DEPARTMENT OF OBSTETRICS, GYNAECOLOGY

& REPRODUCTIVE MEDICINE

FACULTY OF MEDICINE OF SAARLAND UNIVERSITY

HOMBURG/ SAAR, GERMANY

Impact of Tobacco smoking on sperm nuclear proteins genes: H2BFWT, TNP1, TNP2, PRM1, and PRM2 and its influence on male infertility

Dissertation in partial fulfilment of the requirement for the award the degree of ***Doctor Rerum Medicinae (Dr. rer. Med.)***

Conferred by the Faculty of Medicine of the University of Saarland, Germany

2019

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Tag der Promotion:

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ABSTRACT

During fertilization, the spermatozoon task is not limited to just deliver the male genome to the oocyte, it is beyond that. During the last stages of spermatogenesis, the spermatozoa develop to have a highly organized genome. The sperm DNA is compacted by nearly 85% of protamines and 15 % of histones carrying epigenetic signals, together with different kind of RNA molecules and proteins participate in post-fertilization events and mainly in embryo development.

About 50% of infertility cases are referred to as idiopathic infertility, around 15% of it is due to genetic factors and 35% due to environmental factors. Tobacco smoke is one of the lifestyle factors that present a big threat for human health and it is believed to have an influence on male fertility and sperm quality by inducing epigenetic and/or genetic modulations on sperm genome. Thus, this study comes to fulfil a part in the remained gap in the comprehensive understanding of the expression and regulation of the genes in the human spermatozoa.

The purposes of this study were first, to find out the effect of Tobacco smoke on sperm quality determined by standard parameters (WHO, 2010), sperm DNA maturity tested by Chromomycin A3 (CMA3) staining, sperm DNA fragmentation tested by TUNEL assay, and clinic outcomes after ICSI therapy. Second, to quantify the transcript levels of five nuclear proteins genes: H2BFWT, TNP1, TNP2, PRM1, and PRM2 by RT-PCR, correlate them with the previous parameters and determine the smoking effect on this gene expression and regulation. Finally, to determine the single nucleotides polymorphisms in three genes: H2BFWT, PRM1, and PRM2 by Sanger sequencing and their association to smoking and previous parameters.

The study population (n=167) were male partners, randomly collected, of couples undergoing intracytoplasmic sperm injection (ICSI) therapy, in reproductive age (25-49 years). The patients were divided into two groups: heavy-smokers group and non-smokers group.

In heavy-smokers group a significant decrease ($p<0.01$) in standard semen parameters in comparison to non-smokers has been shown: sperm concentration (62.17 ± 51.68 mill/ml

vs. 88.09 ± 63.42 mill/ml), progressive motility (PR) (14.86 ± 10.95% vs. 27.31 ± 21.78%), and sperm normal morphology (4.01 ± 2.88% vs. 10.87 ± 12.11%).

Besides, the mean percentage of protamine deficiency (CMA3 positivity) and sperm DNA fragmentation (sDF) were significantly higher ($p<0.01$) in heavy-smokers than in non-smokers (33.30 ± 23.33% vs. 20.35 ± 13.43% and 26.86 ± 19.77% vs.14.23 ± 13.07% respectively). A significant positive correlation has been found between CMA3 positivity and sDF ($r=0.484$, $p=0.0001$) in the group of heavy-smokers, and no correlation ($r=0.256$, $p=0.098$) between these two parameters in the non-smokers group.

In the present study, by comparing the ICSI results between the heavy-smokers and non-smokers, the pregnancy rate was significantly higher in the group of non-smokers than the heavy-smokers group (0.60 ± 0.49% vs. 0.38 ± 0.48%, $p=0.013$), other parameters showed no significant differences.

Furthermore, the transcript level of each studied gene mRNA (mean delta Ct) was differentially expressed between the heavy-smokers and non-smokers groups and this difference was highly significant ($p<0.01$). H2BFWT, TNP1, TNP2, PRM1 and PRM2 genes were down-regulated in spermatozoa of heavy-smoker compared to non-smoker (Fold change<0.5) and were significantly correlated between each other ($p<0.01$). Moreover, the protamine mRNA ratio, in the current study, was significantly higher in the heavy-smokers group in comparison to the non-smokers group (0.60 ± 1.08 vs. 0.11 ± 0.84, $p=0.001$).

In the non-smokers group, protamine ratio correlated positively with the expression levels of TNP2 ($r=0.349$, $p=0.032$) and PRM2 ($r=0.488$, $p=0.001$). However, in the heavy-smokers group, it correlated positively to TNP2 transcript ($r=0.307$, $p=0.004$), PRM2 transcript ($r=0.445$, $p=0.0001$), and H2BFWT transcript ($r=0.342$, $p=0.001$). Only in the group of heavy-smokers, the protamine ratio significantly correlates with CMA3 positivity ($r=0.413$, $p=0.0001$) and sDF ($r=0.302$, $p=0.003$).

Moreover, the present study demonstrated the absence of a connection between genetic variations founded in H2BFWT gene (rs7885967, rs553509 and rs578953), protamines genes PRM1 (rs737008) and PRM2 (rs2070923 and rs1646022) and fertility alteration in heavy-smoker and non-smoker males.

In conclusion, the results of this study demonstrated that smoking has inverse effects on sperm quality, sperm DNA integrity, mRNA expression levels of H2BFWT, TNP1, TNP2, PRM1and PRM2 genes and protamine mRNA ratio, but has no effect on the nucleotides sequences of these genes. This suggests that the RNA of the studied genes and protamine mRNA ratio in the sperm of male partners of ICSI patients are good predictive factors to evaluate the sperm quality and its fertilizing capacity.

ZUSAMMENFASSUNG

Das Spermium hat während der Befruchtung mehrere Aufgaben. Zum einen muss das männliche Genom zur Eizelle. Des Weiteren entwickeln sich die Spermien in den letzten Stadien der Spermatogenese zu einem hoch organisierten Genom. Die Spermien-DNA wird aus Protaminen (ca. 85%) und Histonen (ca. 15%) gebildet, zusammen mit verschiedenen Arten von RNA-Molekülen und Proteinen. Diese sind vor allem an Prozessen nach der Fertilisation beteiligt und nehmen eine wichtige Rolle in der Embryonalentwicklung ein.

Etwa 50% der Fälle von Unfruchtbarkeit werden als idiopathische Unfruchtbarkeit bezeichnet, davon sind 15% auf genetische Faktoren und 35% auf Umweltfaktoren zurückzuführen. Tabakrauch ist einer der Lebensstilfaktoren, die eine große Bedrohung für die menschliche Gesundheit darstellen. Es wird angenommen, dass Rauchen Einfluss auf die männliche Fruchtbarkeit und Spermienqualität hat, indem es epigenetische und/oder genetische Modulationen auf das Spermiengenom induziert.

Ziel dieser Studie war es, zunächst die Wirkung von Tabakrauch auf die Spermienqualität zu ermitteln.Die Spermienqualität wurde bestimmt durch Standardparameter (WHO, 2010), die DNA-Integrität (gemessen mit Chromomycin A3 Färbung, CMA3) und die DNA-Fragmentierung (gemessen durch den TUNEL-Test). Des Weiteren wurde das klinische Outcome nach ICSI Therapie dokumentiert. Transkriptionswerte von fünf atomaren Proteingenen wurden ebenfalls quantifiziert: H2BFWT, TNP1, TNP2, PRM1 und PRM2 von RT-PCR.Es wurde untersucht, ob die Gene mit den bisherigen Parametern korrelieren und ob es einen Effekt des Rauchens auf die Genexpression, bzw. Genregulation gibt. Um die einzelne Nukleotid-Polymorphismen zu bestimmen, wurden 3 Gene (H2BFWT, PRM1 und PRM2) durch Sanger-Sequenzierung untersucht.Ihre Assoziation mit dem Rauchen, sowie mit den anderen Spermienparametern wurde ebenfalls gemessen.

Die Studienpopulation (n=167) waren männliche Probanden, die sich einer intrazytoplasmatischen Spermieninjektion (ICSI) unterzogen hatten. Das Alter betrug 25-49 Jahre. Die Patienten wurden in zwei Gruppen eingeteilt: Die Gruppe der schweren Raucher und die Nichtraucher-Gruppe.

Bei der Gruppe der Raucher wurde eine signifikante Abnahme ($p<0,01$) bei den Standardparametern im Vergleich zu Nichtrauchern gezeigt: Spermienkonzentration (62,17 ± 51,68 mill/ml vs. 88,09 ± 63,42 mill/ml), progressive Beweglichkeit (PR) (14,86 ± 10,95% vs. 27,31 ± 21,78%) und Spermienmit normaler Morphologie (4,01 ± 2,88% vs. 10,87 ± 12,11%) waren bei Rauchern im Vergleich zu den Nichtrauchern reduziert.

Außerdem war der Mittelanteil von Protamin-Mangel (CMA3-Positivität) und der DNA-Fragmentierung von Spermien (sDF) bei Rauchern signifikant höher ($p<0,01$) als bei Nichtrauchern (33,30 ± 23,33% vs. 20,35 ± 13,43% und 26,86 ± 19,77% vs. 14,23 ± 13,07%). Es wurde eine signifikante, positive Korrelation zwischen die CMA3-Positivität und sDF ($r=0,484$; $p=0,0001$) in der Gruppe der Raucher festgestellt. Dagegen gab es keine Korrelation ($r=0,256$; $p=0,098$) zwischen diesen beiden Parametern in der Nichtraucher-Gruppe.

In der vorliegenden Studie war die Schwangerschaftsrate nach ICSI in der Gruppe der Nichtraucher deutlich höher als die der Raucher-Gruppe (0,60 ± 0,49% vs 0,38 ± 0,48%; $p=0,013$).

Darüber hinaus wurde die Transkriptionsstufe jedes untersuchten Gens mRNA (mean delta ct) zwischen den Rauchern und Nichtrauchern differenziert ausgedrückt.Dieser Unterschied war signifikant ($p<0,01$). Die Gene H2BFWT, TNP1, TNP2, PRM1 und PRM2 wurden in Spermien der Raucher im Vergleich zum Nichtraucher (Foldchange<0,5) herunterreguliert und signifikant miteinander korreliert ($p<0,01$). Zudem lag das Protamin-MRNA-Verhältnis in der Raucher-Gruppe deutlich höher (0,11 ± 0,84 vs. 0,60 ± 1,08, $p=0,001$).

In der Gruppe der Nichtraucher korrelierte das Protaminsverhältnis positiv mit den Ausdruckstufen TNP2 ($r=0,349$; $p=0,032$) und PRM2 ($r=0,488$; $p=0,001$). In der Gruppe der Raucher korrelierte es jedoch positiv mit TNP2-Transkript ($r=0,307$; $p=0,004$), PRM2-Transkript ($r=0,445$; $p=0,0001$) und H2BFWT-Transkript ($r=0,342$; $p=0,001$). Nur in der Gruppe der Raucher korreliert das Protaminsverhältnis signifikant mit CMA3-Positivität ($r=0,413$; $p=0,0001$) und sDF ($r=0,302$; $p=0,003$).

Darüber hinaus hat die vorliegende Studie gezeigt, dass es keinen Zusammenhang zwischen genetischen Variationen gibt, die im H2BFWT-Gen (rs7885967, rs553509 und rs578953), Protaminen-Gen PRM1 (rs737008) und PRM2 (rs2070923 und rs1646022) vorkommen.

Zusammenfassend haben die Ergebnisse dieser Studie gezeigt, dass Rauchen einen negativen Einfluss auf die Spermienqualität hat.Die Spermienqualität, die mRNA-Expressionsstufen der H2BFWT, TNP1, TNP2, PRM1-und PRM2-Gene und das Protamin-mRNA-Verhältnis waren bei Nichtrauchern reduziert, hatten aber keine Auswirkungen auf die Nukleotid-Sequenzen dieser Gene. Dies deutet darauf hin, dass die RNA der untersuchten Gene, sowie das Protamin-mRNA-Verhältnis in den Spermien männlicher Partner von ICSI-Patienten gute Vorhersage-Faktoren sind, um die Spermienqualität und ihre Fertilität zu bewerten.

LIST OF ABBREVIATIONS

8-OhdG	8-Hydroxydeoxyguanosine
AA	Amino acid
AR	Androgen receptor
Arg	Arginine
ART	Assisted Reproductive Technique
Bp	Base pair
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary Deoxyribonucleic acid
Chk1	Checkpoint kinase 1
CMA3	Chromomycin A3
CMA3+	CMA3 positivity
CNVs	Copy number variations
CpG/CG	Dinucleotides Cytosine-Guanine
CRE	cAMP-response elements
CT	Cycle Threshold
CYP1A1	Cytochrome P450, a family 1, subfamily A polypeptide 1
Cys	Cysteine
DFI	DNA fragmentation index
DNA	Deoxyribonucleic acid
DNMT1	DNA Methyltransferase 1
DSBs	double strand breaks DNA
ESR1	Estrogen receptor 1
ESR2	Estrogen receptor 2
FSH	Follicle-stimulating hormone
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GnRH	Gonadotrophin-releasing hormone

GSTs	Glutathione S-transferases
H1	Linker histone H1
H1.T	Histone H1-like protein
H1.t	Testis-specific histone H1
H1.T2	Histone H1-like protein 2
H2A	Histone H2A
H2A.Bbd	H2A Barr body-deficient
H2B	Histone H2B
H2BFWT	H2B histone family, member W, testis-specific
H3	Histone H3
H4	Histone H4
HILS1	Spermatid-specific linker histone H1-like protein
His	Histidine
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
ID	Identifier
Indels	Insertions/deletions
IUI	Intrauterine Insemination
IVF	In Vitro Fertilization
kDa	Kilodalton
LH	Luteinizing hormone
MARs	Matrix attachment regions
MDA	Malondialdehyde
Mg	Microgram
Min	Minute
Mir	microRNA
miRNA	MicroRNA

miRNA	mitochondrial RNA
Ml	Millilitre
mRNA	Messenger Ribonucleic Acid
MTHFR	Methylenetetrahydrofolate reductase
NAT2	N-acetyltransferase-2 gene
ND	Nanodrop
NOS3	Nitric oxide synthase 3
NP	Non-progressive motility
P1	Protamine 1
P2	Protamine 2
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PR	Progressive motility
PR + NP	Total motility
PRM1	Protamine 1 gene
PRM2	Protamine 1 gene
PTM	Postranslational modifications
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
RNPs	Ribonucleoproteins
ROS	Reactive Oxygen species
Rpm	Rounds Per minute
rRNA	Ribosomal ribonucleic acid
RT	Room Temperature
RT-qPCR	Quantitative reverse transcription-Polymerase chain reaction
S	Second
SAGE	Serial Analysis of Gene Expression

SCB	Sertoli cell barrier
SCSA	Sperm chromatin structure assay
SD	Standard deviation
sDF	Sperm DNA fragmentation
SH	Thiol group
snc-RNAs	small non-coding RNAs
SNPs	Single nucleotides polymorphisms
SNVs	single nucleotide variations
SpcI	Spermatocytes type I
SpcII	Spermatocytes type II
SPO11-1	Meiotic recombination protein
SREs	Sperm RNA elements
S-S	Disulfide bridges
SSBs	Single strand breaks
TE	Trophectoderm Epithelium
TH1	A testis-specific variant of histone H1
TH2A	A testis-specific variant of histone H2A
TH2B	A testis-specific variant of histone H2B
TH3	The testis-specific variant of histone H3
TNP1	Transition protein 1 gene
TNP2	Transition protein 2 gene
Topo II	Type II DNA topoisomerase
TP1	Transition protein 1
TP2	Transition protein 2
TSH2B	Testis-specific histone 2B
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UV	Ultraviole**t**

V	Volt
VNTRs	Variable number tandem repeats
WHO	World Health Organization

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1. INTRODUCTION

Infertility is a big problem that many couples worldwide have to face. In fact, it is affecting nearly 15% of the couples who, according to the World Health Organization (WHO, 2010), fail to geta clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (WHO, 2010).

A population based-study carried out by Agarwal *et al*. (2015) to estimate male infertility distribution around the world, has shown that the male factor distribution in the infertility was between 20% and 70% and the proportion of infertile men was on the 2.5% - 12% range.

Infertility or subfertility is related to health problems that can be the consequence of many factors. 50% of cases are referred to as idiopathic infertility and around 15% of these cases are due to genetic factors that include chromosomal aneuploidy, chromosome microdeletions, structural and numerical Karyotype anomalies (Harton & Tempest, 2012), chromosome-linked copy number variations (CNVs), variable number tandem repeats (VNTRs) and single nucleotide polymorphisms (SNPs) associated with genes with an endocrine function (ESR1, ESR2, AR, etc.), specific function in spermatogenesis (PRM1, PRM2, H2BFWT, etc.) or ordinary cell function (CYP1A1, MTHFR, NOS3, etc.) (Krausz *et al*., 2015).

In other idiopathic male infertility cases, environmental and lifestyle factors, such as nutrition, drinking alcohol, physical activity and tobacco smoke, play an important role in the aggravation of infertility problems.

In fact, external exposure to toxicants leads to different alterations during the various phases of spermatogenesis (mitotic, meiotic and post-meiotic) (Beal *et al*., 2017). As a matter of fact, it has been confirmed that male fertility impairment is growing and that this might be associated with environmental factors and lifestyles. In this study, we have focused on tobacco smoke and its effect on the spermatozoa structure, the function nuclear protein gene polymorphism and the sperm potential to fertilize the oocyte by analysing semen samples of heavy-smoker and non-smoker patients undergoing intracytoplasmic sperm injection (ICSI) therapy.

1.1. Sperm chromatin organization

It took 50 years to discover the two major components of the sperm chromatin: DNA and protamine. The beginning of this research was in 1865 when Gregor Mendel established the laws of heredity (Mendel, 1865).

Four years later, Friedrich Miescher began his research about the cells and their chemical composition by isolating the proteins and discovered an acid-insoluble structure that he called the Nuclei (Miescher, 1869). Later on, the nucleus accumulated from the human cells was not enough for his study, so he decided to use salmon sperm cells instead. At this time, he isolated a highly basic protein, which he called protamine. Then, he reported that the major structure of the spermatozoa head was DNA and protamine and that they were attached in a salt-like state (Miescher, 1874).The most research on the protamine molecule was carried out by Kossel and his team for almost 3 decades (Kossel, 1898; Kossel & Dakin, 1904; Kossel & Dakin, 1905; Kossel & Edlbacher, 1913).

1.2. The spermatogenesis

Sexual reproduction is a type of reproduction where two haploid cells called spermatozoon and oocyte fuse together. These cells are obtained through a series of cell phases and divisions including nuclei and chromatin packaging and function changes in a differentiation process called spermatogenesis starting from diploid somatic cells called spermatogonium. This process is the main key to have successful fertilization. It includes several series of cellular, chromatin structural and functional modifications (**Figure 1**).

Spermatogenesis begins with differentiation via the mitosis of spermatogonial cells into primary spermatocytes. Then, the diploid spermatocytes (44 chromosomes, XX or XY) undergo a meiotic phase (meiosis I and meiosis II) to produce haploid spermatids containing only one copy of each chromosome (22, X or Y). Later, the spermatid undergoes a series of morphological changes (Head, midpiece and tail) and their chromatin structure and function change (Balhorn, 2018).

In fact, there are imprinted genes in the male genome, epigenetic changes in the DNA and nucleoproteins that edit the chromatin to make it ready to control the embryonic growth and

development (Canovas & Ross, 2016) and step-by-step the chromatin will be genetically silenced in the spermatozoa (Ren *et al*., 2017).

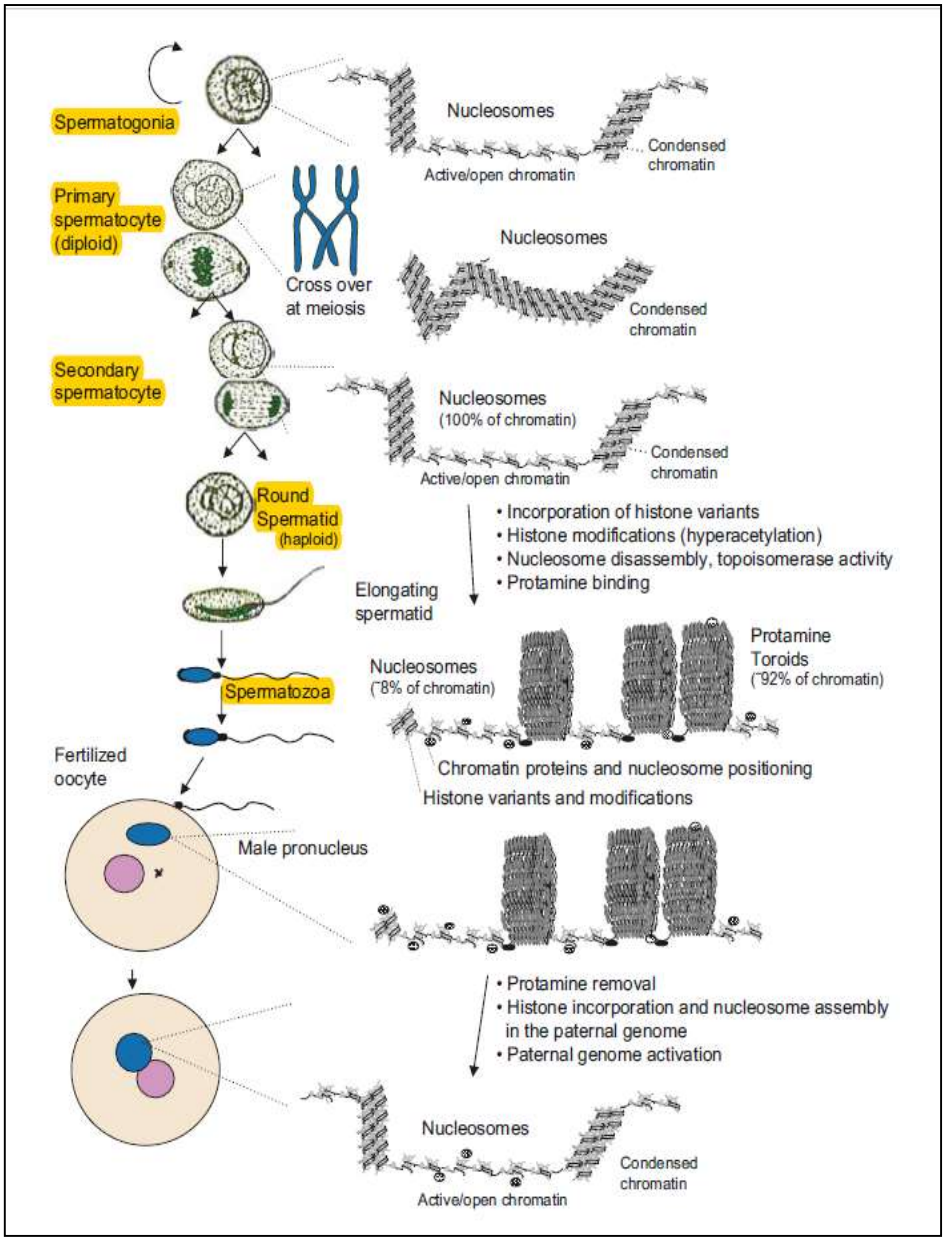

Figure 1: Themajor events in the life of sperm during spermatogenesis and fertilization; Cellular changes (left) and chromatin changes (right). [Adapted from Barrachina *et al*., 2018]

Spermatogenesis is controlled through several hormones. The first control is through a neurological pathway; the gonadotrophin-releasing hormone (GnRH) secreted by the hypothalamus provokes the adenohypophysis to excrete the follicle-stimulating hormone

(FSH) and luteinizing hormone (LH). The LH provokes the Leydig cells to produce the Testosterone, and the FSH assists the Sertoli cells to support the spermatozoa during the different phases of spermatogenesis. Beside FSH and LH there are other hormones, such as prolactin and the growth hormone, which play crucial roles during spermatogenesis (Sharma & Agarwal, 2011).

