2010; Ferfouri *et al*., 2013**). Furthermore, the correlations shown in this thesis between the methylation levels and semen parameters are consistent with the results of other authors who found a high correlation between sperm count, sperm motility, and sperm DNA methylation levels (**Marques *et al*., 2004; Kläver & Gromoll, 2014**). It is worth mentioning that, the following genes *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**). Besides that, the *MLPH* gene expressed with high level in the testes and contributes to the development of spermatid tail, through the intra-manchette transport (IMT) mechanism (**Fukuda *et al*., 2002; Kierszenbaum *et al*., 2003**). Furthermore, expression of the *SMC1β* gene is shown in the M phase of spermatogonia as a checkpoint gene (**Roy *et al*., 2010**), this, in turn, can result in spermatogonial apoptosis, meiotic arrest, or gametes with genomic instability, leading to either infertility or congenital malformations of the offspring (**Kurahashi *et al*., 2012**). As well as the *GAA* gene which plays a role in the maturation of germ cells and fertilization, also the *PDE11A* gene was found in the acrosomal cap and flagellum of spermatozoa (**Raben *et al*., 2000; Baxendale & Fraser, 2005**). In addition, the *ANXA2* is found in the acrosome and flagellum of the sperm cells and could be implicated in different events that are known to be calcium dependents, such as flagellar motility, acrosome reaction and fertilization (**Skrahina *et al*., 2008**).

On the other side, the results of the fourth manuscript indicated that cigarette smoking causes biochemical changes in the sperm DNA methylation in many differentially methylated CpG (DMC), which related to *MAPK8IP* and *TKR* gene amplicon in current smoker group compared to never smoke control group. Several of previous studies showed that the *MAPK8IP* gene is associated with human spermatozoa functions and sperm capacitation (**De Lamirande & Gagnon, 2002**). Besides that, *TKR* proteins play very important roles in controlling cell survival, proliferation and differentiation, spermatogenesis, and phagocytosis (**Manning *et al*., 2002; Hafizi & Dahlbäck, 2006; Xiong *et al*., 2008**). In general, the variation in methylation levels between cases and controls group in the fourth manuscript 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 (**Hammadeh *et al*., 2010; Hayes & Knaus, 2013; Afanas'ev, 2014**). Besides, the correlations those shown in the fourth manuscript are agreement with previous studies that found an association between alterations in the methylation patterns of sperm DNA methylation and sperm parameters of smoker's males (**El Hajj *et al*., 2011, Montjean *et al*., 2013**).

5 Conclusion

The manuscripts included in this thesis identified different CpGs related to the following genes *ALS2CR12, ALDH3B2, PRICKLE2, PTGIR KCNJ5, MLPH, SMC1β, GAA, PRRC2A, PDE11A, ANXA2,* and *MAPK8Ip3* with consistently altered methylation levels in sperm DNA from males with reduced fecundity. In addition, an association between changes in the methylation level at these CpGs and different semen parameters was found. In addition, 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. These data indicate that the identified CpGs play a crucial role in spermatogenesis and the change in the methylation levels has negatively affect semen parameters. So, these results will contribute to increasing the knowledge about sperm methylation process and subfertility problems. Furthermore, to understand the complex relationship between subfertility problems and abnormal DNA methylation in this genes, more studies are needed to clarify the mechanisms relating to these alterations and to discover their significance and functional consequences for male fecundity. Therefore, in this regard, the CpGs related to this gene may have future diagnostic value and shed more light on the molecular mechanisms of male infertility problems.

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