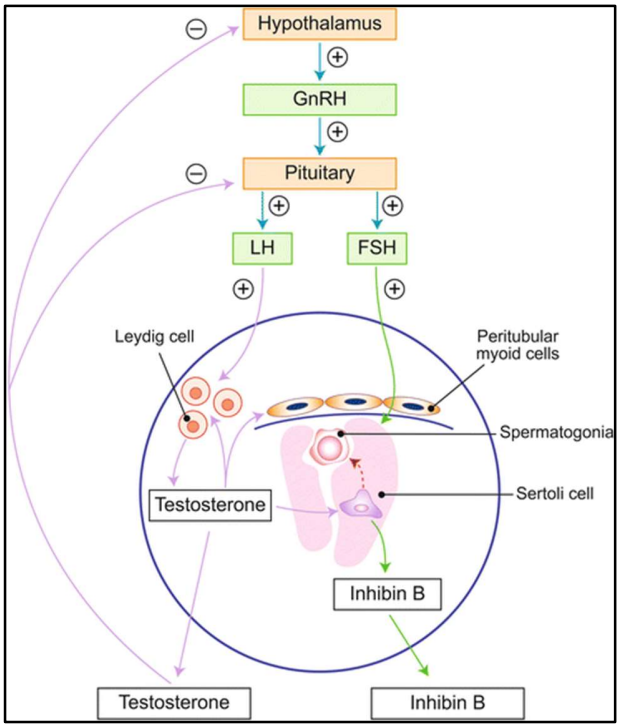

Figure 2: Gonadotropin and steroid hormone control of spermatogenesis. [Adapted from Mitchell *et al.*, 2017]

1.3. The chromatin remodelling during spermiogenesis

In order to obtain a hydrodynamic sperm head and to protect the paternal genome from any modifications in the male orfemale reproductive tracts, the human sperm DNA, in early spermiogenic phases, undergoes major cellular and nuclear changes (Ward & Coffey, 1991).

First, the spermatid undergoes the Golgi phase, which is marked by the formation of polarity. The Golgi apparatus, which evolves later on the acrosome and synthesized proteolytic enzyme, appears in the head. Mitochondria accumulate in the midpiece and the axoneme starts to form the distal centriole. Next, one of the centriole pair elongates to form the tail or flagellum (Russell *et al*., 1993).

Besides, the chromatin in the elongated nucleus becomes ten times more compact than the chromatin in the nucleus of a somatic cell through progressive modifications (Braun, 2001) (**Figure 3**).

So, in early spermiogenic phases, major chromatin packaging takes place. The nucleosome-bound DNA configuration will first be destabilized by hyperacetylation of the canonical histones, which will neutralize the positive charge of lysine, reducing their affinity for DNA and by the DNA topoisomerase II (topo II), which will cause double and single DNA strand breaks to reduce the tension of the DNA (McPherson & Longo, 1993; Laberge & Boissonneault, 2005).

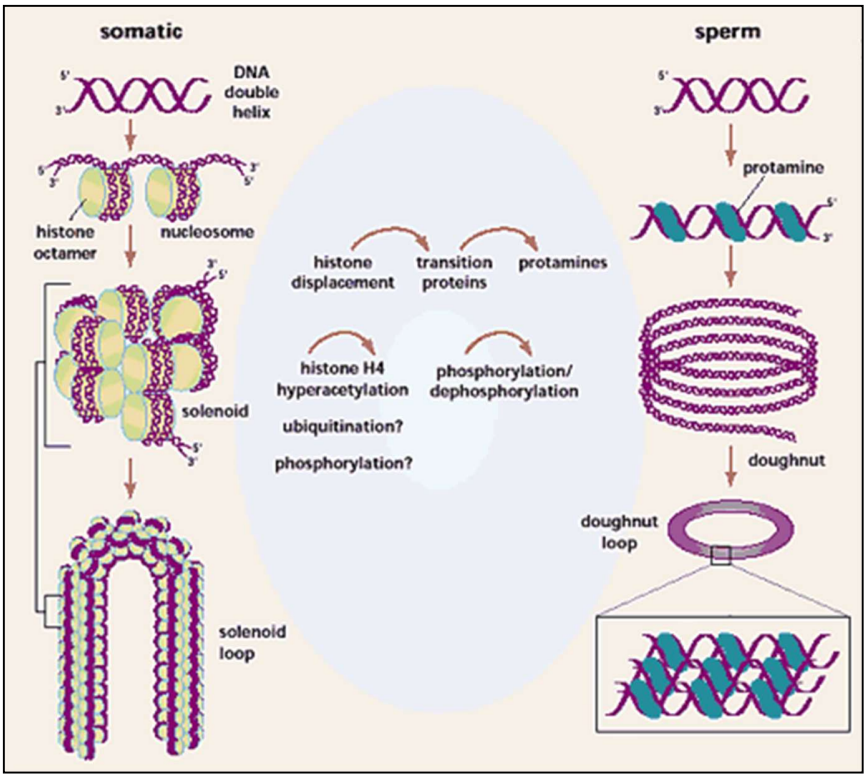

Figure 3: A representation of the difference in the chromatin packaging between a somatic cell (left) and a sperm (right). The chromatin is converted from a nucleo-histone structure (solenoid loop) into a nucleo-protamine structure (Toroid: doughnut loop). Post-translational modifications of the proteins facilitated the transition histone-protamine: acetylation, ubiquitination and phosphorylation of histone H4, phosphorylation and dephosphorylation of the transition proteins. [Adapted from Braun, 2001].

The "canonical histones" are composed of the core histones (H2B, H2A, H3, and H4) and the linker histone (H1), will be replaced by their testis-specific histone counterparts. These histones are called "histones variants" and start to appear during different stages of spermatozoon formation and they are found in lower quantities during the cell cycle (Cheema & Ausió, 2015) (**Figure 4**). Among these histones, there are H2A variants which are major players like H2A.Bbd (González-Romero *et al*., 2008), H2B variants like H2BFWT (Churikov *et al*., 2004a), H3 variants like H3.T (Witt *et al*., 1996), and H1 variants like H1.T and H1.T2 (Tanaka *et al*., 2005). Some of these histone variants are present only in masculine germ cells (Talbert & Hanikoff, 2010).

Churikov *et al.* (2004b) demonstrated that the TH3 histone variant is shown in spermatogonia, TH2A and TH2B, prior to meiosis, combined with the chromatin of spermatocytes. However, TH1 or H1.t is found at the end of the meiotic prophase.

Moreover, there are posttranslational modifications (PTM) of histones that are also important for the good progress of the spermiogenesis (Carrell, 2012; Godmann *et al*., 2007). During the elongated spermatids phase, the H3 and H4 are acetylated and consequently, the interactions between these histones and the sperm DNA are relaxed, especially in the regulatory regions of genes which contribute to embryonic development (Nair *et al*., 2008; Sonnack *et al*., 2002). The H4 methylation increases during the differentiation of the spermatogonia to spermatid (Luense *et al*., 2016), but decreases in the elongated spermatids (Sonnack *et al*., 2002). H3 histone is methylated in the round spermatids and this methylation has been demonstrated to mark the regulatory sites on evolutionary repressed genes, which are important for both gamete differentiation and embryo development (Godmann *et al*., 2007; Hammoud *et al.,* 2009; Khalil *et al*., 2004).

It has been demonstrated that 10-15% of canonical histones and their variants remains bound to DNA in the mature human spermatozoa (Bench *et al.,* 1996; Gatewood *et al*., 1990; Wykes & Krawetz, 2004). It concerns mainly the transcription sites of genes in sperms that are important for the preservation of the paternal genome epigenetics for their later expression during early embryonic development (Carrell & Hammoud, 2009; Ihara *et al*., 2014). The regulatory sequences (Brykczynska *et al.,* 2010; Castillo *et al*., 2014), microRNA clusters, transcription factors, paternally imprinted genes (Hammoud *et al*., 2009), the centromeric and telomeric DNA (Zalenskaya & Zalensky, 2004), retroposons (Pittoggi *et al*., 1999), matrix associated regions (Ward, 2009), and genes that produce rRNA are transcribed at the final stages of spermatogenesis (Sillaste *et al*., 2017).

After spermatocytogenesis, the chromatin structural changes will be more obvious when two smaller more basic proteins (10-20% lysine and arginine) named "Transition proteins" TP1 and TP2 are synthesized and deposed at the mid-stage of spermatid formation (**Figure 4**).

TP2 (13kDa) appears in step 1 and TP1 (6.2kDa) appears in step3 (Steger *et al*., 1998). At this time the majority of the core histones are eliminated and the chromatin structure becomes more condensed.

Pradeepa & Rao (2007) have reported that TP1 plays the main role in the destabilization of the nucleosome structure and the initiation of gene transcription termination when TP2 is

attached to CG rich sequence by zinc fingers. Also, it is important for the chromatin condensation progress (Zhao *et al.,* 2001). It has also been shown that TP1 facilitates the repair mechanism of the DNA strand alterations (Caron *et al*., 2001).

As their name indicates, these proteins stay only for a short period of time attached to the DNA. Therefore, they are modified in arginine and lysine residues by methylation, acetylation, and phosphorylation to lose their ability of attachment to the sperm DNA (Nikhil *et al*., 2015). Moreover, the transition proteins have been shown to be important not only for the chromatin condensation procedure but also for the DNA damage repair caused during the histones replacement (Boissonneault, 2002). The TPs are then replaced by a set of small (5-8 kDa) highly basic proteins called "protamines" in the late spermatid stage (**Figure 4**). The sperm protamines 1 and 2 are encoded each by a single gene (PRM1 for P1 and PRM2 for P2) located in a cluster of genes beside the TNP2 gene.

They are located on chromosome 16 (16p13.13) (Oliva, 2006; Martins & Krawetz, 2007). The mRNA of these proteins is silenced and translated after gradual modulation of sperm nuclear proteins, histones to transition proteins. This step is important to ensure a good process of chromatin repackaging (Chromatin decondensation) after the intrusion or injection of a spermatozoon into the cytoplasm of the oocyte (Hecht, 1989; Kleene & Flynn, 1987; Lee *et al*., 1995).

The sperm protamine 1 (P1) (51 AA) is the first to be synthesized as a mature protein (Green *et al*., 1994; Queralt *et al*., 1995). The protamine 2 is created as a precursor which is twice the size of P1 (101 residues) and undergoes cleavage by proteolysis after its deposition onto sperm DNA in order to eliminate short fragments of the peptide (Martinage *et al*., 1990; Green *et al.,* 1994). These proteins are reported to be expressed in equal quantities (P1/P2 ratio ≈ 1) (Corzett *et al*., 2002; Aoki *et al*., 2005). Nanassy *et al.* (2011) suggested a clinical value of the protamine ratio of between 0.54 and 1.43 for a fertile, normozoospermic man.

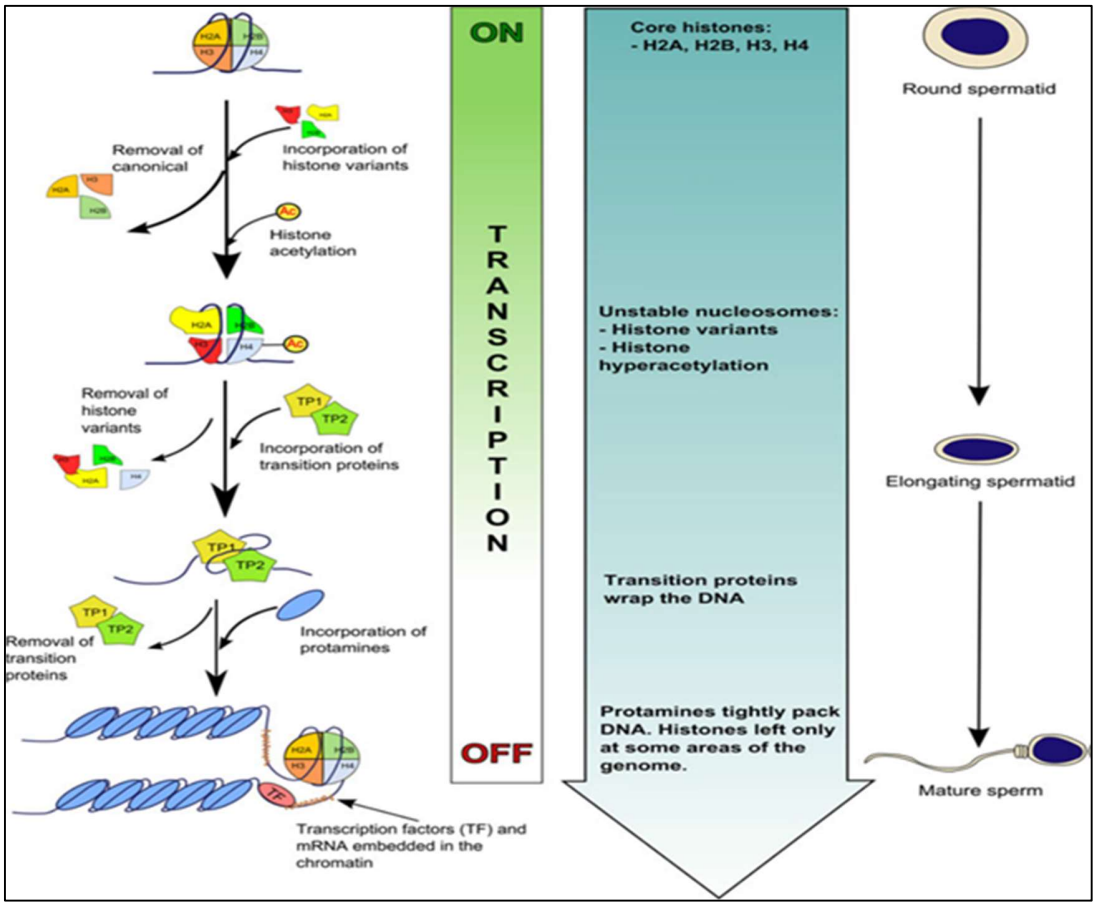

Figure 4: Major chromatin structure remodelling events during spermiogenesis. [Adapted from Teperek & Miyamoto, 2013]

These proteins have a characteristic constitution, mainly composed of arginine (48%) and cysteine residues (**Figure 5**) (Balhorn, 1989; Balhorn, 2007; Oliva & Castillo, 2011). The arginines are highly positive charged residues which form a highly ordered nucleo-protamine complex (Toroid) with the negatively charged sperm DNA (**Figure 6**) (Ward, 2009; Oliva & Castillo, 2011).

The cysteine residues are responsible for the formation of inter- and intra-protamine disulfide bridges (S-S), which stabilizes the nucleo-protamine complex (Lewis *et al*., 2003; Vilfan *et al*., 2004; Balhorn, 2018). It is known that zinc is abundant in human sperm nuclei (Morisawa & Mohri, 1972) and that it is likely to get trapped by the S-S (Bedford *et al*., 1973). The Cys2/His2 motif of P2 is a zinc finger domain, which leads to the appearance of zinc bridges responsible for the high stabilization of the chromatin in the mature

spermatozoa and in the cessation of transcription until the fertilization (Bianchi *et al*., 1992; Björndahl & Kvist, 2009).

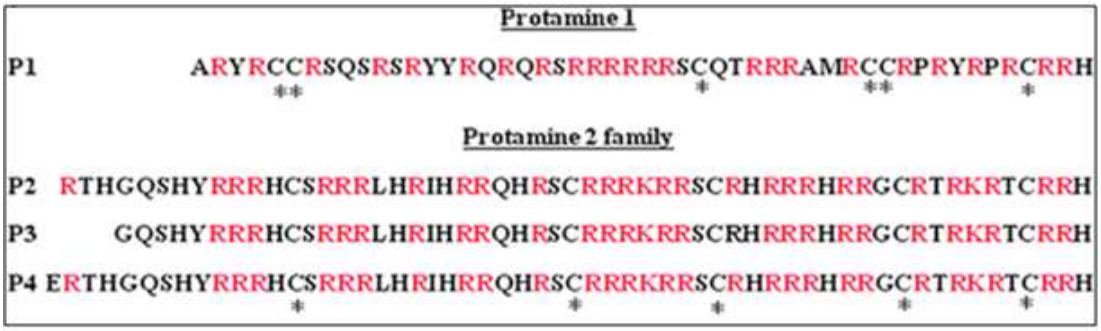

Figure 5: The human sperm protamine sequences. The arginine residues are distributed in clusters (red). The cysteine residues (asterisk) are responsible for the formation of the disulfide bonds (inter-or intramolecular) with molecules that are adjacent to protamine. [Adapted from Oliva & Castillo, 2011]

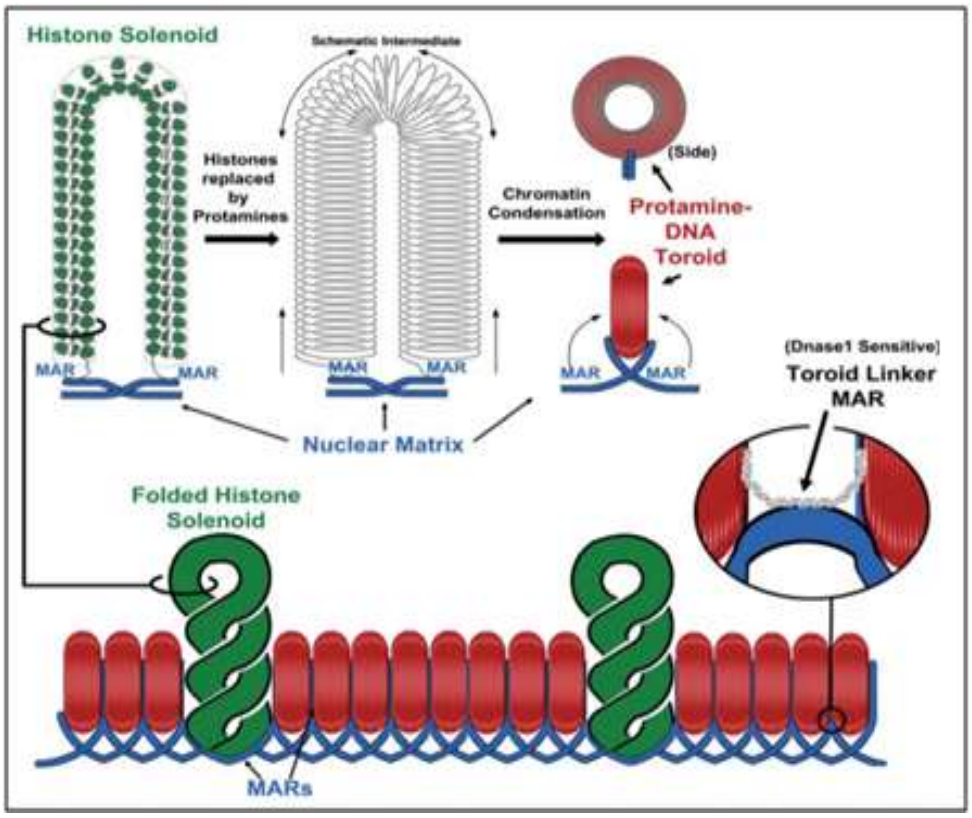

Figure 6:The sperm chromatin remodelling during spermiogenesis: from a histone solenoid structure to a protamine toroid structure. [Adapted from Ward, 2009]

1.4. Sperm DNA alterations and their consequences on male fertility and ART outcomes

Any defect occurring in one of the chromatin remodelling steps may lead to damage of the DNA integrity and may influence the sperm motility and morphology resulting in asthenozoospermia, teratozoospermia and male infertility.

As a result, the sperm will be incapable of penetrating the oocyte and in cases where the sperm is intruded or injected into the oocyte, the fertilization process will fail (Spano *et al*., 2005). Recently, as shown in a large number of cases, such failure can be overcome through different technologies like intrauterine insemination (IUI), *in vitro* fertilization (IVF), and intracytoplasmic sperm injection (ICSI) known as assisted reproductive technologies (ARTs), but the probability of implantation failure and problems during the embryonic development could still be high (Bungum & Oleszczuk, 2018).

The traditional way of diagnosing male infertility is by determining the classical semen parameters, as described in the World Health Organization laboratory (WHO, 2010).

It is obvious that these parameters were not good prognostic factors of a man's fertility and of ART success. This has led to more focus on the genomic side of the sperm. In fact, many studies have revealed that the DNA injuries in sperm may affect the pregnancy results in IUI (Muriel *et al*., 2006; Bungum *et al*., 2007; Bungum & Oleszczuk, 2018). In the last decade, it has also been shown that DNA impairment can affect the fertilization rate, pre-embryo development and thus the pregnancy or miscarriage (Hammadeh *et al.,* 2006; Bakos *et al*., 2008; Frydman *et al*., 2008; Tarozzi *et al*., 2009). Moreover, others have demonstrated that no significant correlation was observed between the sperm chromatin impairments, the fertilization rate (Henkel *et al*., 2003), the embryo quality (Benchaib *et al*., 2003) and the IVF/ICSI outcomes (Chohan *et al*., 2004). Despite that, many studies used different techniques for sperm DNA structure or DNA fragmentation index (DFI) measurement; it is still not used as a standard parameter to test male infertility or as a predictor of ART success.

1.5. Sperm chromatin maturity assessment (Tests)

Several function tests have been proposed to explore sperm fertilizing ability and to predict the rate of ART outcome. However, in the literature there is still controversy about the benefits of using these tests. After semen analysis, normal semen parameters provide no firm conclusion to the clinicians either about the fertility status of the patient or the outcomes of infertility treatment. Various studies demonstrated that abnormalities during the sperm chromatin organization may lead to male infertility (Sakkas & Tomlinson, 2000; Spanò *et al*., 2000; Saleh *et al*., 2002) and may subsequently influence the fertilization and the embryo quality and development (Gannon *et al.,* 2014; Simon *et al*., 2014). This means that sperm DNA analysis with the standard semen analysis may help to reveal hidden sperm DNA abnormality in men with idiopathic infertility. Because of the increasing evidence for DNA integrity and its importance for the ART outcome, many methods have been developed in the last decade to reveal any changes in the sperm chromatin status and maturities, such as fragmentation and protamination. But the use of these techniques as complementary biomarkers besides semen analysis is still controversial.

Many assays are used to evaluate the sperm chromatin status, each one concentrating on a specific point on the sperm DNA (**Table 1**).

Table 1: Sperm chromatin abnormalities assessment assays (according to Agarwal *et al.*, 2017)

Assessment Test	**Variable**	**Method**
Acidic aniline blue (Hammadeh *et al.,* 1996)	Nuclear maturity	Optical microscopy
Toluidine blue stain (Erenpreisa *et al*., 2003)	DNA fragmentation	Optical microscopy
Chromomycin A3 (Manicardi *et al*., 1995)	Nuclear maturity (DNA protein composition)	Fluorescent microscopy
Sperm chromatin dispersion (Fernandez *et al*., 2003)	DNA fragmentation	Fluorescent microscopy
DNA breaking detection-fluorescent *in situ* hybridization (Fernandez *et al.,* 2000) *In situ* nick translation (Gorczyza *et al.,* 1993)	DNA fragmentation (ssDNA)	 Fluorescent microscopy Fluorescent microscopy Flow cytometry
Acridine orange (Hammadeh *et al.,* 2001)	DNA denaturation (acid)	Fluorescent microscopy Flow cytometry
TUNEL (Barroso *et al*., 2000)	DNA fragmentation	Optical microscopy Fluorescent microscopy Flow cytometry
Comet (neutral) (Singh *et al*., 1988) (alkaline) (Singh *et al*., 1989)	DNA fragmentation (dsDNA) DNA fragmentation (ssDNA/dsDNA)	Fluorescent microscopy
Sperm chromatin structure (Evenson *et al.,* 1991)	DNA denaturation (acid/heat)	Flow cytometry
8-OHdG measurement (Shen & Ong, 2000)	8-OHdG	High-performance liquid chromatography

8-OHdG, 8-hydroxy-2-deoxyguanosine; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA

1.6. Gene expression in human spermatozoa

The spermatozoon is a unique differentiated cell that resulted from extremely ordered processes. Transcription is a remarkable process preserved during the first steps of spermiogenesis until the evolution of the round spermatids. At this step, the mRNAs are synthesized intensively under the control of a set of specific transcription factors (Grégoire & Boissonneault, 2011).

Then, the transcripts which are needed to complete in the assistance of the mature spermatozoa evolution are kept as ribonucleoproteins (RNPs). At the same time, the majority of cytoplasm with its constituents, including RNA, will be eliminated (Cooper, 2005), and the histones are replaced by protamines, as previously described, which leads to a highly compacted DNA. This event leaves the sperm cell transcriptionally inert and lacking a translation process due to ribosomal RNA (rRNA) cleavage (Johnson *et al., 2011*).

The remaining transcripts, shown by RNA sequencing (RNA-Seq), are coding and non-coding RNAs which contain both fragmented and submissively non-degenerated mRNAs, mi (micro), si (small interfering), lnc (long non-coding), and pi (Piwi-interacting)-RNAs (**Figure 7**) (Jodar *et al*., 2013; Sendler *et al*., 2013).

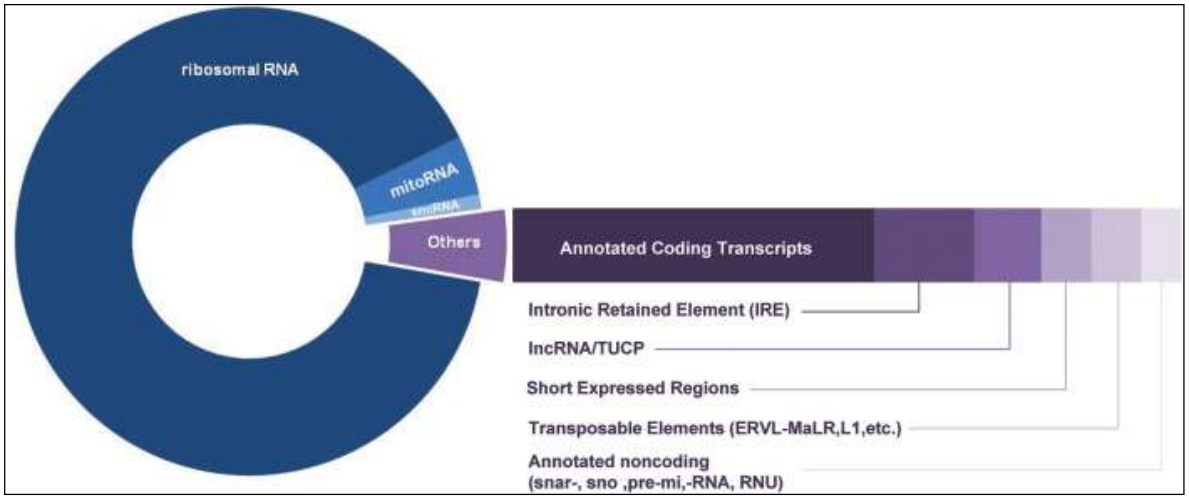

Figure 7: Spermatozoal RNAs population in normal sperm samples. The rRNA class was the most abundant followed by the mitochondrial RNA (mitoRNA), small non-coding RNAs (snc-RNAs) and others. [Adapted from Jodar*et al*., 2013]

The composition of a mammal's spermatozoal RNA, like in humans, is the most distinguishable structure. The majority of germ cell transcriptome studies that provided a general view of the spermatozoal RNA population were performed on the microarray (cDNA/ Oligonucleotide). Ostermeier *et al.* (2002) found that almost 3000-7000 different coding RNAs existed in human mature spermatozoa. In another study, they demonstrated that sperm transcripts originating from the earlier phases of spermatogenesis reflect testicular events that had happened previously (Ostermeier *et al*., 2005).

Recently, as a result of various studies on the RNA content in mature sperm, an alternative way has evolved of diagnosing male fertility problems. A number of infertility phenotypes have been associated with altered transcripts; Oligozoospermia (Ferlin *et al*., 2010; Montjean *et al*., 2012), Asthenozoospermia (Jedrzejczak *et al*., 2007; Kempisty *et al*., 2007; Chen *et al*., 2012; Jodar *et al*., 2012; Abu-Halima *et al*., 2013; Yatsenko *et al*., 2013; Bansal *et al*., 2015), oligoasthenozoospermia (Li *et al*., 2011; Zheng *et al*., 2011; Abu-Halima *et al*., 2013; Shen *et al*., 2015).

Thus, it is obvious that sperm RNA profile depends on the testicular microenvironment and consequently provides an idea of how environmental and pathological agents can affect this microenvironment.

Others have tried to find a correlation between the presence of the remaining RNA in spermatozoa with regulative functions and fertilization, early embryo development (Jodar *et al*., 2013; Sendler *et al*., 2013), and progeny phenotype (Ostermeier *et al.,* 2004, Jodar *et al.,* 2013).

Furthermore, Garrido *et al*. (2009) and García-Herrero *et al.* (2011) pointed out the possibility of using a group of molecular biomarkers from the spermatozoal RNA profile as a predictor of ART's outcomes.

Jodar *et al.* (2015) identified 648 Sperm RNA elements (SREs), derived from 285 genes, that can be used to make a prediction about the fertility treatment for idiopathic sub-fertile couples and its success rate, as demonstrated, for example, in the live birth rate.

A similar study conducted by Burl *et al.* (2018) on the same SREs concluded that RNAs predict not only live birth success but also could even indicate the human health situation.

1.7. Tobacco smoke as a lifestyle factor influence on male infer tility

Tobacco consumption represents one of the biggest worldwide threats to human health. Despite efforts worldwide to make people aware of its consequences on health, there are still 1.1 billion smokers worldwide (WHO, 2018).

Because of its deadly toxic chemical combinations of at least 7000 chemicals, tobacco smoke causes the deaths of over 6 million people per year (Drope *et al*., 2018). More than 70 of these chemicals are carcinogens, 40 are malignant and more than 400 chemicals, such as cadmium, nicotine, cotinine, lead, and carbon monoxide, are toxic (Kumar *et al*., 2011; Drope *et al*., 2018).

This could cause damage in almost all the human organs, for example, in the lungs, heart, circulatory system, immune system, and male and female reproductive systems (Drope *et al*., 2018).

1.7.1. The relationship between tobacco smoke and male fertility alterations

Various studies have been conducted to evaluate the effects of tobacco smoke on human sperm parameters: approximately 113 studies on sperm count and concentration, 94 studies on sperm morphology and 25 studies on sperm vitality (Beal *et al*., 2017). But none of them have definitely confirmed the influence of smoking on male fertility.

Contradictory findings have in fact been reported concerning the influence of tobacco smoke on standard semen parameters. Some studies have found that smoking is correlated with a decline in semen volume, count, motility and morphology (Hammadeh *et al*., 2010; Zhang *et al*., 2013; Hamad *et al*., 2014; Al-Turki, 2015) but others have not found this association (Davar *et al*., 2012; Kumar *et al*., 2014; Moretti *et al*., 2014).

In addition, it has been reported a dose-response relationship between cigarette smoking and the quantity and morphology of spermatozoa (Zhang*et al*., 2013), and semen volume (Anifandis*et al*., 2014).

Other researchers compared the hormone levels between smokers and non-smokers and found that smokers have a higher concentration of FSH and LH and a nadirlevel of testosterone, compared to non-smokers (Mitra *et al*., 2012; Jeng *et al*., 2014).

Al-Matubsi *et al.* (2011) demonstrated that the concentrations of testosterone and LH were higher in smokers but the FSH level was not different between non-smokers and smokers. These studies showed that tobacco could affect both the Leydig and Sertoli cells, which influence the spermatogenesis process.

Moreover, smoking can also cause hypoxia (lack of oxygen) (Jensen *et al*., 1991), which affects the important metabolic pathways and consequently the different stages of spermatogenesis, causing impairments in testis function (Harlev *et al*., 2015).

1.7.2. The relationship between tobacco smoke and damage to the DNA of sperm

Temporary sperm DNA single-strand breaks (SSBs) and double-strand breaks (DSBs) are genetically programmed and are needed to facilitate both the initiation of the chromosomal recombination (crossing-over) throughout the meiotic prophase I (Bannister &Schimenti, 2004) and the sperm chromatin repackaging. These breaks are observed in the round and elongating spermatids and are important for the alleviation of torsion stress in the DNA strand, which will facilitate the histone-protamine transition (Bench *et al*., 1998; Carrell & Liu, 2001).

These breaks are created and ligated by an enzyme, named type II DNA topoisomerase (Topo II) (De Yebra *et al*., 1993; Kierszenbaum, 2001). Bakshi *et al*. (2001) have studied the hormonal regulation of Topo II and have concluded that its activity is androgen-dependent.

Moreover, Tweed *et al.* (2012) demonstrated that nicotine is an endocrine disruptor because it affects the hypothalamic-pituitary-end organ axes. These endocrine disruptors may alter the activity of Topo II, which may have a consequence on DNA integrity.

A second process leading to sperm DNA damage is the formation of the DNA adduct that are constituted after the DNA has bound to a chemical, such as acetaldehyde and benzo[a]pyrene in cigarette smoke, that causes cancer (Zenzes *et al*., 1999 (a & b); Bengum, 2012; Phillips & Venitt, 2012). A number of studies have shown that there is a correlation between altered sperm hormones and high DNA adduct levels (Horak *et al*., 2003; Gaspari *et al*., 2003).

It has also been demonstrated that this adduct might not be repaired by the oocyte and may be transferred to the zygote (Perrin *et al*., 2011).

Another reason for sperm DNA damage is oxidative stress (**Figure 8**). The cigarette smoke contains an elevated number of reactive oxygen species (ROS), such as free radicals, hydroxyl (OH), superoxide (O2-), and hydroperoxyl (HO2) radicals, and non-radical species, such as hydrogen peroxide (H2O2) (Thomas *et al.,* 2008). On the other hand, spermatozoa are vulnerable and can be damaged because of their plasma membrane being rich in polyunsaturated fatty acid that constitutes a substrate for ROS production and reduces the amount of scavengers in the cytoplasm, such as the ROS-metabolizing enzymes, ascorbate and uric acid (Alvarez & Storey, 1995; De Lamirande & Gagnon, 1995).

Moreover, the exchange between histone-protamine during spermiogenesis is also important because an incomplete protamination will expose the sperm DNA to an elevated risk of oxidative attack, which will damage it (Giwercman & Spanó, 2018). A correlation has been shown between tobacco smoke, altered spermatogenesis and variations in protein levels, such as protamine (protamine ratio) (Hammadeh *et al*., 2010; Hamad *et al*., 2017) and the protamine-to-histone ratio (Hamad *et al*., 2014; Yu *et al*., 2014).

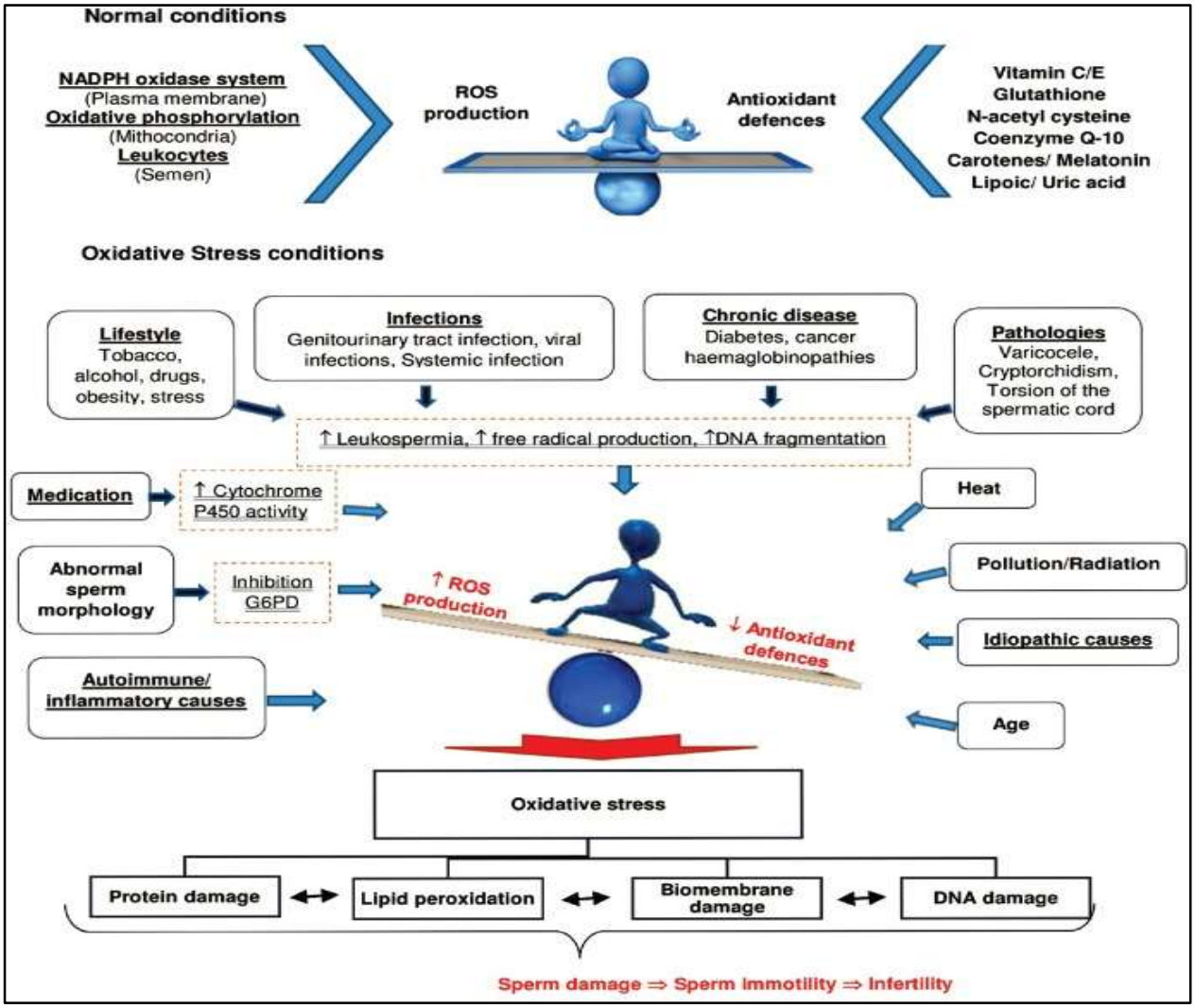

Figure 8: Diagram presenting the main causes of oxidative stress. (G6PD: glucose-6-phosphate dehydrogenase; NADPH: nicotinamide adenine dinucleotide phosphate). [Adapted from Pereire *et al.*, 2017]

1.7.3.The relationship between tobacco smoke and molecular alterations (aneuploidy, genetic and epigenetic) in correlation to male infertility

The Sertoli cell barrier (SCB) known as the blood-testis is thought to be a protective barrier for the germ cells from the majority of toxicants (Smith & Braun, 2012). However, a contradictory finding has been reported (Yauk *et al*., 2015). The genotoxic agents included in cigarette smoke pass through the testis-barrier (Pacifici *et al*., 1993; Vine *et al*., 1993; Pacifici *et al*., 1995; Zenzes *et al*., 1999 (a)) and reach the spermatozoa genome in different stages of spermatogenesis (**Figure 9**).

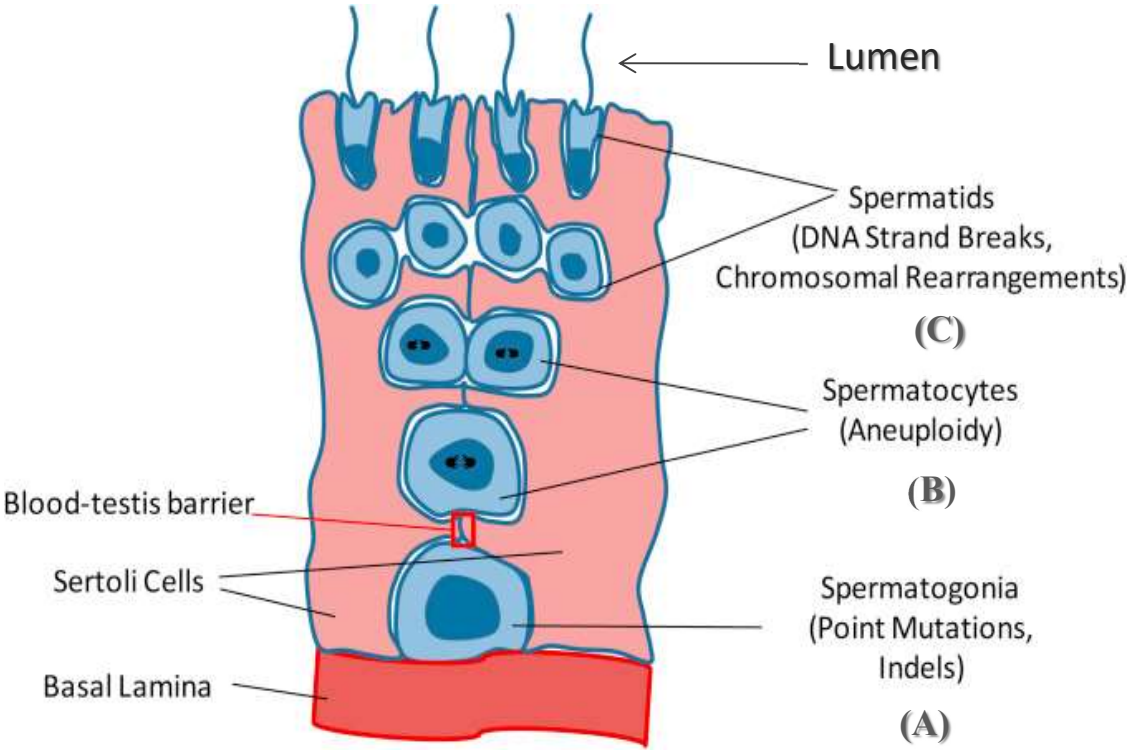

Figure 9: Divergent mutations that can occur during spermatogenesis after exposure to an external disturbance. [Adapted from Beal *et al*., 2017].(A) because of her position (open side of the blood-testis Barrier), the spermatogonia are more vulnerable to external exposure like toxicant which is leading to an increase in the insertions/deletions (indels) and single nucleotides polymorphisms (SNPs).(B) When she passes the testis barrier, the spermatogonia divide into two spermatocytes type I (spcI) which go throughout two meiotic divisions. At this stage, chromosomal recombination may lead to chromosomal perturbations and aneuploidies. (C) During his differentiation of formed spermatids to spermatozoon, the sperm DNA became more compacted which lead to a deficiency in DNA may repair which leads to DNA strand breaks and chromosomal rearrangement (post-fertilization).

Furthermore, many designed studies have examined the association between tobacco smoke and the frequency of the mutations in spermatogonia. Most of the studies were carried out on rodents and showed that both mainstream and sidestream cigarette smoke increased the mutation frequency in the spermatogonial stem cell (Stoichev *et al*., 1993; Yauk *et al*., 2007; Marchetti *et al*., 2011).

In a human study, Linschooten and his colleagues demonstrated that the risk of the transmission of mutations, type tandem repeat minisatellite, to offspring are four-times higher in smokers than in non-smokers (Linschooten *et al*., 2013).

Furthermore, the meiosis process during spermatogenesis is important and any alteration could lead to numerical and/or structural chromosomal changes. It has been reported that growing amniocytes in media containing nicotine led to abnormalities in the structure and

number of chromosomes 22, 21, 20, 15, and 8 (Demirhan *et al*., 2011). Another study showed that the spermatozoa of male smokers contain a high frequency of disomy in chromosomes 3 and XY (Pereira *et al*., 2014).

Chromatin remodelling during spermiogenesis is also an epigenetic mechanism, characteristic of spermatozoa. Male smoking has been associated with histone-to-protamine transition deficiency in sperm, alteration in protamine (P1 to P2) ratio (Hammadeh *et al*., 2010; Yu *et al*., 2014; Hamad *et al*., 2017), and alterations in the histone (H2B)-to-protamine ratio (Hamad *et al*., 2014).

A correlation was demonstrated between the variations in the xenobiotic metabolism genes, such as glutathione S-transferases (GSTs), N-acetyltransferase (NAT2) (Yarosh *et al*., 2015), the enzyme Cytochrome P450 (CYP1A1) (Yarosh *et al.,* 2013) and idiopathic infertility in smokers.

What is more, tobacco smoke is one of the main environmental factors that modify DNA methylation (Joubert *et al*., 2012; Philibert *et al*., 2012; Shenker *et al*., 2013). In our own laboratory in fact, a correlation between methylation and infertility in male smokers has been demonstrated (Laqqan *et al*., 2017; Alkhaled *et al*., 2018).

1.8. The purpose of the study

The histone-protamine transition in sperm chromatin is very important for sperm maturity. Thus, in this particular study, we had the following aims:

1. to investigate the effects of tobacco smoke on standard sperm parameters, sperm maturity and sperm DNA integrity;
2. to determine the gene expression of some primordial nucleoproteins in sperm chromatin condensation: H2B histone family, H2BFWT testis-specific encoded by H2BFWT gene, spermatid nuclear transition protein 1 encoded by TNP1 gene, spermatid nuclear transition protein 2 encoded by TNP2 gene, protamine 1 encoded by PRM1, protamine 2 encoded by PRM2, in semen samples of heavy-smoker and non-smoker patients undergoing intracytoplasmic sperm injection (ICSI) therapy;
3. to find out the influence of tobacco smoke on the paternal genome by studying the variation in the DNA sequence of three of these investigated protein genes, namely, H2BFWT, PRM1 and PRM2.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Study population

Semen samples (n= 167) were collected randomly from male partners of couples undergoing ICSI treatment. Each patient had a physical examination and was interviewed following an organized questionnaire (Medical history, Job, Lifestyle and tobacco smoke). The participant who smokes more than one pack/day for 10 years or 2 pack/ day for 5 years was considered as heavy-smoker, and the participant who did not smoke was considered as a non-smoker. The individuals were in reproductive age (25-49 years).

The inclusion criteria of patients were males who did not have cryptorchidism, present or past cancer treatment, genetic abnormalities (Klinefelter's syndrome or Y-chromosome microdeletion), hypogonadotropic hypogonadism (hormonal disorder), drug abuse, varicocele, and/or recent fever episode, and female partners without any history of female-related cause of subfertility (endometriosis, tubal occlusion, or ovulatory disturbance) and no surgical or medical infertility treatment in the last three months before undergoing ICSI.

2.1.2. Reproduction and Andrology laboratory materials

Reagent or chemical	Company
Combur 2 Test LN	Roche, Switzerland
Embryogen (Medium for Embryo)	Origio, Denmark
Eosin G (Sperm staining)	Merck, Germany
G-1 Plus (Culture medium)	Vitrolife, Sweden
G-2 Plus (Culture medium)	Vitrolife, Sweden
G-IVF Plus (Culture medium)	Vitrolife, Sweden
G-MOPS (Culture medium)	Vitrolife, Sweden
G-TL Plus (Long term culture medium)	Vitrolife, Sweden
SynVitro Hyadase (Denudation)	Origio, Denmark

Immersion Oil	Merck, Germany
Liquid paraffin	Origio, Denmark
PureSperm 100 (Sperm preperation)	Nidacon international AB, Sweden
PVP clinical grade (Sperm immobilization)	Origio, Denmark
Sodium chloride (NaCl) 0,9%	B.Braun, Germany
Disposables	
1 ml insulin Syringe sterile U-40	BD Medical, USA
5 ml Syringe sterile	B.Braun, Germany
5 Well culture Dish	Vitrolife, Sweden
Accu-jet pro pipette controller	BrandTech Scientific, USA
Biosphere Filter tips (10-20-200-100 ml)	Sarstedt, Germany
Centre Well Dish	Vitrolife, Sweden
Centrifuge tube (15 ml)	Vitrolife, Sweden
Centrifuge tube (50 ml)	Vitrolife, Sweden
Collection Dish 90mm	Vitrolife, Sweden
Coverslips	R. Langenbrinck, Germany
Culture Dish 40mm	Vitrolife, Sweden
Culture Dish 60mm	Vitrolife, Sweden
Embryo Transfer Catheter Set	Cook Medical, USA
EmbryoSlide culture dish	Vitrolife, Sweden
Flexipet denuding pipette (140 µm, 170 µm, 300 µm)	Cook Medical, USA
Handling micropipettes	Origio, Denmark
Handling pipette for assisted reproduction	MTG Medical, Germany
ICSI micropipettes	Origio, Denmark
IVF ICSI Dish	Thermo Scientific Nunc, Denmark
Microscope Slides	R. Langenbrinck, Germany

Oocyte collection tube (14 ml)	Vitrolife, Sweden
OOsafe sperm collection cup (80ml)	SparMed, Denmark
Pipettes	Eppendorf, Germany
Serological Pipette (2 ml)	Vitrolife, Sweden
Serological Pipette (5 ml)	Sarstedt, Germany
Spinal needle	BD Medical, USA
Steripette (60 mm)	Minitüb, Germany
Instruments	
Bench-top centrifuge	Sigma-Aldrich, Germany
Binocular light microscope	Olympus, Japan
Blockthermostat	Labotect, Germany
CO2 incubator (C200)	Labotect, Germany
EmbryoScope time-lapse incubator	Vitrolife, Sweden
Heating systems for microscopes	Minitüb, Germany
Heraeus horizontal laminar flow cabinet	Heraeus, Germany
Hot Plate 062	Labotect, Germany
Incubator C16	Labotect, Germany
Inverted microscope: ZEISS Axio Observer	ZEISS, Germany
Makler Counting Chamber	Origio, Germany
Micromanipulation system	Narishige, Japan
Microscope SMZ18 Normal	Nikon, Japan
Vortex-Genie 2	Scientific industries, USA

2.1.3. Reagents, chemicals, kits and equipment in the experimental part

Reagent or chemical	**Company**
Absolute Ethanol	Merck, Germany
Agarose tablets (DNase/RNase free)	Bioline, UK
Chromomycin A3 (CMA3)	Merck, Germany
DAPI (4′,6-Diamidine-2′-phenylindole dihydrochloride)	Merck, Germany
DNA Ladder (10 kb)	New England BioLabs, USA
Ethidium Bromide	New England BioLabs, USA
Glacial acetic acid	Fluka, Germany
Glycerol	Merck, Germany
Methanol	Merck, Germany
Nuclease-free water	Qiagen, Germany
Paraformaldehyde (PFA) 4% in PBS	Morphisto, Germany
Phosphate buffer saline (PBS)	Sigma-Aldrich, Germany
Sodium citrate	Merck, Germany
Tris-Acetate-EDTA buffer (TAE)	Sigma-Aldrich, Germany
Tris-EDTA (TE)	Sigma-Aldrich, Germany
Triton X-100	Sigma-Aldrich, Germany
β-Mercaptoethanol	Merck, Germany
Kits	
In Situ cell death detection kit, Fluorescein (TUNEL)	Roche Diagnostics, Germany
Isolate II RNA/DNA/Protein Kit (Phenol free)	Bioline, UK
miScript II RT kit (Hiflex Buffer)	Qiagen, Germany
MyTaq^{TM}HS RedMix Kit	Bioline, UK
PCR primers (H2BFWT, PRM1, PRM2)	MicrosynthSeqLab, Germany
QuantiTect primer assay (200), GAPDH	Qiagen, Germany

QuantiTect primer assay (200), H2BFWT	Qiagen, Germany
QuantiTect primer assay (200), PRM1	Qiagen, Germany
QuantiTect primer assay (200), PRM2	Qiagen, Germany
QuantiTect primer assay (200), TNP1	Qiagen, Germany
QuantiTect primer assay (200), TNP2	Qiagen, Germany
QuantiTect SYBR Green PCR kit	Qiagen, Germany
Instruments	
7500 Fast Real-Time PCR system	Applied Biosystems, USA
Balance Mettler PM200	Mettler Toledo, USA
Centrifuge CM-6MT	ELMI, Latvia
Consort EV 243 Electrophoresis power supply	Sigma-Aldrich, Germany
EasyCast B2 Mini Gel Electrophoresis System	Thermo Scientific, USA
Eppendorf Bench-top centrifuge	Eppendorf, Germany
Fluorescence Microscope	Olympus, Japan
Freezer, -20°C	Liebherr, Germany
Freezer, -80°C	Thermo Scientific, USA
Freezer, 8°C	Liebherr, Germany
Laboratory timer	Qiagen, Germany
Light Microscope	Carl Zeiss Microscopy, Germany
Manual counter	Karl Hecht "Assistent", Germany
Microcentrifuge	VWR international, USA
Miniprep PCR-purification HT	Qiagen, Germany
MolecularImager Gel Doc XR & System with Image Lab Software	Bio-Rad, Germany
Nanodrop spectrophotometer ND-2000c	Thermo Scientific, USA

PCR workstation pro (peqlab)	VWR international, USA
Single Read HT	Qiagen, Germany
Thermal Cycler C100	Bio-Rad, Germany
Thermomixer comfort	Eppendorf, Germany
Vortex-Genie 2	Scientific industries, USA
Disposables	
96-well PCR Plate 0.2 ml, non-skirted	Nippon Genetics Europe, Germany
Biosphere Filter tips (10-20-200-100 ml)	Sarstedt, Germany
Biosphere plus SafeSeal Micro Tubes (1,5 mL/ 2 ml)	Sarstedt, Germany
Eppendorf Conical Tubes, 15 mL	Eppendorf, Germany
Flat Cap Strips	Nippon Genetics Europe, Germany
MicroAmp Fast Optical 96-Well Reaction Plate with Barcode (0.1ml)	Applied Biosystems, USA
Multiply-Pro cup 0.2ml, PP	Sarstedt, Germany
Optical Adhesive Covers (DNA/RNase/Inhibitors Free)	Applied Biosystems, USA
Parafilm	American National Can, USA
PCR Soft tubes, 0.2 ml (DNA, DNase, RNase free)	Biozym, Germany
Pipettes	Eppendorf, Germany
Racks	Sarstedt, Germany
Single Scale Graduated Cylinders	VWR international, USA
Storage boxes	Sarstedt, Germany

2.2. Methods

2.2.1. Sperm collection and handling

The samples were analysed in the IVF laboratory at the department of Obstetrics and Gynecology in the Saarland University clinic.

All samples were collected, by masturbation, after a period of sexual abstinence betwen two to five days.

The specimen container was kept on the heating stage or incubator (37°C) for 30-60 minutes for liquefaction. Then, macroscopic (ejaculate appearance, viscosity, pH, and volume) and microscopic (The spermatozoa concentration, motility, vitality, aggregation, and morphology in semen) evaluations were done according to the WHO laboratory manual (WHO, 2010) and table 2 illustrated the lower reference limits.

Table 2: Semen characteristics according to the WHO (2010)

Parameters (Unit)	Reference value
Semen volume (ml)	1.5
Total sperm number (10^6per ejaculate)	39
Sperm concentration (10^6 per ml)	15
Total motility (PR + NP, %)	40
Progressive motility (PR, %)	32
Vitality (live spermatozoa, %)	58
Sperm morphology (normal forms, %)	4

*PR: progressive motility; NP: non-progressive motility.

For CMA3 and TUNEL staining, additional semen smears were prepared.

Before DNA and RNA isolation, the total of semen samples was treated (purification step) to remove the cells other than spermatozoa by loading each sample onto 40%–80% discontinuous Puresperm gradients (Nidacon International, Sweden) and then centrifuged at (500 x g/20 min) at room temperature.

2.2.2. Sperm Chromatin condensation assay (Chromomycin A3 assay)

For the sperm DNA condensation assessment, the Chromomycin A3 (CMA3) assay as described previously by Manicardi et al, (1995) was used. CMA3 is aguanine–cytosine-specific fluorochrome which unveils unwell packaged chromatin in human spermatozoa by competing with the protamines for the sameDNA binding sites.

The first step was the fixation by putting the slides1 hour in glacial acetic acid-methanol (1:3) for and then left them to air dry. To each slide, 25µl of CMA3 stain solution was added and incubated in the dark for 30 minutes at room temperature (RT). After Wash with Phosphate buffer saline (PBS), slides were mounted then kept overnight at 4°C in the dark. On each slide, 200 spermatozoa were evaluated using fluorescence Microscope (Olympus, Japan): a bright green spermatozoon presents a low protamination state (CMA3 positive) and dull green spermatozoa (CMA3 negative) (**Figure 10**).

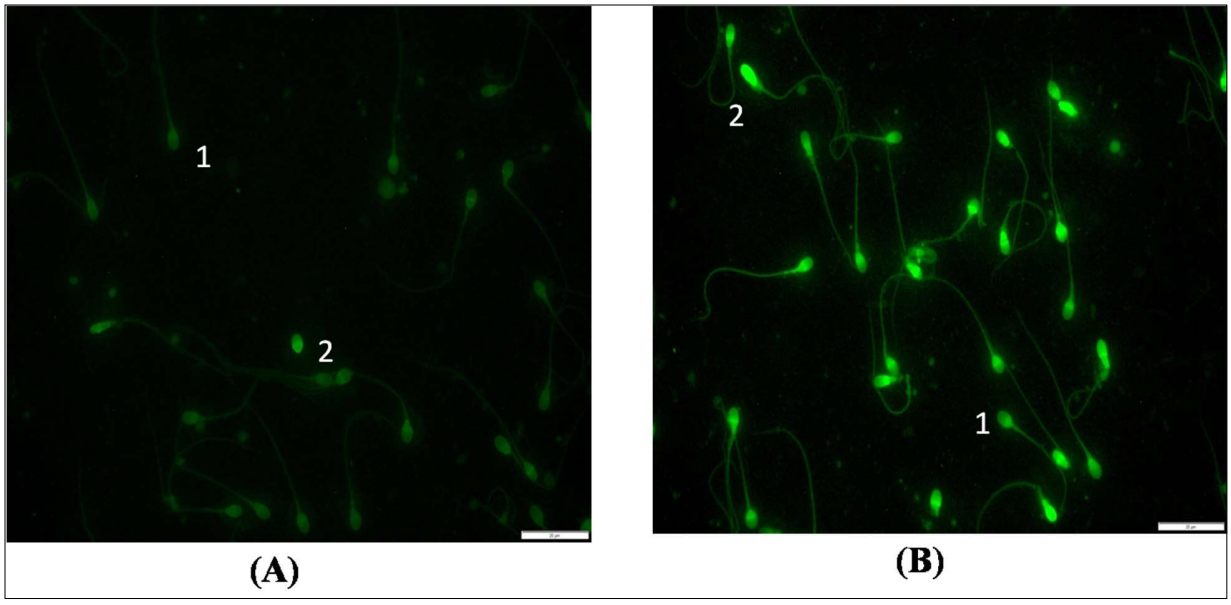

Figure 10: Identification of the sperm chromatin condensation by the chromomycin A3 (CMA3): (1) Spermatozoa with condensed chromatin (CMA3 negative) and (2) Spermatozoa with non-condensed chromatin (CMA3 positive); (A) Example of non-smoker semen sample and (B) Example of the heavy-smoker semen sample.

2.2.3. Sperm DNA fragmentation (Terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL))

To assess the fragmentation in the sperm DNA, TUNEL test was used as previously described by Borini *et al*. (2006). The test is based on the quantification of the integrationof dUTP at single- and double-strand DNA breaks. This reaction is catalyzed by an enzyme terminal deoxynucleotidyl transferase (TdT) provided in the kit of in situ cell death detection kit fluorescein (Roche Diagnostics GmbH, Germany).

The first step is the fixation of smears in slides with 4% paraformaldehyde (Sigma-Aldrich, Germany) for two hours at room temperature. Then in the permeabilisation step, the smears were incubated for 15 min with 0.1% Triton at RT. To each slide, 25µl of the TdT-labelled nucleotide mixture was added then incubated overnight at 37°C in a humidified chamber. Then the slides were washed with PBS. After that, 25µl of DAPI (Sigma-Aldrich, Germany) was added to each slide as a counterstain. On each slide, 200 spermatozoa were evaluated using a fluorescence Microscope (Olympus, Japan). A mixture of exciter dichromic barrier filter of BP 436/10: FT 580: LP 470 was used: Green stained spermatozoa are TUNEL-positive and blue stained spermatozoa are TUNEL-negative (**Figure 11**).

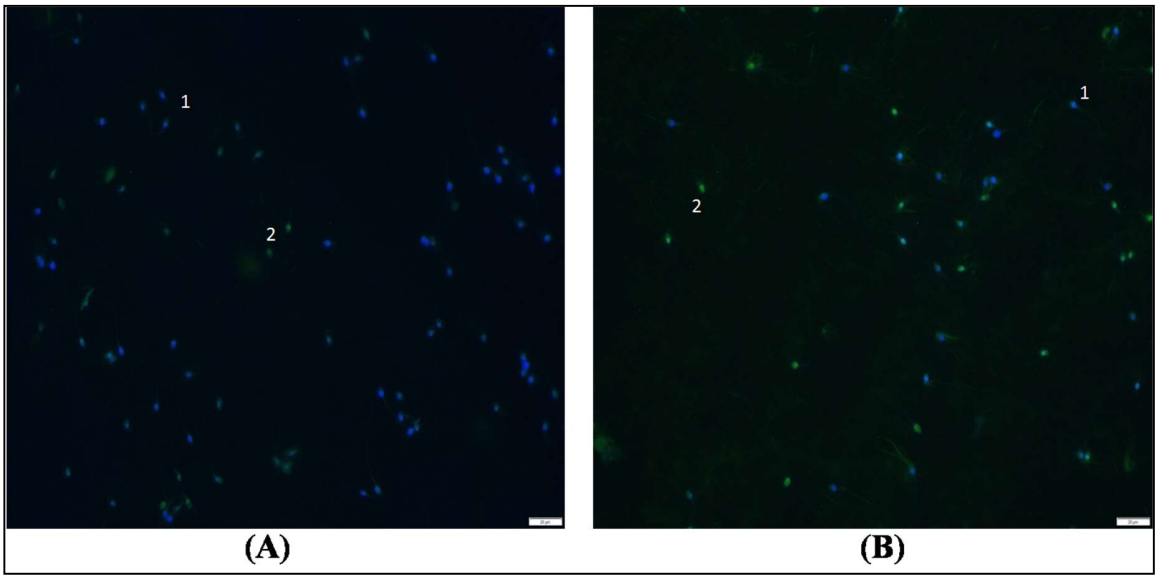

Figure 11: Identification of the sperm DNA fragmentation by the TUNEL assay: (1) spermatozoa without fragmented DNA (TUNEL-negative) and (2) spermatozoa with fragmented DNA (TUNEL-positive); (A) example of non-smoker semen sample and (B) Example ofthe heavy-smoker semen sample.

2.2.4. Genomic DNA and total RNA purification from semen samples

Genomic DNA and total RNA were isolated from the purified semen samples according to a modified protocol of the Isolate II RNA/DNA/Protein Kit (Phenol-free) (Bioline, UK). First, the washing buffers (W1 and W2), Lysis buffer TX and DNase I were prepared as follow:

Wash buffer (W1)	Wash buffer W1 concentrate diluted in 90ml ethanol (100%) and stored at 25°C
Wash buffer (W2)	Wash buffer W2 concentrate diluted in 15ml ethanol (100%) and stored at 25°C
Lysis buffer TX	10µl β-mercaptoethanol in 1ml buffer TX, stored at 25°C
DNase I (RNase free)	15µl DNase I in 100µl DNase reaction buffer (DRB), stored at -20°C

Then, the lysate was prepared for each sample by adding to each 100µl of the semen sample, 300µl of lysis buffer TX followed by vortexing (15s) until the mixture turned to be transparent.

2.2.4.1. Purification of genomic DNA

The next steps summarized the protocol for DNA isolation from the semen sample:

1. The providedDNA column was assembled with thecollection tube

2. 600µl of the prepared lysate was applied onto the column, thencentrifuged (14,000 x g/ 1 min) until the total volume passed through.

3. The flow-through in the collection tube was retained and stored on ice for later total RNA isolation.

4. The column was reassembled with another collection tube.

5. Genomic DNA in the column was washed two times by applying 500µl of wash buffer W1 then 500µl of wash buffer W2 and each time the column was centrifuged (14,000 x g/ 1 min) and the flow through was discarded.

6. The column was dried by spin for 2 min at 14,000 x g.

7. 50µl of DNA elution buffer was applied to the column placed into a fresh elution tube and centrifuged first 2 min at 300 x g, then 1 min at 14,000 x g.

8. The extracted DNA was stored at -80°C for later use.

2.2.4.2. Purification of total RNA

The flow-through stored earlier for total RNA purification was processed as follows:

1. 60µl of 100% ethanol was added to each 100µl of flow-through and mixed by vortex.

2. The provided RNA column was assembled with thecollection tube and 600µl of lysate was applied onto it and centrifuged (3,500 x g/ 1 min).

3. Total RNA in the column was washed three times by applying 400µl of wash buffer W1 and each time the column was centrifuged (14,000 x g/ 1 min) and the flow through was discarded.

4. The column was dried by spin (14,000 x g/ 2 min).

5. 50µl of RNA elution buffer was applied to the column placed into a fresh elution tube and centrifuged first 2 min at 300 x g, then 1 min at 14,000 x g.

6. The extracted RNA was stored at -80°C for later use.

2.2.4.3. Analysis of RNA and DNA concentrations and purity

The purity and the quantity of the isolated DNA and RNA were checked with the Nanodrop spectrophotometer ND-2000c (Thermo Scientific, USA) and they were later held at -80°C for later use. The purity of DNA and RNA was assessed by the 260/280 ratio. For pure DNA, a ratio equal to 1.8 was accepted and for pure RNA, a ratio equal to 2.0 was accepted. The elution buffer was used for the blank measurement.

2.2.5. Identification of SNPs in the H2BFWT, PRM1 and PRM2

A standard polymerase chain reaction (PCR) technique was used to amplify the Human H2BFWT, PRM1 and PRM2 genes. The primers were designed based on the reference sequence for the three candidate genes downloaded from GenBank using Primer3 (Untergasser et al., 2012) (**Table 3**).

Table 3: Primer pairs used for the amplification of H2BFWT, PRM1 and PRM2 gene

Gene	**Primer Name**	**Sequence 5'-3'**	**Amplicon Length**
H2BFWT	H2BFWT_F01	tggcatggatcagctgagaa	1462
	H2BFWT_R01	ggacactccctaagcctact	
PRM1	PRM1_F01	cctttgccctcacaatgacc	710
	PRM1_R01	aacaaaacccagcgtgacaa	
PRM2	PRM2_F01	ccaacagtaacaccaagggc	883
	PRM2_R01	gccaggtttgtgtgattcgt	

A 30 µl PCR reaction mixture was prepared using MyTaq^{TM}HS Red Mix Kit (Bioline, UK) according to the manufacturer's instructions:

DNA template	(20 ng/µl)
Primers (20µM each)	0.6µl
MyTaqHS Red Mix, 2x	15µl
Nuclease-free water	Up to 30µl

The Thermocycler (C1000^{TM} Thermal cycler, Bio-Rad, United States) program was set as follow: 95°C for 3 min, after that40 cycles of 95°C for 20s; annealing temperature for 20s (H2BFWT: 64°C ; PRM1 and PRM2: 66°C), extension of 72°C for 1:40 min, thena final hold for 1 min at 72°C. To check the amplification, 5 µl of each PCR product for each gene was run in a 2% agarose gel mixed with Ethidium Bromide (Biolabs, United States) for visualization (EasyCast B2 Mini Gel Electrophoresis System, Thermo Scientific, United States) (**Figures 12, 13 and 14**).

The rest of the PCR products were purified with Miniprep PCR-purification HT (Qiagen, Germany) and sequenced using the Sanger sequencing technique and 2 single Read HT (Qiagen, Germany) were made for each gene.

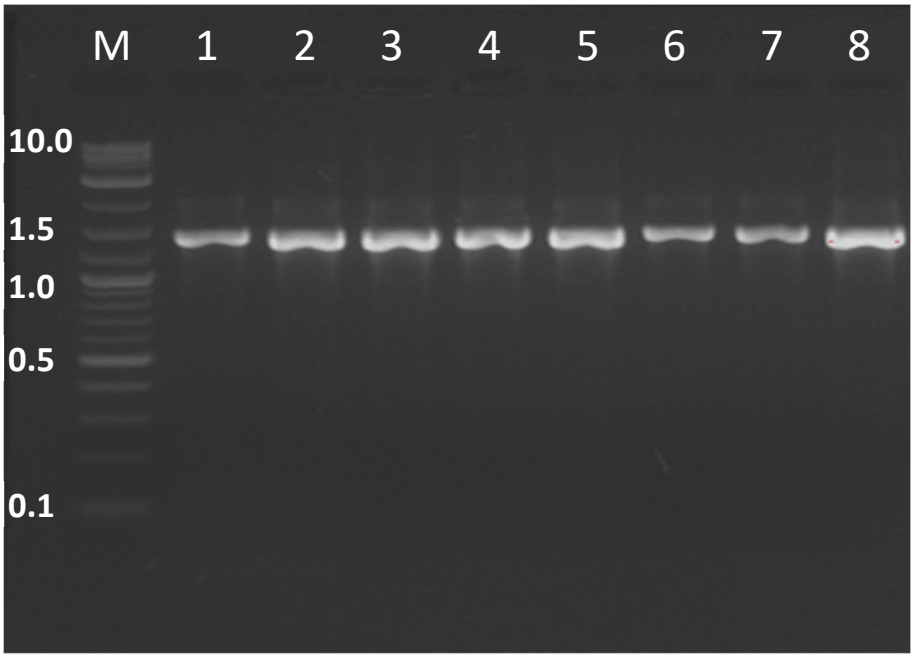

Figure 12: Representative gel electrophoresis on agarose gel (2%) of PCR products for the amplification of the H2BFWT gene (1.46 Kb). Lane M: DNA Ladder (0.1-10.0 kb) (NE Biolabs, USA), Lane 1-8: PCR samples products. The Fragments of DNA were separated onan electric field of 70 V/ 90 min. For the visualization of the gel after DNA migration, the Molecular Imager® Gel Doc™ XR& System with Image Lab™ Software (BIO-RAD, USA) were used.

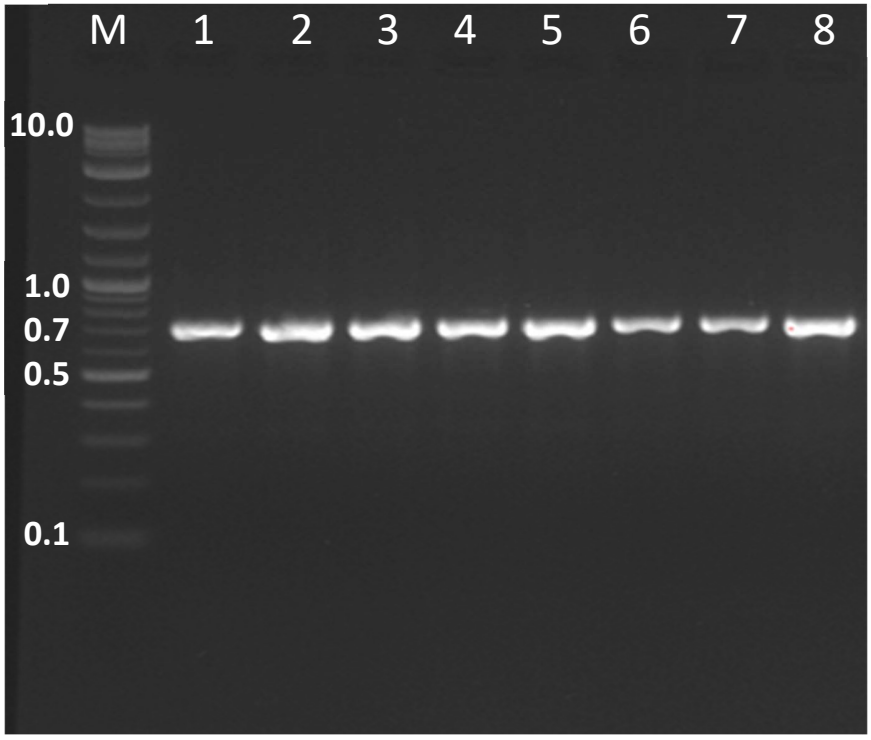

Figure 13: Representative gel electrophoresis on agarose gel (2%) of PCR products for the amplification of the PRM1 gene (0.71 Kb). Lane M: DNA Ladder (0.1-10.0 kb) (NE Biolabs, USA), Lane 1-8: PCR samples products. The Fragments of DNA were separated on an electric field of 70 V/ 90 min. For the visualization of the gel after DNA migration, the Molecular Imager® Gel Doc™ XR& System with Image Lab™ Software (BIO-RAD, USA) were used.

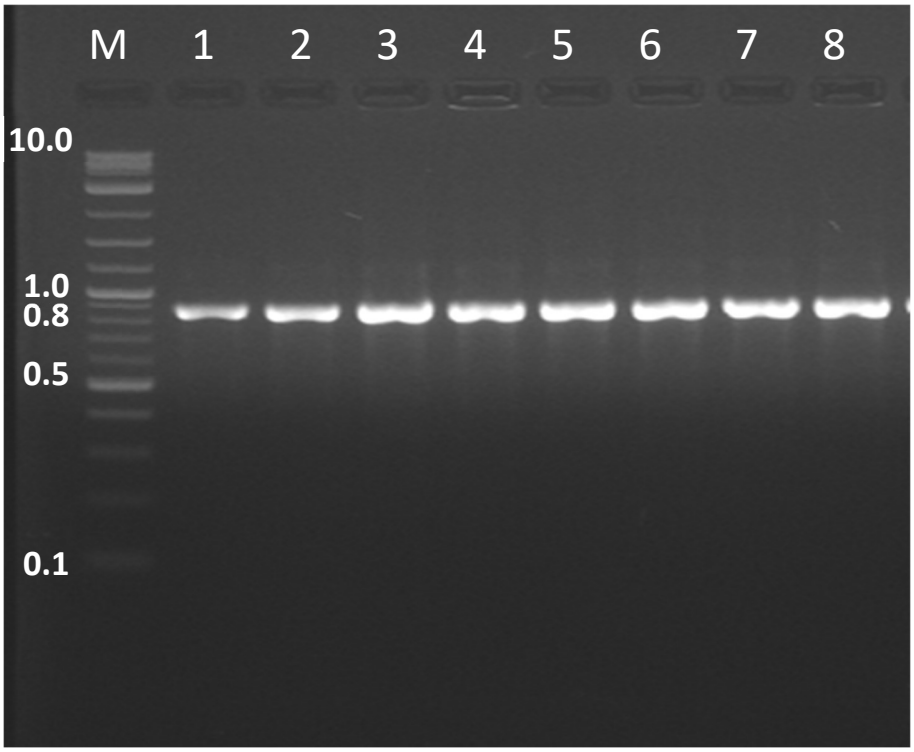

Figure 14: Representative gel electrophoresis on agarose gel (2%) of PCR products for the amplification of the PRM2 gene (0.88 Kb). Lane M: DNA Ladder (0.1-10.0 kb) (NE Biolabs, USA), Lane 1-8: PCR samples products. The Fragments of DNA were separated on an electric field of 70 V/ 90 min. For the visualization of the gel after DNA migration, the Molecular Imager® Gel Doc™ XR& System with Image Lab™ Software (BIO-RAD, USA) were used.

2.2.6. Reverse transcription and quantitative PCR (RT-qPCR)

RT-qPCR technique was used for the quantification of the expression level of the five studied genes:H2B histone family member W, testis-specific (H2BFWT), transition protein 1 (TNP1), transition protein 2 (TNP2), protamine 1 (PRM1), protamine 2 (PRM2), and the reference gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

The extracted sperm RNA was converted into cDNA in a 20µl reaction volume via miScript reverse transcription kit (Qiagen, Germany), following the kit recommendations:

1. The isolated RNA (250-300 ng) was mixed with 4µl of miScript HiFlex Buffer (5x), 2µl of miScript Reverse transcriptase mix, 2µl of miScript nucleic mix and RNase free water until 20µl.

2. The mix was incubated in a thermocycler for 60 min at 37° C, then 5 min at 95°C to inactivate the transcriptase mix.

3. The purity and quantity of cDNA was determined with Nanodrop spectrophotometer ND-2000c (Thermo Scientific, USA)

4. The cDNA was held at -20°C for later use.

QuantiTect SYBR Green PCR Kit (Qiagen, Germany) was usedfor real-time quantitative PCR

A 96 well block real-time cycler (7500 Fast Applied Biosystems, United States) was used to measure the emitted fluorescence during the binding of the SYBR Green to double-stranded DNA molecules.

Then, according to the manufacturer's recommendations, the cDNA was used as a template to prepare along with QuantiTect primer assay (Qiagen, Germany) a PCR reaction plate mix as follow:

1. For each sample, a reaction mixes of 10µl SYBR Green, 2 µl of QuantiTect primer assay (for each studied gene) and 6µl of RNase free water were prepared and added into a well of MicroAmp Fast Optical 96-Well Reaction Plate with Barcode (Applied Biosystems, United States)

2. A 2µl of undiluted cDNA was added to this mix in the well.

3. Each sample was run in triplicate and no template control and no reverse transcriptase control were included in each run.

4. With an optical adhesive cover (Applied Biosystems, United States), the plate was closed and put in a centrifuge (1500 rpm/ 1 minute) to spin the mix down and eliminate air bubbles.

5. The plate was placed in the Applied Biosystems 7500 and the cycling program was: Initial activation (95°C for 15 min), after that3 step cycling (Denaturation (94°C for 15 s), annealing (55°C for 30 s) and extension (72°C for 15 s) for 40 cycles.

6. Data from real-time PCR are Ct or threshold cycles which refer to the cycle number at which detectable signal is achieved.

2.2.7. Protocol of embryo culture and Time-Lapse Imaging

After 3-4 hours of oocytes retrieval, a decoronation of the cumulus-corona oocyte cell complex was performed using hyaluronidase (SynVitro Hyadase, Origio, Denmark) and the intracytoplasmic sperm injection (ICSI) is effectuated onoocytes in metaphase II stage using a micromanipulation system (Narishige, Japan) and an inverted microscope (Zeiss, Germany) (**Figure** 15).

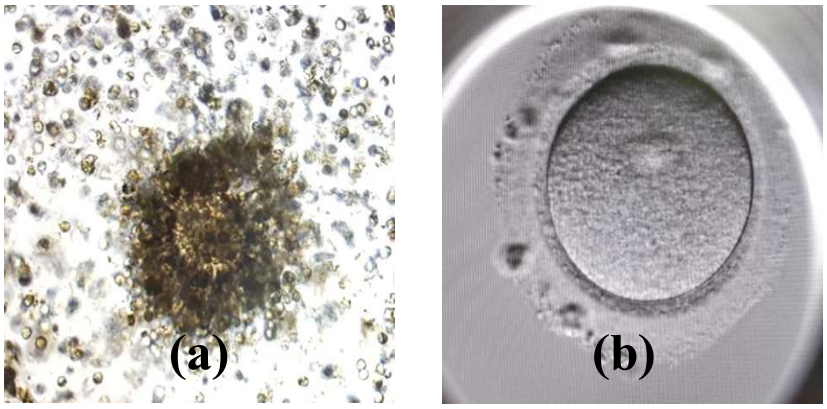

Figure 15: Intracytoplasmic sperm injection (ICSI) Day; (a) A cumulus-corona oocyte cell complex, (b) Denuded Metaphase II (MII) Oocyte.

For embryo culture and assessment, the microdrop culture system and the Embryo Scope time-lapse incubator (Vitrolife, Sweden) were used. After injection, the oocytes were distributed in an Embryo Slide culture dish (Vitrolife, Sweden) that can hold 12 embryos. In each well of the dish, 25 µl of global total culture medium (Life Global, Canada) was added. The incubation conditions were: Temperature = 37°C, 5.5% Oxygen (O_2) and 5.5% carbon dioxide (CO_2).

The embryo quality grade was assessed on day 3 after injection (Cleavage stage) according to the division symmetry, cytoplasmic fragmentation proportion (Grade 1: 0-10% of cytoplasm fragmented, Grade 2: 11-20% of cytoplasm fragmented, grade 3: >20 of cytoplasm fragmented) (Depa-Martynow *et al.*, 2012) using the EmbryoViewer Software (Vitrolife, Sweden) (**Figure16**).

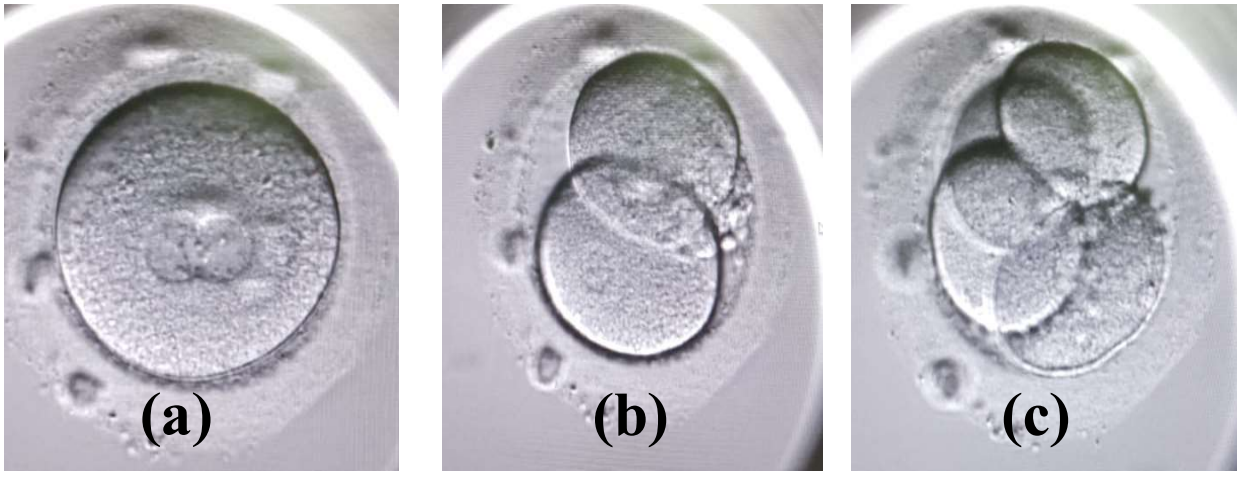

Figure 16: Embryo cleavage stages after ICSI; (a) Fertilized oocyte (Zygote) with two polar bodies and two central equal pronuclei (PNs) (18h post-ICSI), (b): A 2-blastomeres human embryo (Day 1 post-ICSI), (c): A 4-blastomeres human embryo (Day 2 post-ICSI), (d): A 8-blastomeres human embryo (Day 3 post-ICSI).

Also, the embryo quality is assessed on day 4 (Morula stage) (**Figure17**) and day 5 (Blastocyst stage) using Gardner's blastocyst grading scaleincluding expansion, inner cell mass (ICM) and Trophectoderm Epithelium (TE) (Gardner *et al*., 2007) (**Figure 18**).

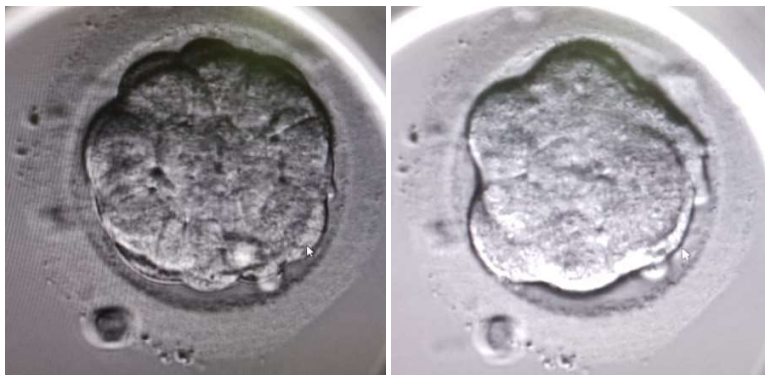

Figure 17: Progressive compaction of the human embryo on day 4 post-ICSI leading to the formation of morula.

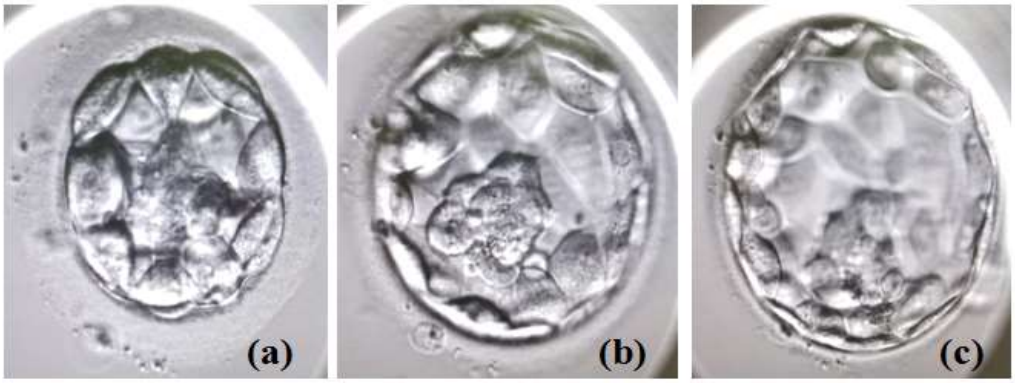

Figure 18: Blastocyst formation (Day 4-5 post-ICSI); (a) Human embryo with early cavitation, (b) Early blastocyst, (c) Expanded blastocyst.

In 30% of cases, the embryo is transferred in the cleavage stage and in 70% in the blastocyst stage. The average of embryo transfer is 2 embryos/ patient.

2.2.8. Statistical Analysis

IBM SPSS for Windows software package version 24.0 (SPSS Inc., USA) was used to analyse the data obtained in the current study. After application of the skewness test, Kurtosis test, Z-value and Shapiro test, it has been demonstrated that the samples were notnormally distributed. Thus, for the comparison of the quantitative variables between the heavy-smokers and non-smokers groups, the Mann- Whitney U-test was used and the Spearman correlation test was applied to determine the correlation between the different studied parameters. The p-value$\leq$0.05 was interpreted as statistically significant and $p<0.01$ was interpreted as statistically highly significant.

For the analysis of the real-time qPCR data, the relative quantification, based on the use of a reference gene, was used. The relative amount of each gene mRNA to the GAPDH gene was determined by the Livak or $\Delta\Delta$Ct method (Livak & Schmittgen, 2001). The protamine PRM1: PRM2 mRNA ratio was calculated as described previously by Steger *et al.*, 2003.

The tool Tracy (https://github.com/gear-genomics/tracy) was used to obtain the allelic sequences for each gene. The aligner bwa (Li & Durbin, 2009), the samtools mpileup (Li *et al.*, 2009) and WhatsHap (Ebler *et al.*, 2018, Patterson *et al.*, 2015) were used for the variant calling. From the set of all SNPs resulting from the previous steps, all positions with an allele frequency above 5 % across all studied individuals were selected and tested for Hardy-Weinberg Equilibrium using Fisher's Exact Test. To test the association between

SNP alleles and each of the studied groups of heavy smokers and non-smokers, Fisher's Exact Test was performed to find significant differences in allele distributions among the two groups (Heavy-smokers and non-smokers) and corrected for multiple testing by performing a Benjamini Hochberg correction (alpha=0.05).

3. RESULTS

3.1. Investigated parameters for all study patients

Table 4: Descriptive statistic of studied parameters for all the population undergoing ICSI therapy (N=167)

Parameters	M ± SD	Median	Mini-Max
Semen volume (ml)	3.26 ± 1.54	3.00	0.7 - 9.0
Sperm concentration (10^6x1 ml)	70.56 ± 56.87	56.00	1.0 - 286.0
Total motility (PR + NP. %)	42.26 ± 20.46	44.00	2 - 91
Progressive motility (PR. %)	18.89 ± 16.32	16.00	0 - 80
Morphologically normal spermatozoa (%)	6.23 ± 7.93	4.00	0 - 53
Protamine deficiency (CMA3 positivity) (%)	30.30 ± 19.96	25.50	0 - 98
Sperm DNA fragmentation (sDF)(%)	24.25 ± 18.99	20.00	0 - 97
Number of collected oocytes	11.72 ± 6.82	12.00	1 - 37
Number of injected oocytes	8.81 ± 5.42	8.00	1- 24
Number of fertilized oocytes	6.91 ± 4.62	6.00	0 - 22
Fertilization rate (%)	77.75 ± 20.46	83.17	0 - 100
Number of cleaved embryos	6.60 ± 4.47	5.00	0 - 20
Number of grade 1 embryos (G1)	2.23 ± 2.53	2.00	0 - 13
Number of grade 2embryos (G2)	2.79 ± 2.36	2.00	0 - 14
Embryos grade score	1.88 ± 0.56	2.00	0 - 4

M: mean; **Mini**: minimum; **Max**: maximum; **SD**: standard deviation

Table 4 illustrates the descriptive statistics: mean ± standard deviation, median, and minimum-maximum of the different studied parameters. The mean of the sperm parameters: semen volume (ml), sperm concentration (10^6 per ml), total motility (PR + NP. %), progressive motility (PR. %), and morphologically normal spermatozoa (%) were (3.26 ± 1.54; 70.56 ± 56.87; 42.26 ± 20.46; 18.89 ± 16.32; 6.23 ± 7.93 respectively).

For those patients where the protamine deficiency (CMA3 positivity) was determined by CMA3 staining it ranged between 0 and 98% with a mean of 30.30 ± 19.96%, and the sperm DNA fragmentation (sDF), determined by a TUNEL assay, it was in the range (0-97) with a mean of 24.25 ± 18.99%.

Concerning the clinical parameters after ICSI, the mean number of collected oocytes was 11.72 ± 6.82, injected oocytes was 8.81 ± 5.42, and fertilized oocytes was 6.91 ± 4.62. The fertilization rate has a mean of 77.75 ± 20.46%.

The mean number of grade 1 (G1) and 2 (G2) embryos ranged between 0 and 14 and their mean percentage together (G1+G2) was 77.54 ± 27.57%. The mean of the embryos grade score mean was 1.88 ± 0.56.

Table 5: Comparison of the semen analysis parameters between control group (non-smokers) and case groups (heavy-smokers)

Parameter (Unit)	Non-smoker (n=54) M± SD	Heavy-smoker (n=113) M± SD	P-value
Semen volume (ml)	3.56 ± 1.76	3.12 ± 1.40	**0.325**
Sperm concentration (10^6 x1 ml)	88.09 ± 63.42	62.17 ± 51.68	**0.009****
Total motility (PR + NP. %)	46.20 ± 21.92	40.38 ± 19.54	**0.070**
Progressive motility (PR. %)	27.31 ± 21.78	14.86 ± 10.95	**0.001****
Morphologically normal spermatozoa (%)	10.87 ± 12.11	4.01 ± 2.88	**0.002****

M: mean; **n**: Number; **SD**: standard deviation

The ICSI patients were divided into the following two groups: a control group (non-smokers, n=54) and a case group (heavy-smokers, n= 113).

By comparing the classical semen parameters between these two investigated groups, the mean percentage of sperm concentration, progressive motility (PR), and morphologically normal spermatozoa were significantly elevatedin the non-smokers group (p= 0.009, p=0.001, and p=0.002 respectively) (**Table 5**).

Table 6: Comparison of the grade of protamine deficiency in sperm DNA (CMA3 positivity) and sperm DNA fragmentation (sDF) between the control group (non-smokers) and the case group (heavy-smokers)

Parameter (Unit)	Non-smokers (n=54) M± SD	Heavy-smokers (n=113) M± SD	P-value
CMA3 positivity (%)	23.50 ± 14.70	33.58 ± 21.34	**0.003****
Sperm DNA fragmentation (sDF) (%)	17.41 ± 14.59	27.55 ± 20.01	**0.0001****

M: mean; **n**: Number; **SD**: standard deviation

Protamine deficiency (CMA3 positivity) and sperm DNA fragmentation (sDF) were significantly higher in the heavy-smokersgroup in comparison to the non-smokersgroup (33.58 ± 21.34% vs.23.50 ± 14.70%, p=0.003 and 27.55 ± 20.01% vs. 17.41 ± 14.59%, p= 0.0001 respectively) (**Table 6**).

Table 7: Comparison of the ICSI-outcomes between the control group (non-smokers) and case groups (heavy-smokers)

Parameter (Unit)	Non-smoker (n=54) M± SD	Heavy-smoker (n=113) M± SD	P-value
Number of collected oocytes	11.11 ± 7.44	11.99 ± 6.54	**0.357**
Number of injected oocytes	9.00 ± 5.86	8.72 ± 5.24	**0.931**
Number of fertilized oocytes	6.95 ± 5.09	6.89 ± 4.41	**0.791**
Number of cleaved embryos	6.64 ± 4.71	6.58 ± 4.37	**0.908**
Fertilization rate (%)	77.96 ± 19.30	77.65 ± 21.35	**0.943**
Number of grade 1 embryos (G1)	2.68 ± 2.89	2.03 ± 2.33	**0.265**
Number of grade 2 embryos (G2)	2.45 ± 2.29	2.94 ± 2.38	**0.140**
Embryos grade score	1.98 ± 0.60	1.84 ± 0.53	**0.450**

M: mean; **n**: Number; **SD**: standard deviation

The clinical data of patients who had had ICSI therapy and whose husbands were heavy-smokers (n=113) and non-smokers (n= 54) showed no significant difference. The mean number of collected, injected, fertilized oocytes and the fertilization rate were similar in both investigated groups. In addition, the mean of the grade 1 embryos, the grade 2 embryos and the embryos grad score did not differ significantly from each other (**Table 7**).

3.2. The study of the gene expression level of H2BFWT, PRM1, PRM2, TNP1 and TNP2

3.2.1. The characteristic of the study population

141 samples out of 167were used for the evaluation of the gene expression levels of some of the key nuclear proteins in the sperm chromatin remodelling: H2BFWT, TNP1, TNP2, PRM1, and PRM2 in order to identify their correlation to the classical sperm parameters and ICSI outcomes using two of the common tests of the sperm's chromatin integrity, namely, CMA3 and a stability TUNEL Assay.

Table 8: Descriptive statistics of studied parameters for the gene expression study patients (n=141)

Parameters	M± SD	Median	Mini-Maxi
Semen volume (ml)	3.33 ± 1.57	3.00	0.70 - 9.00
Sperm concentration (10^6 per ml)	79.03 ± 59.68	63.00	2.00 - 286.00
Total motility (PR + NP %)	42.80 ± 20.999	44.00	2.00 - 91.00
Progressive motility (PR %)	20.55 ± 17.17	17.00	0 - 80.00
Morphologically normal spermatozoa (%)	6.94 ± 8.40	4.00	0 - 53.00
Protamine deficiency (CMA3 positivity) (%)	29.35 ± 20.86	24.00	0 - 98
Sperm DNA fragmentation (sDF)(%)	22.89 ± 18.85	15.00	0 - 97
Fertilization rate (%)	79.04 ± 19.85	83.00	0 - 100
Number of cleaved embryo	6.65 ± 4.74	5.00	0 - 24.00
Number of grade 1embryos (G1)	2.34± 2.56	2.00	0 - 13.00
Number of grade 2embryos (G2)	2.83±2.48	2.00	0 - 14.00
Embryos grade score	1.87±0.56	1.94	0 - 4.00
Pregnancy rate (%)	0.45 ± 0.5	-	-

M: mean;**Mini**: minimum; **Max**: maximum; **SD**: standard deviation

Table 8 provides a summary of the statistical analysis of the sperm parameters, protamine deficiency, sperm DNA fragmentation, and ICSI outcomes. The means ± SD of the sperm volume was 3.33 ± 1.57 (ml), sperm concentration was 79.03 ± 59.68 (10^6/ml), total motility was 42.80 ± 20.99%, progressive motility was 20.55 ± 17.17%, and morphologically normal spermatozoa was 6.94 ± 8.40%.

The CMA3 positivity ranged between 0 and 98% and had a mean value of 29.35 ± 20.86%.The sDF ranged between 0 and 97% and had a mean of 22.89±18.85%.

The fertilization rate was 79.04 ± 19.85%. The number of cleaved embryos ranged between (0-24), grade 1 embryos (G1) ranged between (0-13), and grade 2 embryos (G2) ranged between (0-14). The mean embryos grade score was 1.87 ± 0.56 and the mean percentage of pregnancy rate was 0.45 ± 0.5%.

Table 9: Descriptive statistic of the expression level (Delta Ct (ΔCt)) of the studied genes for all the investigated patients (n=141) in the gene expression study

Parameters	M± SD	Median	Mini-Max
ΔCt H2BFWT	12.51±4.75	12.43	1.55 - 23.52
ΔCt TNP1	0.80±2.04	1.48	-4.87 - 4.35
ΔCt TNP2	8.33±5.56	8.20	-32.09 - 20.00
ΔCt PRM1	0.26±2.42	0.55	-6.38 - 7.39
ΔCt PRM2	0.71±2.86	1.00	-5.58 - 9.85
Protamine (P1-P2) mRNA ratio	0.45±1.04	0.54	-2.88 - 4.78

M: mean; **Mini**: minimum; **Max**: maximum; **SD**: standard deviation

The relative quantification of each gene of interest expression (H2BFWT, TNP1, TNP2, PRM1 and PRM2) was done by performing the normalized expression analysis method and indicated by delta Ct value (ΔCt). The reference gene was the GAPDH and the non-smokers group was the control group.

Table 9 represents the mean ± standard deviation, the median, minimum and maximum of the relative mRNA amount of the 5 studied genes. For the H2BFWT gene, the mean ± SD was 12.51 ± 4.75. For TNP1 and TNP2, the expression level means were 0.80 ± 2.04 and 8.33 ± 5.56 respectively. Besides, the delta Ct mean value ± SD for protamin1 gene was 0.26 ± 2.42 and for protamine 2 was 0.71 ± 2.86. The Protamine mRNA ratio (P1-P2) has a mean value of 0.45 ± 1.04.

3.2.2. Non-smokers versus heavy-smokers

The patients were later divided according to smoking status into two groups: A group of non-smokers (n=43) and a group of heavy-smokers (n=98).

Table 10: Comparison of the semen analysis parameters between the control group (non-smokers) and case group (heavy-smokers) in the gene expression study

Parameter (Unit)	**Non-smokers (n=43) M ± SD**	**Heavy-smokers (n=98) M ± SD**	**P-value**
Semen volume (ml)	3.71 ± 1.76	3.17 ± 1.46	**0.181**
Sperm concentration (10^6 per ml)	98.56 ± 64.63	70.46 ± 55.59	**0.014***
Total motility (PR + NP. %)	48.42 ± 21.83	40.34 ± 20.25	**0.026***
Progressive motility (PR. %)	31.42 ± 22.24	15.78 ± 11.66	**0.0001****
Morphologically normal spermatozoa (%)	12.91 ± 12.76	4.32 ± 2.93	**0.0001****

M: mean; **n**: Number; **SD**: standard deviation

By comparing the semen parameters between the two groups (**Table 10**), we found that the mean concentration and the total motility were significantly elevatedin the group of non-

smokers (p=0.014, and p= 0.026 respectively) and similarly noticed for the mean percent of the progressive motility and normal morphology (p= 0.0001).

Table 11: Comparison of the grade of protamine deficiency (CMA3 positivity) in sperm DNA and sperm DNA fragmentation (sDF) between the control group (non-smokers) and the case group (heavy-smokers) in the gene expression study

Parameter (Unit)	**Non-smokers (n=43) M±SD**	**Heavy-smokers (n=98) M± SD**	**P-value**
CMA3 positivity (%)	20.35 ± 13.43	33.30 ± 22.33	**0.001****
Sperm DNA fragmentation (sDF)(%)	14.23 ± 13.07	26.68 ± 19.77	**0.0001****

M: mean; **n**: Number; **SD**: standard deviation

Furthermore, the mean percentage of CMA3 positivity in the group of non-smokers was significantly lower in comparison to heavy-smokers (20.35 ± 13.34 vs. 33.30 ± 22.33, p=0.001). The mean percentage of sDF revealeda marked difference between the non-smoker and heavy-smoker groups (14.23 ± 13.07 vs. 26.68 ± 19.77, p= 0.0001) (**Table 11)**.

Table 12: Comparison of the ICSI-outcomes between the control group (non-smokers) and the case group (heavy-smokers) in the gene expression study

Parameter (Unit)	Non-smokers (n=43) M± SD	Heavy-smokers (n=98) M± SD	P-value
Fertilization rate (%)	78.23 ± 19.48	79.40 ± 20.10	0.691
Number of the cleaved embryo	6.70 ± 4.75	6.63 ± 4.76	0.923
Number of grade 1 embryos (G1)	2.67 ± 2.93	2.19 ± 2.39	0.567
Number of grade 2 embryos (G2)	2.49 ± 2.31	2.98 ± 2.55	0.195
Embryos grade score	1.99 ± 0.61	1.82 ± 0.54	0.229
Pregnancy rate (%)	0.60 ± 0.49	0.38 ± 0.48	**0.013****

M: mean; **n**: Number; **SD**: standard deviation

Moreover, except for the pregnancy rate, which was significantly elevatedin the group of non-smokers than in the heavy-smokers group (p=0.013), there were no significant dissimilarities in the other investigated parameters, namely, fertilization rate, number of

cleaved embryos, number of grade 1 (G1), grade 2 (G2) embryos and the embryo grade score, between the two groups (**Table 12**).

Table 13: Comparison of the mRNA relative amount of the studied genes between the control group (non-smokers) and the case group (heavy-smokers) in the gene expression study

Gene expression level (ΔCt)	**Non-smoker (n=43)** **M ± SD**	**Heavy-smoker (n=98)** **M ± SD**	**P-value**
H2BFWT	10.75 ± 4.90	13.35 ± 4.45	**0.001****
TNP1	-0.04 ± 1.954	1.17 ± 1.98	**0.001****
TNP2	5.61 ± 7.54	9.52 ± 3.92	**0.0001****
PRM1	-0.64 ± 2.248	0.66 ± 2.41	**0.002****
PRM2	-0.53 ± 2.61	1.25 ± 2.82	**0.001****
Protamine (P1–P2) mRNA ratio	0.11 ± 0.84	0.60 ± 1.08	**0.001****

M: mean; **n**: Number; **SD**: standard deviation

The relative amounts of the investigated genes mRNA (mean delta ct) (H2BFWT, TNP1, TNP2, PRM1, and PRM2) were differentially expressed between the compared groups. This difference between the group of heavy smokers and the group of non-smokers was highly significant ($p<0.01$) (**Table 13**). Furthermore, the protamine mRNA ratio was also significantly elevatedin the case group in comparison to the control group (0.60 ± 1.08 vs. 0.11 ± 0.84, $p=0.001$) (**Table 13)**.

Table 14: Mean expression levels (delta Ct) of H2BFWT, TNP1, TNP2, PRM1 and PRM2 genes from spermatozoa in the case group (heavy-smokers) compared to control group (non-smokers)

Genes	Mean delta Ct Non-smokers	Mean delta Ct Heavy-smokers	Fold change (FC)	Log2 fold change	Regulation
H2BFWT	10.75	13.35	0.16	-2.6	Down
TNP1	-0.04	1.17	0.43	-1.21	Down
TNP2	5.61	9.52	0.06	-3.91	Down
PRM1	-0.64	0.66	0.40	-1.3	Down
PRM2	-0.53	1.25	0.29	-1.78	Down

Since the correlation between delta Ct (ΔCt) and the gene expression level is contradictory, the higher delta Ct values indicate that the gene expression is decreased. This was demonstrated in **table 14** where the H2BFWT, TNP1, TNP2, PRM1 and PRM2 were down-regulated (Fold change <0.5).

3.2.3. Correlation between the investigated sperm parameters and ICSI outcomes

Table 15: Correlation between sperm parameters and ICSI outcomes in the heavy-smokers group (n=98)

		Fertilization rate (%)	Number of cleaved embryos	number of grade 1 embryos (G1)	number of grade 2 embryos (G2)	Embryos grade score
Semen volume (ml)	r	-0.012	-0.112	-0.160	-0.031	0.035
	p	0.904	0.271	0.115	0.760	0.735
Sperm concentration (10^6 per ml)	r	0.033	-0.075	-0.027	-0.082	-0.018
	p	0.744	0.465	0.790	0.427	0.860
Total motility (PR + NP. %)	r	0.035	-0.078	-0.138	0.008	-0.121
	p	0.729	0.444	0.175	0.940	0.237
Progressive motility (PR. %)	r	0.071	-0.002	-0.079	0.114	-0.049
	p	0.489	0.983	0.441	0.268	0.630
Morphologically normal spermatozoa (%)	r	0.174	0.049	-0.103	0.123	0.023
	p	0.087	0.631	0.315	0.231	0.823

In the heavy-smokers group, the ejaculate volume, sperm concentration, motility and morphologically normal spermatozoa showed no correlation with the ICSI outcomes (fertilization rate, number of cleaved, grade 1 (G1) and, grade 2 (G2) embryos and, the embryos grade score) (**Table 15**).

Table 16: Correlation between sperm parameters and ICSI outcomes in the non-smokers group (n=43)

		Fertilization rate (%)	**Number of the cleaved embryos**	**Number of grade 1embryos (G1)**	**Number of grade 2embryos (G2)**	**Embryos grade score**
Semen volume (ml)	r	-0.092	-0.033	0.006	-0.110	0.019
	p	0.556	0.834	0.969	0.482	0.905
Spermconcentration (10^6 per ml)	r	-0.083	-0.088	-0.154	0.010	0.166
	p	0.598	0.576	0.324	0.948	0.288
Total motility (PR + NP. %)	r	-0.064	**-0.522****	**-0.391****	**-0.511****	**0.338***
	p	0.685	**0.0001**	**0.009**	**0.0001**	**0.027**
Progressive motility (PR. %)	r	0.081	**-0.584****	**-0.425****	**-0.585****	**0.351***
	p	0.605	**0.0001**	**0.004**	**0.0001**	**0.021**
Morphologically normal spermatozoa (%)	r	0.209	**-0.535****	**-0.447****	**-0.433****	**0.368***
	p	0.178	**0.0001**	**0.003**	**0.004**	**0.015**

On the contrary, for the non-smokers group (**Table 16**), the mean value of cleaved embryos correlated as being negatively significant (p=0.0001) with the mean percentage of sperm parameters: total motility (r=-0.522), progressive motility (r=-0.584), and morphologically normal spermatozoa) (r=-0.535). Moreover, similar correlations were observed between the same parameters (total motility, progressive motility, and morphologically normal spermatozoa) and the number of grade 1embryos (r=-0.391, p=0.009; r=-0.425, p=0.004; r=-0.447, p=0.003 respectively), the number of grade 2 embryos (r=-0.511, p=0.0001; r=-0.585, p=0.0001; r=-0.433, p=0.004 respectively). Moreover, the embryos grade score correlated positively with the investigated sperm parameters (Total motility progressive motility and morphologically normal spermatozoa) (r=0.338, p=0.027; r=0.351, p=0.021; r=0.368, p=0.015 respectively) (**Table 16**).

3.2.4. Correlation between the different sperm parameters, protamine deficiency and sperm DNA fragmentation

Table 17: Correlation between the investigated sperm parameters, protamine deficiency (CMA3 positivity) and sperm DNA fragmentation (sDF) in the heavy-smokers group (n=98)

		Semen volume (ml)	Sperm concentration (10^6 per ml)	Total motility (PR + NP. %)	Progressive motility (PR. %)	Morphologic-ally normal spermatozoa (%)	CMA3 positivity(%)	sDF(%)
Semen volume (ml)	r	1.000	-0.042	0.004	-0.024	0.097	-0.117	-0.029
	p		0.681	0.965	0.812	0.343	0.251	0.776
Sperm concentration (10^6 per ml)	r	-0.042	1.000	**0.294****	**0.515****	**0.531****	**-0.233***	**-0.263****
	p	0.681		**0.003**	**0.0001**	**0.0001**	**0.021**	**0.009**
Total motility (PR + NP. %)	r	0.004	**0.294****	1.000	**0.677****	**0.439****	0.133	0.101
	p	0.965	**0.003**		**0.0001**	**0.0001**	0.193	0.321
Progressive motility (PR. %)	r	-0.024	**0.515****	**0.677****	1.000	**0.583****	-0.097	-0.063
	p	0.812	**0.0001**	**0.0001**		**0.0001**	0.340	0.534
Morphological-ly normal spermatozoa (%)	r	0.097	**0.531****	**0.439****	**0.583****	1.000	-0.140	-0.177
	p	0.343	**0.0001**	**0.0001**	**0.0001**		0.169	0.081
CMA3 positivity (%)	r	-0.117	**-0.233***	0.133	-0.097	-0.140	1.000	**0.484****
	p	0.251	**0.021**	0.193	0.340	0.169		**0.0001**

In the heavy-smokers group (**Table 17**), the mean percentage of the sperm concentration correlated positively with the mean percentages of total motility (r= 0.294, p= 0.003), progressive motility (r=0.515, p= 0.0001), and morphologically normal spermatozoa (r=0.531, p= 0.0001) and correlated negatively with the protamine deficiency (r=-0.233, p=0.021) and the sDF (r=-0.263, p=0.009). The total and progressive motility showed a high positive correlation with the mean of morphologically normal spermatozoa (r=0.439,

r=0.583 respectively; p=0.0001). In addition, a significant correlation (r= 0.484, p=0.0001) between CMA3 positivity and sDF was shown (**Table 17**).

Table 18: Correlation between the different sperm parameters, protamine deficiency (CMA3 positivity) and sperm DNA fragmentation (sDF) (%) in the non-smokers group (n=43)

		Semen volume (ml)	Sperm concentration (10^6 per ml)	Total motility (PR + NP. %)	Progressive motility (PR. %)	Morphologica-lly normal spermatozoa (%)	CMA3 positivity (%)	sDF(%)
Semen volume (ml)	r	1.000	**-0.338***	0.184	0.242	0.227	0.147	-0.113
	p		**0.027**	0.237	0.117	0.142	0.345	0.469
Sperm concentration (10^6 per ml)	r	**-0.338***	1.000	0.159	0.107	0.110	-0.240	-0.297
	p	**0.027**		0.309	0.494	0.481	0.121	0.053
Total motility (PR + NP. %)	r	0.184	0.159	1.000	**0.874****	**0.663****	0.037	**-0.304***
	p	0.237	0.309		**0.0001**	**0.0001**	0.815	**0.048**
Progressive motility (PR. %)	r	0.242	0.107	**0.874****	1.000	**0.830****	0.018	**-0.304***
	p	0.117	0.494	**0.0001**		**0.0001**	0.907	**0.047**
Morphologic-ally normal spermatozoa (%)	r	0.227	0.110	**0.663****	**0.830****	1.000	-0.146	**-0.361***
	p	0.142	0.481	**0.0001**	**0.0001**		0.350	**0.017**
CMA3 positivity (%)	r	0.147	-0.240	0.037	0.018	-0.146	1.000	0.256
	p	0.345	0.121	0.815	0.907	0.350		0.098

Table 18 illustrates the correlations between the mean percentage of the different sperm parameters, the protamine deficiency (CMA3 positivity) and sperm DNA fragmentation (sDF) in the non-smokers group. The semen volume correlated negatively with the concentration (r=-0.338, p=0.027) and the mean percentage of total motility correlated positively with the mean percentage of morphologically normal spermatozoa (r=0.663,

p=0.0001) and negatively with the mean percentage of sDF (r=-0.304, p=0.048). In addition, the progressive motility correlated positively with morphologically normal spermatozoa (r=0.830, p=0.0001) and negatively with the mean percentage of sDF (r=-0.304, p=0.047). In addition, the mean percentage of morphologically normal spermatozoa showed a significant negative correlation with the mean percentage of sDF (r=-0.361, p=0.017).

3.2.5. Correlation between protamine deficiency, sperm DNA fragmentation and ICSI results

Table 19: Correlation between protamine deficiency (CMA3 positivity), sperm DNA fragmentation (sDF) and ICSI results in the non-smokers group (n=43)

		Fertilization rate (%)	**Number of the cleaved embryos**	**Number of grade 1 embryos**	**Number of grade 2 embryos**	**Embryos grade score**
CMA3 positivity (%)	r	0.039	-0.037	0.186	-0.235	-0.107
	p	0.805	0.813	0.232	0.130	0.496
sDF (%)	r	0.077	**0.394****	**0.341***	**0.316***	-0.045
	p	0.624	**0.009**	**0.025**	**0.039**	0.773

In the non-smokers group (**Table 19**), the mean percentage of the sperm DNA fragmentation correlated positively with the number of cleaved embryones (r=0.394, p=0.009), number of grade 1 (r=0.341, p=0.025), and number of grade 2 embryos (r=0.316, p=0.039). The remaining parameters showed no significant difference.

Table 20: Correlation between protamine deficiency (CMA3 positivity), sperm DNA fragmentation (sDF) and ICSI results in the heavy-smokers group (n=98)

		Fertilization rate (%)	**Number of the cleaved embryos**	**Number of grade 1 embryos**	**Number of grade 2 embryos**	**Embryos grade score**
CMA3 positivity (%)	r	0.152	0.082	0.009	0.061	0.145
	p	0.135	0.421	0.929	0.550	0.153
sDF (%)	r	0.050	0.117	0.086	-0.007	0.034
	p	0.625	0.252	0.402	0.946	0.736

Table 20 showed that in the heavy-smokers group, neither the mean percent of CMA3 positivity nor the sDF correlate with the clinical parameters after ICSI

3.2.6. Correlation between the expression levels of the different studied genes and sperm parameters

Table 21: Correlation between the mRNA relative amount of the studied genes and sperm parameters in the control group (non-smokers, n=43)

Expression level		Semen volume (ml)	Sperm concentration (10^6 per ml)	Total motility (PR + NP. %)	Progressive motility (PR. %)	Morphologically normal spermatozoa (%)
ΔCt PRM1	r	-0.142	-0.035	-0.062	-0.146	-0.143
	p	0.363	0.821	0.693	0.352	0.362
ΔCt PRM2	r	-0.197	-0.036	-0.072	-0.164	-0.166
	p	0.205	0.817	0.645	0.293	0.288
ΔCt TNP1	r	-0.184	0.079	-0.044	-0.175	-0.241
	p	0.237	0.615	0.778	0.262	0.119
ΔCt TNP2	r	-0.176	0.064	0.013	-0.077	-0.120
	p	0.290	0.704	0.937	0.645	0.473
ΔCt H2BFWT	r	-0.177	-0.075	0.179	0.251	0.150
	p	0.274	0.644	0.270	0.119	0.356
Protamine (P1–P2) mRNA ratio	r	**-0.463****	0.100	-0.036	-0.108	-0.180
	p	**0.002**	0.525	0.820	0.489	0.248

Table 21 shows that in the non-smokers group none of the investigated spermatozoa parameters (Semen volume, sperm concentration, total motility, progressive motility, and morphologically normal spermatozoa) correlates with the relative expression levels (ΔCt) of the PRM1, PRM2, TNP1, TNP2 and H2BFWT. However, the protamine mRNA ratio had a significant negative correlation with the semen volume (r=-0.463, p=0.002).

Table 22: Correlation between the mRNA relative amount of the studied genes and sperm parameters in the case group (heavy-smokers, n=98)

Expression level		Semen volume (ml)	Spermconcentration (10^6 per ml)	Total motility (PR + NP. %)	Progressive motility (PR. %)	Morphologically normal spermatozoa (%)
ΔCt PRM1	r	-0.008	**0.335****	**0.203***	**0.337****	**0.214***
	p	0.940	**0.001**	**0.045**	**0.001**	**0.035**
ΔCt PRM2	r	-0.028	**0.329****	0.187	**0.338****	0.166
	p	0.782	**0.001**	0.066	**0.001**	0.103
ΔCt TNP1	r	-0.076	**0.391****	**0.238***	**0.361****	**0.294****
	p	0.458	**0.0001**	**0.018**	**0.0001**	**0.003**
ΔCt TNP2	r	-0.027	**0.369****	0.197	**0.359****	**0.303****
	p	0.805	**0.0001**	0.067	**0.001**	**0.004**
ΔCt H2BFWT	r	-0.135	0.058	0.064	**0.230***	0.084
	p	0.199	0.581	0.543	**0.027**	0.421
Protamine (P1–P2) mRNA ratio	r	-0.073	0.012	**0.228***	0.128	-0.030
	p	0.478	0.910	**0.024**	0.210	0.771

In the heavy-smokers group (**Table 22**), the mean of the relative protamine 1 (PRM1) and transition protein 1 (TNP1) level correlated positively with the sperm concentration (r=0.335, p= 0.001 ; r=0.391, p= 0.0001 respectively), total motility (r=0.203, p= 0.045;r=0.238, p= 0.018 respectively), progressive motility (r=0.337, p=0.001; r=0.361, p=0.0001respectively), and morphologically normal spermatozoa (r=0.214, p=0.035; r=0.294, p=0.003 respectively), while the expression level of PRM2 showed a significant positive correlation with the sperm count (r=0.329, p=0.001) and the progressive motility (r=0.338, p=0.001).

The mean of the relative expression level of TNP2 showed a high positive correlation with the mean of the sperm count, the mean percentage of the progressive motility and the morphologically normal sperm form (r=0.369, p=0.001; r=0.359, p=0.001; r=0.303, p=0.004 respectively) (**Table 22**).

Besides, significant positive correlations between the relative amount of H2BFWT mRNA and the mean percent of sperm with progressive motility (r=0.230, p=0.027) and between the protamine mRNA ratio and the mean percent of total motility (r=0.228, p=0.024) were found (**Table 22**).

3.2.7. Correlation between the investigated gene expression levels and clinical parameters after ICSI

Table 23: Correlation between the mRNA relative amount of the studied genes and clinical parameters after ICSI in the controlgroup (non-smokers, n=43)

		ΔCt PRM1	ΔCt PRM2	ΔCt TNP1	ΔCt TNP2	ΔCt H2BFWT	Protamine (P1–P2) mRNA ratio
Fertilization rate (%)	r	-0.281	-0.258	**-0.401****	-0.310	0.011	0.002
	p	0.068	0.094	**0.008**	0.058	0.948	0.988
Number of cleaved embryos	r	0.070	0.064	0.095	0.005	-0.253	0.046
	p	0.654	0.682	0.543	0.976	0.115	0.771
Number of grade 1 embryos (G1)	r	-0.053	-0.045	-0.105	-0.106	-0.168	0.085
	p	0.733	0.776	0.504	0.526	0.301	0.589
Number of grade 2 embryos (G2)	r	0.159	0.158	0.204	0.113	-0.190	0.078
	p	0.310	0.313	0.189	0.499	0.241	0.617
Embryos grade score	r	0.092	0.068	0.132	0.035	0.170	-0.100
	p	0.556	0.666	0.400	0.836	0.293	0.522

In all the investigated parameters there was no significant correlation between the relative expression levels of PRM1, PRM2, TNP1, TNP2 and H2BFWT genes and clinical parameters after intracytoplasmic sperm injection (ICSI). However, the relative amount of the TNP1 mRNA correlated negatively with the fertilization rate (r=0.401, p=0.008) in the non-smokers group (**Table 23**).

Table 24: Correlation between the mRNA relative amount of the studied genes and ICSI outcomes in the casegroup (heavy-smokers, n=98)

		ΔCt PRM1	ΔCt PRM2	ΔCt TNP1	ΔCt TNP2	ΔCt H2BFWT	Protamine (P1–P2) mRNA ratio
Fertilization rate (%)	r	0.043	0.081	-0.014	0.093	0.086	0.195
	p	0.675	0.427	0.894	0.393	0.413	0.055
Number of cleaved embryos	r	-0.043	-0.013	-0.120	-0.021	0.003	-0.016
	p	0.674	0.898	0.240	0.849	0.979	0.878
Number of grade 1 embryos (G1)	r	-0.059	-0.083	-0.051	-0.008	0.002	-0.135
	p	0.564	0.416	0.619	0.943	0.987	0.185
Number of grade 2 embryos (G2)	r	0.109	0.105	-0.002	0.085	0.052	-0.048
	p	0.288	0.307	0.985	0.436	0.619	0.641
Embryos grade score	r	0.110	0.119	-0.003	0.052	-0.119	0.047
	p	0.282	0.244	0.979	0.636	0.256	0.647

Furthermore, no correlations were observed between the clinical parameters after ICSI (fertilization rate, number of cleaved embryos, number of grade 1 embryos, number of grade 2 embryos, embryos grade score) and the relative expression levels of H2BFWT, TNP1, TNP2,PRM1, and PRM2 genes in the heavy-smokers group (**Table 24**).

3.2.8. Correlation between the expression levels of the investigated genes and protamine deficiency (CMA3 positivity) and sperm DNA fragmentation (sDF)

Table 25: Correlation between the mRNA relative amount of the studied genes, protamine deficiency (CMA3 positivity) and sperm DNA fragmentation (sDF) in the control group (non-smokers, n=43)

		CMA3 positivity(%)	sDF (%)
ΔCt PRM1	r	-0.135	0.222
	p	0.387	0.153
ΔCt PRM2	r	-0.134	0.230
	p	0.390	0.138
ΔCt TNP1	r	-0.179	0.133
	p	0.250	0.397
ΔCt TNP2	r	-0.171	0.039
	p	0.305	0.814
ΔCt H2BFWT	r	-0.038	-0.023
	p	0.814	0.887
Protamine (P1–P2) mRNA ratio	r	-0.091	0.100
	P	0.560	0.524

By examining the correlation between the mRNA relative amount of the studied genes (TNP1, TNP2, PRM1, PRM2, and H2BFWT), and protamine deficiency (CMA3 positivity) as well as sperm DNA fragmentation (sDF), no correlation has been shown either in non-smokers (**Table 25**) or in heavy-smokers (**Table 26**), a correlation could not be found.

Table 26: Correlation between the mRNA relative amount of the studied genes, protamine deficiency (CMA3 positivity) and sperm DNA fragmentation (sDF) in the case group (heavy-smokers, n=98)

		CMA3 positivity (%)	**Sperm DNA fragmentation (sDF) (%)**
ΔCt PRM1	r	0.036	-0.055
	p	0.722	0.587
ΔCt PRM2	r	0.165	0.069
	p	0.104	0.499
ΔCt TNP1	r	0.002	-0.008
	p	0.982	0.935
ΔCt TNP2	r	0.029	-0.039
	p	0.789	0.721
ΔCt H2BFWT	r	0.076	-0.002
	p	0.469	0.983
Protamine (P1–P2) mRNA ratio	r	**0.413****	**0.302****
	p	**0.0001**	**0.003**

Nevertheless, in the heavy smokers' group (**Table 26**), the protamine mRNA ratio showed a high positive correlation with both protamine deficiency (CMA3 positivity) and sperm DNA fragmentation (sDF) (r= 0.413, p=0.0001; r=0.302, p=0.003 respectively).

3.2.9. Correlation between the relative expression levels of the studied genes: H2BFWT, TNP1, TNP2, PRM1 and PRM2

Table 27: Correlation between the mRNA relative amount of the studied genes in the control group (non-smokers, n=43)

		ΔCt PRM1	ΔCt PRM2	ΔCt TNP1	ΔCt TNP2	ΔCt H2BFWT	Protamine (P1–P2) mRNA ratio
ΔCt PRM1	r	1.000	**0.961****	**0.920****	**0.731****	0.257	0.299
	p		**0.0001**	**0.0001**	**0.0001**	0.110	0.052
ΔCt PRM2	r	**0.961****	1.000	**0.887****	**0.709****	0.237	**0.488****
	p	**0.0001**		**0.0001**	**0.0001**	0.141	**0.001**
ΔCt TNP1	r	**0.920****	**0.887****	1.000	**0.737****	0.236	0.294
	p	**0.0001**	**0.0001**		**0.0001**	0.142	0.055
ΔCt TNP2	r	**0.731****	**0.709****	**0.737****	1.000	**0.487****	**0.349***
	p	**0.0001**	**0.0001**	**0.0001**		**0.003**	**0.032**
ΔCt H2BFWT	r	0.257	0.237	0.236	**0.487****	1.000	0.229
	p	0.110	0.141	0.142	**0.003**		0.155

In the non-smokers group (**Table 27**), the relative expression level of the H2BFWT correlated positively with the relative amount of TNP2 mRNA (r=0.487, p=0.003).

Moreover, the relative expression levelof TNP1 showed a highly positive correlation (p=0.001) with the expression levels of TNP2, PRM1, and PRM2 (r=0.737, r=0.920, r=0.887).

The TNP2 relative expression level correlated positively (p=0.0001) with the relative amount of PRM1 and PRM2 (r=0.731, r=0.709 respectively).

The correlation between the PRM1 and PRM2 expression levels was a high positive significant correlation (r=0.961, p=0.0001).

The protamine mRNA ratio correlated positively with the expression levels of TNP2 (r=0.349, p=0.032) and PRM2 (r=0.488, p=0.001).

Table 28: Correlation between the mRNA relative amount of the studied genes in the case group (heavy-smokers, n=98)

		ΔCt PRM1	ΔCt PRM2	ΔCt TNP1	ΔCt TNP2	ΔCt H2BFWT	Protamine (P1–P2) mRNA ratio
ΔCt PRM1	r	1.000	**0.926****	**0.859****	**0.903****	**0.254***	0.150
	p		**0.0001**	**0.0001**	**0.0001**	**0.014**	0.139
ΔCt PRM2	r	**0.926****	1.000	**0.822****	**0.887****	**0.398****	**0.445****
	p	**0.0001**		**0.0001**	**0.0001**	**0.0001**	**0.0001**
ΔCt TNP1	r	**0.859****	**0.822****	1.000	**0.814****	**0.357****	0.186
	p	**0.0001**	**0.0001**		**0.0001**	**0.0001**	0.066
ΔCt TNP2	r	**0.903****	**0.887****	**0.814****	1.000	**0.354****	**0.307****
	p	**0.0001**	**0.0001**	**0.0001**		**0.001**	**0.004**
ΔCt H2BFWT	r	**0.254***	**0.398****	**0.357****	**0.354****	1.000	**0.342****
	p	**0.014**	**0.0001**	**0.0001**	**0.001**		**0.001**

In the heavy-smokers group (**Table 28**), the H2BFWT expression level showed a significant positive correlation with the transition proteins 1 (TNP1) and 2 (TNP2) relative expression levels (r=0.357, p= 0.0001; r=0,354, p=0.001 respectively).

Moreover, a positive correlation with protamines 1 (PRM1) and 2 (PRM2) mRNA relative amount was found (r=0.254, p=0.014; r=0.398, p=0.0001 respectively).

The relative amount of TNP1 gene mRNA demonstrates a highly significant positive correlation with the following mRNA relative amounts of TNP2, PRM1 and PRM2 genes (r=0.814, r=0.859, r=0.822; p<0.001 respectively).

Similarly, the TNP2 gene mRNA relative amount showed a highly positive correlation (p=0.0001) to PRM1 and PRM2 expression levels (r=0.903, r=0.887 respectively).
PRM1 and PRM2 expression levels also correlated as positively significant with each other's (r=0.926, p=0.0001).
In contrast to the group of non-smokers, the protamine mRNA ratio correlated significantly positive to TNP2 mRNA relative amount (r=0.307, p=0.004), PRM2 mRNA relative amount (r=0.445, p=0.0001), and to H2BFWT mRNA relative amount (r=0.342, p=0.001) (**Table 28**).

3.3. Investigations of single nucleotide polymorphisms (SNP) and mutations in H2BFWT, PRM1, and PRM2 genes

3.3.1. Variant calling

For each sample, primary and secondary sequences were extracted from the chromatogram (.ab1) files using the tool Tracy (https://github.com/gear-genomics/tracy) to obtain the allelic sequences for each gene. The aligner bwa (Li & Durbin, 2009) was used to map the resulting FASTA-reads to the hg19 reference genome. Since for each individual forward and reverse Sanger reads were provided, a BAM file containing four reads was produced for each individual in this way. Next, a set of potential SNP positions was generated. We used samtools mpileup (Li *et al*., 2009) to report all positions in the alignment files where at least one read carried an allele different from the reference sequence. All resulting SNP candidates were then genotyped in all individuals using WhatsHap (Ebler *et al*., 2018, Patterson *et al*., 2015). The variant calling process is illustrated in Figure 19 below.

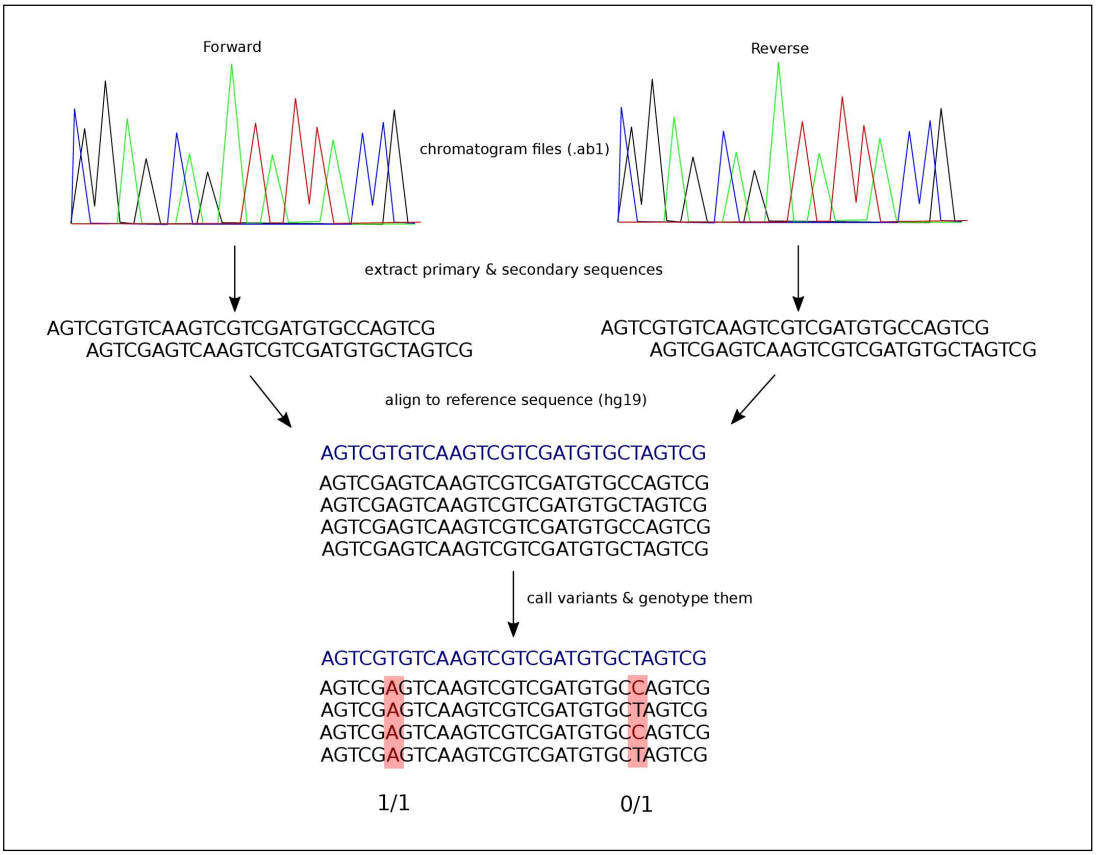

Figure 19: Variant Calling. Allelic sequences were extracted from the chromatogram files and aligned to a reference genome. Next, variants were called and genotyped in all samples.

3.3.2. Quality Control

From the set of all SNPs resulting from the previous steps, all positions with an allele frequency above 5 % across all studied individuals were selected. A set of nine SNP positions resulted. Three of them were located on chromosome X. Since all sequenced individuals were males, one would expect all of these positions to be genotyped as either 0/0 or 1/1 since each individual carry only one X chromosome. Indeed, our genotyping algorithm genotyped all SNPs located on the X chromosome as 0/0 or 1/1 in all individuals. The remaining six SNPs were located on chromosome 16. They were tested for Hardy-Weinberg Equilibrium using Fisher's Exact Test. Four of these six SNPs showed no significant deviation from HWE. We concluded that the two other SNPs, for which the test reported a significant deviation from HWE, might not be real variants and excluded them from further analysis. Hence, our final set of SNP calls contained 7 SNPs.

Corresponding positions (wrt. to reference sequence hg19) and alleles of these SNPs are shown below (**Table 29**).

Table 29: Detected SNPs and their allele frequencies

Genomic Position (hg19)	ID	Reference allele	Alternative allele	Allele Frequency (across all samples)
Chromosome 16 11374866	rs737008	G	T	0.47
Chromosome 16 11369534	rs424908	G	A	0.99
Chromosome 16 11369855	rs2070923	G	T	0.50
Chromosome 16 11369930	rs1646022	C	G	0.30
Chromosome X 103267865	rs553509	C	T	0.72
Chromosome X 103268241	rs7885967	G	A	0.60
Chromosome X 103268333	rs578953	G	A	0.09

Furthermore, we observed that our SNP calls had been reported previously by the 1000 Genomes project (1000 Genomes Project Consortium, 2012). As an additional quality control, we compared the allele frequencies that we obtained for these SNPs to the ones reported by 1000 Genomes and observed that they matched very well. The results are shown in Figure 2. The blue dots correspond to the allele frequencies that we have observed across our samples, and the boxplots show the distribution of allele frequencies for these variants that were observed across several populations studied in the 1000 Genomes project.

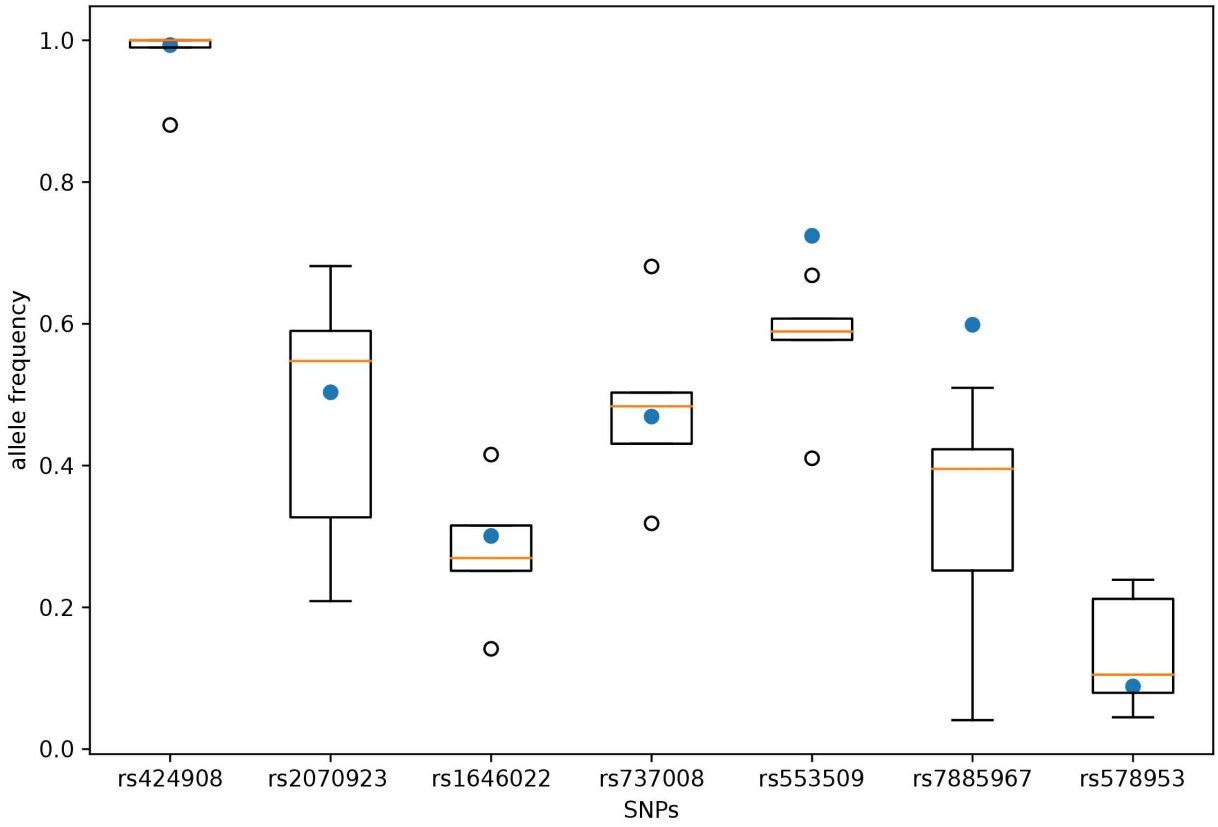

Figure 20: Comparison to 1000 Genomes allele frequencies. We compared the allele frequencies for our detected SNPs (blue) to the ones previously reported by the 1000 Genomes project (1000 Genomes Project Consortium, 2012) for these variants across several populations

3.3.3. Distribution of SNPs in H2BFWT, PRM1 and PRM2 genes among the groups of heavy-smokers and non-smokers

For each group heavy smokers and non-smokers, we computed a contingency table for each SNP by counting the number of reference and alternative alleles among the individuals belonging to the different groups. The goal was to test the association between SNP alleles and each of the classes. We then performed Fisher's Exact Test to find significant differences in allele distributions among the two groups and corrected for multiple testing by performing a Benjamini Hochberg correction (alpha=0.05). None of the SNPs was reported as being significant. All tested SNPs and corresponding allele frequencies are shown in **Tables 30-32**.

3.3.3.1. Single nucleotides polymorphisms (SNP) in the H2BFWT gene

The H2BFWT gene is located in the X chromosomes (q22.2 band). Our patients were male so the detected SNPs: rs7885967, rs553509 and rs578953 were homozygous SNPs. The same observation as for the previous genes, there were no significant differences in allele distribution among the heavy-smokers and non-smokers. SNP rs7885967 is located in the 5 prime untranslated regions. The variant rs553509 was located in a coding region (Exon 1) and it was qualified as a missense mutation that caused an amino acid change of arginine to histidine. For those 2 SNPs, the alternative allele frequency was higher than the reference allele one (**Table 29**), so the number of heavy smokers and non-smokers with the homozygous minor-type was higher than with major homozygous one (**Table 30**). The opposite was observed for the SNP rs 578953, which is an upstream gene variant. For this variant, the reference allele frequency was higher than the alternative allele frequency (0.09). So, 8 heavy smokers and 5 non-smokers were minor homozygous (A/A), and 103 heavy smokers and 27 non-smokers were major homozygous (G/G).

Table 30: Recapitulation of results obtained by direct sequencing of PCR products including the H2BFWT gene (*Genomic and allelic frequencies are denoted*)

SNP	Gene region	AA change	ID	Heavy-smokers		Non-smokers		p-value
				Genotype	Allele	Genotype	Allele	
chrX g.103268241G>A	5 prime UTR	NA	rs7885967	A/A (65) 0.59 G/A (0) 0.0 G/G (46) 0.41	A= 0.59 G= 0.41	A/A (20) 0.63 G/A (0) 0.0 G/G (12) 0.37	A= 0.63 G= 0.37	NS
chrX g.103267865C>T	Exon 1	R/H	rs553509	T/T (77) 0.7 C/T (0) 0.0 C/C (33) 0.3	T= 0.7 C= 0.3	T/T (25) 0.81 C/T (0) 0.0 C/C (6) 0.19	T= 0.81 C= 0.19	NS
chrX g.103268333G>A	upstream	NA	rs578953	A/A (8) 0.07 G/A (0) 0.0 G/G (103) 0.93	A= 0.07 G= 0.93	A/A (5) 0.16 G/A (0) 0.0 G/G (27) 0.84	A= 0.16 G= 0.84	NS

UTR: untranslatedregion

NA: not applicable, NS: no significant differentiation was distinguished by comparing the genotype and allele frequencies between the heavy-smoker and non-smoker groups.

3.3.3.2. Single nucleotides polymorphisms (SNP) in the PRM1 gene

Table 31: Recapitulation of results obtained by direct sequencing of PCR products including thePRM1 gene (*Genomic and allelic frequencies are denoted*)

SNP	Gene region	AA change	ID	Heavy-smokers		Non-smokers		p-value
				Genotype	Allele	Genotype	Allele	
chr16 g.11374866G>T	Exon 2	None	rs737008	T/T (30) 0.28 G/T (46) 0.43 G/G (32) 0.29	T= 0.49 G= 0.51	T/T (7) 0.21 G/T (11) 0.33 G/G (15) 0.46	T= 0.38 G= 0.62	NS

NS: no significant differentiation was distinguished by comparing the genotype and allele frequencies between the heavy-smoker and non-smoker groups.

One SNP has been identified in the PRM1 gene in the study population: rs737008. This SNP is a synonym variant that is located in the coding region of PRM1 (Exon 2) at position g.11374866G>T. There was no significant dissimilarity in allele distribution betweenthe heavy-smokers and non-smokers (**Table 31**). Thirty-two heavy smokers and fifteen non-smokers are homozygous major G/G types, 46 heavy-smokers and 11 non-smokers were heterozygous (G/T), and 30 heavy smokers and 7 non-smokers were the homozygous minor type (T/T) (**Table 31**).

3.3.3.3. Single nucleotides polymorphisms (SNP) in the PRM2 gene

Table 32 shows that 3 SNPs have been found in the PRM2 gene. Two changes were detected in the intronic region (rs2070923 and rs1646022) and one was located in the 3 prime untranslated regions (rs424908). There were no significant differences in allele distribution among the heavy-smokers and non-smokers. For the first variant, in the intronic region, at position g.11369930 G>C, 15 heavy-smokers and 4 non-smokers were minor homozygous (G/G), 38 Heavy smokers and 9 non-smokers were heterozygous (C/G), and 58 heavy smokers and 19 non-smokers were major homozygous (C/C). The second intronic variant was also distributed in three groups: 31 patients of the heavy-smokers group and 12 of non-smokers ones were minor homozygous (T/T), 47 heavy smokers and 11 non-smokers were heterozygous (G/T), and 33 heavy smokers and 9 non-smokers were major homozygous (G/G). The SNP rs424908 was minor homozygous SNPs (A/A) with an

allele frequency of 0.99 across all samples (**Table 29**) and only one patient for each of the studied groups was heterozygous (G/A).

Table 32: Recapitulation of results obtained by direct sequencing of PCR products including the PRM2 gene (*Genomic and allelic frequencies are denoted*)

SNP	Gene region	AA change	ID	Heavy-smokers		Non-smokers		p-value
				Genotype	Allele	Genotype	Allele	
chr16 g.11369930 C>G	Intron	NA	rs1646022	G/G (15) 0.14 C/G (38) 0.34 C/C (58) 0.52	G= 0.31 C= 0.69	G/G (4) 0.13 C/G (9) 0.28 C/C (19) 0.59	G= 0.27 C= 0.73	NS
chr16 g.11369855 G>T	Intron	NA	rs2070923	T/T (31) 0.28 G/T (47) 0.42 G/G (33) 0.3	T= 0.49 G= 0.51	T/T (12) 0.38 G/T (11) 0.34 G/G (9) 0.28	T= 0.54 G= 0.46	NS
chr16 g.11369534 G>A	3 prime UTR	NA	rs424908	A/A (110) 0.99 G/A (1) 0.1 G/G (0) 0.0	A= 1.0 G= 0.0	A/A (31) 0.97 G/A (1) 0.03 G/G (0) 0.0	A= 0.98 G= 0.02	NS

UTR: untranslatedregion

NA: not applicable, NS: no significant differentiation was distinguished by comparing the genotype and allele frequencies between the heavy-smoker and non-smoker groups.

3.3.4. Association between SNPs and standard sperm parameters, protamine deficiency (CMA3 positivity), sperm DNA fragmentation, and clinical outcomes after ICSI therapy

In order to investigate the association between SNPs and standard sperm parameters, protamine deficiency (CMA3 positivity), and sperm DNA fragmentation (sDF), a Wilcoxon rank-sum test was performed for each combination of SNP and phenotype. The goal was to find out whether the distribution of phenotype values is the same for individuals with different genotypes.

For each SNP position and each sperm parameter, we first compared the distribution of values for individuals with genotype 0/1 and 1/1 to the distribution of values for individuals with genotype 0/0. Next, we compared distributions for genotypes 0/0 and 0/1 to the distributions for individuals with genotype 1/1. We again corrected for multiple testing by applying a Benjamini Hochberg correction (alpha=0.05). None of the SNPs showed a significant association with any of the phenotypes and the resulting p-values for each parameter are shown in Supplementary Tables 33-43.

4. DISCUSSION

According to WHO, a significant proportion of the population is affected by infertility and subfertility (WHO, 2017). For this reason, more research is needed, especially in developing countries, to improve infertility and subfertility diagnosis, regulation and therapy.

Infertility is a complicated condition affected by particular or several physiological and physical factors. The highest proportion of human infertility can be correlated with a person's age, lifestyle, environmental factors and physical state (Das *et al.,* 2017). To recognize the connection between the reasons for infertility, which are responsible for the regulation of the expression of genes involved in fertility, genetic factors, and aberrant epigenetic mechanism may lead to an understanding of as yet unidentified reasons for reproductive infertility (Bjornsson *et al*., 2004; Liu & Tollefsbol, 2008; Bunkar*et al*., 2016).

Several lifestyle behaviours, such as nutrition, sport, drinking alcohol and tobacco smoking are reported to have an influence on both male and female reproductive health, and, in a number of cases, they may have an effect on epigenetic mechanism alterations, which may be associated with major common human maladies (Alegría-Torres *et al*., 2011; Sharma *et al*., 2013).

Around 46 % of males of reproductive age between 20- and 39-years-old are cigarette smokers (Ng *et al*., 2014). Almost 75% of men that are daily smokers are living in countries that have a medium or high human development index (Drope *et al*., 2018).

For this reason, we focused, in this current study, on the influence of tobacco smoke on the male reproductive function: sperm parameters, sperm DNA quality and integrity and the possible genetic and epigenetic alterations by focusing on H2BFWT, TNP1, TNP2, PRM1 and PRM2 genes. Moreover, we investigated the consequences of this on ICSI outcomes.

4.1. Smoking and semen parameters

Burning tobacco is highly dangerous for almost every organ system in the human body. It causes the release of more than 7000 toxic chemicals, mutagenic elements, and at least 70 familiar carcinogens (Drope*et al*., 2018). Nicotine is one of these chemicals that makes

someone addicted to tobacco smoke. In male smokers, the serum and seminal levels of cotinine and trans-3′-hydroxycotinine (nicotine metabolites) (Zhu *et al*., 2013), are approximately similar, whereas the serum nicotine levels were lower than the seminal levels (Pacifi *et al*., 1993). Pacifi and his colleagues demonstrated the the levels of cotinine in seminal plasma had a positive correlation with the sperm motility type progressive (PR) but negatively with the total sperm motility (Pacifi *et al*., 1993)**.** Chen and Kuo (2007) demonstrated that cotinine levels in semen had negative effects on sperm count, motility and normal morphology.

Another study showed that cotinine in seminal plasma reduces sperm parameters. It can therefore be used as a biomarker for smoking by patients that have fertility problems (Hammadeh *et al*., 2010).

However, heavy metals, like lead and cadmium, are the crucial compounds in tobacco thataffect the sperm parameters. It was reported that blood and seminal cadmium levels depend on the number of cigarettes smoked and showed a positive correlation with the number of daily smoked cigarettes per year (Oldereid *et al*., 1994). Moreovers, in abnormal spermatozoa, the cadmium and lead levels in seminal plasma had a negative correlation with sperm parameters (concentration, motility and abnormal morphology) (Pant *et al*., 2015).

In the current study, we found in our heavy-smokers group (n=113) a significant decrease ($p<0.01$) in standard semen parameters, namely, sperm concentration, progressive motility (PR), and sperm normal morphology, in comparison to our non-smokers group (62.17 ± 51.68 mill/ml vs. 88.09 ± 63.42 mill/ml, 14.86 ± 10.95% vs. 27.31 ± 21.78%, and 4.01 ± 2.88% vs. 10.87 ± 12.11% respectively). However, semen volume and total motility (PR+NP) were not significantly reduced in heavy-smokers in comparison to non-smokers **(Table 5).**

The present results are in accordance with various other studies that reported the toxic elements in tobacco smoking to have negative effects on semen quality (El-Melegy & Ali, 2011; Joo *et al.,* 2012; Anifandis *et al*., 2014; Hamad *et al*., 2014). The Practice Committee of the American Society for Reproductive Medicine also disclosed in 2012 that the relationship between tobacco consumption and reduced sperm parameters is a dose-

response relationship (Practice Committee of the American Society for Reproductive Medicine, 2012). In a meta-analysis, Sharma *et al*. (2016) concluded that smoking had a generally negative influence on standard semen parameters and this was generally more obvious in infertile male patients than in the common population because the spermatozoa are probably more sensitive to the inhaled toxic chemicals (Sharma *et al.,* 2016).

Nevertheless, other studies reported that smoking had no meaningful effect on conventional sperm parameters (Saleh *et al*., 2002b; Trummer *et al*., 2002; Martini *et al*., 2004; Sepaniak *et al*., 2006). Therefore, further studies at the molecular level are needed to find out how tobacco smoking affects sperm function and to shed more light on the clinical condition.

4.2. Smoking and sperm DNA damage

Spermatozoa chromatin has a specific composition in comparison to somatic cell chromatin and the gradual transition of a histone to a protamine during spermatogenesis is a critical process for normal sperm function. In fact, chromatin tight packaging in spermatozoa is essential to protect the paternal genome against endogenous and exogenous agents, such as genotoxic or mutagenic agents, free radicals and nucleases, and to deliver it safely to the oocyte (Rathke *et al*., 2014).

Any defect occurring at any of the steps of spermatogenesis and leading to chromatin remodelling may cause damage to DNA integrity, which may in turn influence the sperm morphology and motility, and consequently lead to male infertility. The sperm will then be rendered incapable of intruding into the oocyte or fail to fertilize it (Spano *et al*., 2005). However, it has also been demonstrated that the vitality, motility and morphology of spermatozoa with DNA damage can be normal and enable the sperm to fertilize the oocyte (Yamauchi *et al*., 2012). This fertilization depends on both the oocyte quality and the DNA damage degree to the spermatozoa (Ménézo *et al*., 2010). In the event of the oocyte not being able to effectively repair sperm DNA damage, the probability of fertilization is low or nil and the risk of embryonal abnormality development will be higher (Bungum & Oleszczuk, 2018).

Thus, there are always discussions about the use of the various techniques for measuring sperm DNA fragmentation and/or compaction as a supplementary test to predict the results

of such assisted reproductive techniques (Bach & Schlegel, 2016; Amiri-Yekta *et al.,* 2017).

A number of studies have demonstrated that spermatozoa in the ejaculates of infertile men showed more DNA fragmentation (accumulation of several double or single-stranded DNA breaks) than was the case in fertile men (Irvine *et al*., 2000; Lewis *et al*., 2013).

The main causes of DNA alteration were reported to be the defective repair of double or single-stranded DNA breaks caused by topoisomerase II during chromatin remodelling (Leduc *et al*., 2008), abortive apoptosis (Sakkas *et al*., 2004), aberrant protamination (Aoki *et al*., 2005; Steger *et al*., 2007; Castillo *et al*., 2011), abnormal expression of transition proteins (Meistrich *et al*., 2003; Suganuma *et al*., 2005), interaction between toxic chemicals and/or heavy metals with protamines (Quintanilla-Vega *et al*., 2000), and oxidative stress (Aitken *et al*., 2014).

In the last decade, more studies were focussed on the mechanisms by means of which environmental and lifestyle factors, especially smoking, have an influence on the sperm genome and epigenome (Harlev *et al*., 2015) and have a potential effect on the developing embryo (Beal *et al*., 2017; Donkin & Barrès, 2018).

Tobacco smoking is in fact associated with high levels of seminal reactive oxygen species (ROS) causing oxidative DNA damage (Hammadeh *et al*., 2010; Kumar *et al*., 2015; La Maestra *et al*., 2015; Opuwari & Henkel, 2016). It has also been reported that tobacco smoke contents are correlated with DNA adduct formation leading to DNA damage (Perrin *et al*., 2011; Phillips & Venitt, 2012).

The following two techniques were used in the current study to evaluate the sperm DNA integrity: terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) for sperm DNA fragmentation (sDF) assessment, and chromomycin A3 (CMA3) staining for protamine deficiency (CMA3 positivity) assessment. In the heavy-smokers group, the mean percentage of sDF and CMA3 positivity were significantly higher than that of the non-smokers group (26.86 ± 19.77% vs.14.23 ± 13.07%, p = 0.0001; 33.30 ± 23.33% vs. 20.35 ± 13.43%, p = 0.001 respectively) (**Table 11**).

These results are in line with a number of studies that used different techniques for the determination of sperm DNA fragmentation. Most of the human researches using a TUNEL

assay demonstrated that the levels of DNA fragmentation in smokers was higher than in non-smokers (Sepaniak *et al*., 2006; Hammadeh *et al*., 2010; Aydin *et al*., 2013). Similar results were found in other studies using different techniques to evaluate DNA fragmentation (Mitra *et al*., 2012; El-Melegy & Ali, 2011; Taha *et al*., 2014; Cui *et al*., 2016). Contradictory studies, however, have concluded that smoking and DNA damage had no correlation (Bojar *et al*., 2013; Bounartzi *et al*., 2016).

On the other hand, a significant positive correlation has been found between protamine deficiency (CMA3 positivity) and sperm DNA fragmentation (sDF) (r=0.484, p=0.0001) in the group of heavy-smokers (**Table 17**), but there was no correlation (r=0.256, p=0.098) between these two parameters in the non-smokers group (**Table 18**). Similar results have been demonstrated by other groups who reported that abnormal protamination leads to abnormal chromatin condensation and raises the sensitivity of sperm DNA to external stress causing an oxidative attack (Hammadeh *et al*., 2010; Ni *et al*., 2014; Hammadeh *et al*., 2016; Ni *et al*., 2016).

In the heavy-smokers group (**Table 17**), the mean percentage of the sperm concentration correlated negatively with the CMA3 positivity (r=-0.233, p=0.021) and the sDF (r=-0.263, p=0.009). A similar situation was observed in the non-smokers group (**Table 18**), the sperm DNA fragmentation sDF being correlated negatively with the mean percentage of total motility (r=-0.304, p=0.048), the mean of progressive motility (r=-0.304, p=0.047) and the mean percentage of morphologically normal spermatozoa (r=-0.361, p=0.017). These results support the claim that the DNA damage in sperm influences negatively the sperm quality, thereby confirming earlier studies (Arabi, 2004; Tarrozi *et al*., 2009; Smit *et al*., 2010).

However, a number of studies have not succeeded in demonstrating a relationship between traditional seminal parameters and sperm DNA damage (Henkel *et al*., 2004; Sepaniak *et al*., 2006; Hammadeh *et al.,* 2010).

Moreover, the use of different techniques to measure alterations in sperm DNA has been a controversial issue in the field of assisted reproduction field. Bungum *et al.* (2006) demonstrated that the pregnancy rate after intrauterine insemination (IUI) decreased when the DNA fragmentation index (DFI) was higher than 20%. In other studies, it was concluded that couples who did not become pregnant after IVF treatment had a DNA

fragmentation higher than 25% and their embryo quality correlated negatively with sDF (Simon *et al*., 2011). According to a study conducted by Oleszczuk *et al.* (2016), DFI above 40% is correlated with a danger of early miscarriage.

In the present study, by comparing the clinical investigated parameters between the heavy-smokers group and non-smokers group, the pregnancy rate was seen to be significantly elevatedin the group of non-smokers in comparison to the heavy-smokers group (0.60 ± 0.49% vs. 0.38 ± 0.48%; p=0.013) but other parameters showed no significant differences (**Table 12**). This is similar to the results of a study done on couples that had a normal conception but the male partner was a smoker; this study demonstrated that smoking has negative effects on fecundity and the time-to-pregnancy in a dose-dependent manner (Hassan & Killick, 2004).

Low fecundity in smokers has also been reported to be related to acrosin activity (Mulla *et al*., 1995). The correlation between smoking and the sperm's ability to fertilize the oocyte was also studied by Sofikitis and his colleagues (2000), who demonstrated that smoking correlates negatively with the sperm's potential to fertilize the oocyte (Sofikitis *et al*., 2000).

Furthermore, toxic elements resulting from tobacco combustion have been proved to decrease the mitochondrial activity and cause impairment in chromatin organization and, as a result, altering the fertilization capability (Calogero *et al*., 2009; Sharma *et al*., 2013).

In the present study, in the heavy-smoker group, the fertilization rate, the number of cleaved embryos, the number of grade 1 embryos, the number of grade 2 embryos, and the embryos' grade score had a correlation neither with the mean percent of protamine deficiency (CMA3 positivity) nor with sperm DNA fragmentation (sDF) (**Table 20**).

These results are in agreement with other studies, which reported no significant correlation between the fertilization rate, the quality of the embryo and pregnancy rates after IVF or ICSI (Chohan *et al*., 2004; Hammadeh *et al*., 2006; Zini *et al*., 2011; Zhang *et al*., 2015).

Henkel and his team (2004) showed that there was no correlation between DNA fragmentation (TUNEL assay) and the fertilization rate. Similar results had been found earlier by Benchaieb *et al.* (2003), who found no correlation between DNA fragmentation

(TUNEL assay) and embryo quality. In both groups, however, it was shown that sperm DNA damage has an adverse effecton pregnancy, and more recently Amiri-Yekta *et al. (*2017) came to the same conclusion.

In contrast, in the non-smokers group, the mean percentage of the sperm DNA fragmentation (sDF) correlated positively with the number of cleaved embryos (r=0.394, p=0.009), the number of grade 1 embryos (r=0.341, p=0.025), and the number of grade 2 embryos (r=0.316, p=0.039) (**Table 19**). This coincides with the results from a study conducted by Payne and his colleagues in which they demonstrated that the higher the sperm DNA fragmentation is, the higher the pregnancies result is (Payne *et al*., 2005). This contradiction can be explained by the fact that, after fertilization, a good quality oocyte is capable of repairing the sperm DNA damage (Ménézo *et al*., 2010) but, if not, this can have a negative impact on the embryo development (Giwercman *et al.,* 2010).

The current study strongly suggests that CMA3 staining and TUNEL measuring the sperm DNA alterations (compaction and fragmentation respectively) caused by various factors, such as tobacco smoking, may be useful as supplementary test before any ART treatment to ensure a good prognosis especially in cases of idiopathic infertility and repetitive miscarriage.

4.3. Tobacco smoking and epigenetic changes in studied genes: H2BFWT, TNP1, TNP2, PRM1 and PRM2

Epigenetic modifications in the sperm epigenome are additional evidence of the possible influence of environmental factors on sperm chromatin reorganization. Epigenetics is defined as modifications in gene expression originated by processes different from modifications in the encoding DNA sequence (Li, 2002). Such modifications may be transferred during cell divisions (Boissonnas *et al.,* 2013).

There is growing evidence that some lifestyle factors, such as nutrition, stress, alcohol consumption, and tobacco smoking, induce epigenetic modulation affecting male fertility (Alegría-Torres *et al*., 2011; Sharma *et al*., 2013).

Spermatogenesis is an epigenetically elevated controlled process and any interruption at any phase might be a reason of male infertility (Das *et al*., 2017). Progressive protamination of the sperm genome during spermiogenesis leads to the elimination of

histones carrying epigenetic signals. Thus, protamination plays a part in the epigenetic regulation of the spermatozoa and any environmental factor affecting protamination may be considered as an epigenetic signal, such as DNA methylation and histone modification, influencing the transcription regulation after fertilization (Donkin & Barrès, 2018).

In addition, human spermatozoa carry a different kind of mRNA molecules (>5000 types) containing at least 100 miRNAs (Güneş & Kulaç, 2013; Castillo *et al*., 2015). Until now, the exact function of these mRNA molecules is still not clear because the protein synthesis is disabled in spermatozoa (Savadi-Shiraz *et al*., 2015). A number of studies have demonstrated that the sperm transcript accompanies the paternal genome throughout fertilization and consequently affects the early embryo development (Jodar *et al*., 2013; Sendler *et al*., 2013).

Testis-specific histones or histone variants, such as H2BFWT, transition proteins TP1 and TP2 and protamines P1 and P2 present the main nuclear proteins that have a crucial role in the morphology and function of mature spermatozoa. We have, therefore, focused on the relative quantification of H2BFWT, TNP1, TNP2, PRM1 and PRM2 gene expression by quantifying the mRNA or transcript levels of each gene of interest, in order to determine if these transcripts may be used as biomarkers to understand more fully and to evaluate the sperm function. At the same time, we investigated the influence of tobacco smoking on the transcript level of each of these investigated nuclear proteins.

The first step in spermatogenesis takes place in round spermatids and includes the substitution of nearly 85% of somatic histones with testis-specific histone variants that are themselves supplanted by TP1 and TP2. Secondly, the protamines P1 and P2 take the place of the transition proteins in elongating spermatids leading to a highly compacted and transcriptionally silent sperm chromatin (Carrell *et al*., 2007).

H2BFWT is one of two H2B variants that are present in the male gamete. The only information known about H2BFWT is that this histone variant's main function is the facilitation of the transition histone-protamine and the epigenetic control of gene transcription and its association with telomeres, which suggests a putative role in early chromatin remodelling at fertilization (Churikov*et al*., 2004a).

Moreover, the transition nuclear proteins play an important role as intermediaries in histone-protamine replacement in sperm chromatin. TNP1 is localized on chromosomes 2,

and code for transition protein TP1, which is important in DNA repair mechanisms, the reduction of the interaction DNA-nucleosome core (Singh & Rao, 1988), in addition to TP2, are all needed for the complete editing of protamine P2 (Meistrich *et al*., 2003). In contrast to TNP1, TNP2 expression has been demonstrated to be different between mammals (Steger *et al*., 1998). TP2 is a DNA-compacting protein necessary to initiate the binding of protamine to the DNA (Kundu & Rao, 1996).

The protamine genes PRM1 and PRM2 are located beside the transition protein 2 gene TNP2 on chromosome 16: 16p13.3 forming a multigenic cluster (Oliva, 2006). This cluster is bordered by the matrix attachment regions (MARs) containing repetitive alanine elements that present sites of methylation. These MARs are important for the appropriate regulation of protamine genes expression, independent of the methylation state (Schmid *et al*., 2001).

Gene silencing is mediated by methylation and its activation is mediated by hypomethylation, which permits the binding between the nuclear matrix and chromatin, thereby retaining a targeted opening of the chromatin domains known as potentiation (Schmid *et al*., 2001). It has been reported that in late pachytene spermatocytes, the protamine cluster is potentiated and then transcribed later in round spermatids (Martins & Krawetz, 2007b). PRM1-PRM2-TNP2 gene loci include a TATA-box, which is important for the initiation of transcription by facilitating the binding of transcription factors to the promoter. The transcription is also regulated by binding between cAMP-response elements (CRE) and a number of CRE proteins (Tamai *et al*., 1997). Finally, the transcriptional suppression or activation is directed by binding between the upstream regulatory sequences in the promoter region and different trans-regulatory transcription factors (Queralt & Oliva, 1995).

Once transcribed, protamine transcripts are saved as ribonucleoproteins (RNPs), which are translationally repressed and translated later in elongated spermatids (Oliva *et al*., 1988).

4.3.1. The relative expression level of the studied genes

In the non-smokers group, we found that the relative expression level of the H2BFWT correlated positively with the relative amount of TNP2 mRNA (r=0.487, p=0.003). The

relative expression level of TNP1 showed a highly positive correlation (p=0.001) with the expression levels of TNP2, PRM1, and PRM2 (r=0.737, r=0.920, r=0.887) (**Table 27**).

The TNP2 relative expression level correlated positively (p=0.0001) with the relative amount of PRM1 and PRM2 (r=0.731, r=0.709 respectively). Furthermore, the PRM1 and PRM2 expression levels showed a highly positive significant correlation (r=0.961, p=0.0001) (**Table 27**).

This was the first study to examine the relative amount of the H2BFWT gene and its correlation to the transition proteins and protamines.

These findings are in agreement with other studies which reported that H2BFWT is synthesized in the human testes and combined to sperm nuclei during spermatogenesis (Chrikov *et al*., 2004a; Wu *et al*., 2015) and associated with chromatin compaction during spermiogenesis (Gineitis *et al*., 2000). The correlation between the H2BFWT and TNP2 gene expression levels indicate an association between these two proteins and the probability that this testis-specific histone may be replaced by TP2 during chromatin condensation, but further studies are needed to fully understand this synchronization.

The correlation between the expression levels of TNP1, TNP2, PRM1 and PRM2 are in accordance with the results of other studies indicating that during chromatin remodelling the transition proteins TP1 and TP2 are supplanted by the protamines P1 and P2 (Oliva & Dixon, 1991; Balhorn, 2007; Rathke *et al*., 2014; Bao & Bedford., 2016).

In fact, transition protein and protamine mRNA are similarly expressed in high quantities in round spermatids (Oliva, 2006; Balhorn, 2007) and their protein shows a significant presence in the nuclei of elongating spermatid (Wu *et al*., 2000; Meistrich *et al*., 2003). Moreover, a deletion of TNP1 or TNP2 leads to an alteration in PRM2 and consequently a defect in chromatin condensation (Yu *et al*., 200; Zhao *et al*., 2001).

The relative proportion of protamine quantities P1 and P2 has been proposed in many studies as a biomarker of the maturity and integrity of the sperm chromatin (Oliva, 2006; Hammadeh *et al*., 2010; Amor *et al*., 2017).

The protamine mRNA ratio, in the non-smokers group, correlated positively with the expression levels of TNP2 (r=0.349, p=0.032) and PRM2 (r=0.488, p=0.001) (**Table 27**). This result is in agreement with other studies, demonstrating that the protamine ratio

(P1/P2) deregulation is correlated to P2, more than P1 deregulation (Aoki *et al*., 2005; Hammadeh *et al.,* 2010; Barrachina *et al*., 2018).

The correlation between the protamine the mRNA ratio and TNP2 can be clarified by the reality that the TNP2 gene and PRM2 gene are simultaneously regulated and expressed because they are in the same locus (Schlüter *et al*., 1992). It is also believed that the PRM2 gene is a homologue to the TNP2 gene and has similar functions (Kramer *et al*., 1998).

By examining the relative amounts of the studied genes mRNA in the heavy-smokers group (**Table 28**), we found that the H2BFWT expression level showed a significant positive correlation to the transition proteins 1 (TNP1) and 2 (TNP2) relative expression levels (r=0.357, p= 0.0001; r=0,354, p=0.001 respectively) and to protamines 1 (PRM1) and 2 (PRM2) mRNA relative amount (r=0.254, p=0.014; r=0.398, p=0.0001 respectively).

The relative amount of TNP1 gene mRNA demonstrated a highly significant positive correlation with the following mRNA relative amounts of TNP2, PRM1 and PRM2 genes (r=0.814, r=0.859, r=0.822; p<0.001 respectively) (**Table 28**).

Similarly, the TNP2 gene mRNA relative amount showed a highly positive correlation to PRM1 and PRM2 expression levels (r=0.903, r=0.887 respectively; p=0.0001) and the same type of association was observed between the PRM1 and PRM2 expression levels (r=0.926, p=0.0001) (**Table 28**).

Notwithstanding, the protamine mRNA ratio correlated positively to TNP2 mRNA relative amount (r=0.307, p=0.004), PRM2 mRNA relative amount (r=0.445, p=0.0001), and H2BFWT mRNA relative amount (r=0.342, p=0.001) (**Table 28**).

The relative amounts of each studied gene mRNA (mean delta ct) were differentially expressed between the heavy-smokers and non-smokers groups and this difference was highly significant (p<0.01) (**Table 13**).Moreover, H2BFWT, TNP1, TNP2, PRM1 and PRM2 genes were down-regulated in the spermatozoa of heavy-smokers compared to that of non-smokers (Fold change <0.5) (**Table 14**).

Moreover, the protamine mRNA ratio, in the current study, was significantly higher in the heavy-smokers group in comparison to the non-smokers group (0.11 ± 0.84 vs. 0.60 ± 1.08, p=0.001) (**Table 13**). Unlike the group of non-smokers (**Table 25**), in the group of heavy-smokers, the protamine mRNA ratio significantly correlated with the protamine deficiency

(CMA3 positivity) (r=0.413, p=0.0001) and sperm DNA fragmentation (sDF) (r=0.302, p=0.003) (**Table 26**).

Our results confirm the previous studies in our laboratory carried on by Hammadeh *et al.* (2010), who disclosed that the protamine 2 (P2) was under-expressed in the group of smokers in comparison to the non-smokers group and that the protamine ratio (P1/P2) was significantly elevated in the smokers' group.

Furthermore, they found that the levels of 8-OHdG, ROS, MDA and cotinine correlated significantly and were significantly higher in the group of smokers in comparison to the non-smokers. Protamine P2 concentration and 8-OHdG correlated negatively and protamine ratio (P1/P2) correlated positively with 8-OHdG, confirming the association between DNA oxidative damage caused by smoking and protamination abnormalities in sperm chromatin (Hammadeh*et al*., 2010). Hamad *et al*. (2017) demonstrated also that smoking has negative effects on protamine PRM1 and that the PRM2 gene expression and protamine transcript ratio was also significantly higher in the smoker group (Hamad et al., 2017).

Other studies have demonstrated that PRM1 and PRM2 transcripts levels were low in asthenozoospermic and oligozoospermic men (Aoki *et al*., 2006; Kempisty *et al*., 2007) in teratozoospermic men (Savadi-Shiraz *et al*., 2015) and generally in infertile men (Jodar *et al*., 2013) in comparison to normozoospermic men. Moreover, others are of the opinion that the protamine transcript ratio can be adapted to discriminate between fertile and infertile men (Depa-Martynow *et al.,* 2012; Rogenhofer *et al.,* 2013) and the ratio (P1/P2) was high in patients having fertility problems (Ni *et al*., 2016) and correlated with high sperm DNA fragmentation (Castillo *et al*., 2011; Simon *et al*., 2011; Hammadeh*et al*., 2010; Ribas-Maynou *et al*., 2015; Amor *et al*., 2018).

This study is unique as it is the first, to our knowledge, to investigate the influence of smoking on the histone variant H2BFWT and the expression of the transition proteins TNP1 and TNP2. A number of studies have examined the correlation between these genes' expression level and male infertility. They have found that TNP1, TNP2, and HILS1 mRNA levels are lower in the spermatozoa of asthenozoospermic patients compared to normozoospermic ones (Jedrzejczak *et al*., 2007), but another study, by Savadi-Shiraz *et al*.

(2015) found that the TNP2 transcript level is higher in teratozoospermic patients compared to normal ones. Moreover, the downregulation of H2BFWT was found to be related to altered spermatogenesis (Van Roijen *et al*., 1998; Churikov *et al*., 1998).

Hamad et al. (2014) concluded that male smoking is related to a high histone-(H2B)-to-protamine ratio, causing an alteration in sperm DNA. Yu *et al*. (2014) confirmed this previous study and added that the histone-protamine transition and the transcription of protamine genes can be affected by tobacco smoking. An earlier study reported that TSH2B was expressed differently in infertile patients (Van Roijen *et al*., 1998). De Yebra*et al*. (1993) and Zhang *et al*. (2006) showed a high ratio of histone levels to total nuclear protein in infertile patients.

Selit *et al*. (2012) reported that sperm DNA and RNA are negatively affected by smoking. Moreover, tobacco smoking is responsible for the down-regulation of mir-469, mir-466, mir450-b, mir-421, and mir-34b (Dashwood & Ho, 2007; Izzotti *et al*., 2011; Wei *et al*., 2015).

Cui and his colleagues (2016) pointed out that the smoking habit is associated with an alteration of DNA integrity and inhibits DNA repair. They found that smoking was associated with sperm DNA fragmentation and reduced amounts of Check point kinase 1 (Chk1) transcripts ($p<0.05$), which are important for the repair of sperm DNA damage.

Nevertheless, tobacco smoke is generally considered to be one of the stronger environmental factors affecting DNA methylation (Lee & Pausova, 2013). It has been reported that nicotine adheres to nicotine acetylcholine receptors and raises the intracellular calcium, causing the downstream activation of the cAMP response element-binding protein, the main transcription factor of a great number of genes, (Shen & Yakel, 2009) including the genes investigated in this study (H2BFWT, TNP1, TNP2, PRM1, and PRM2).

Satta *et al.* (2008) reported that nicotine downregulates the expression of a number of DNA methyltransferase (DNMT1) and other proteins of mouse neurons.

In addition, smoking is a major source of ROS, which leads to oxidative stress and the cysteine and thiol groups (2SH) of protamine are an easy target for oxidative stress constituents. Cotinine, on the other hand, has a negative effect on intra- and intermolecular

disulfide bond formation, leading to less chromatin compaction in sperm and a high percentage of DNA fragmentation (Kemp *et al*., 2008)

Benzopyrene and vinyl chloride, other components of cigarette smoking, increase the linking of DNA adducts, which then participate in improper DNA replication and inaccurate protein synthesis (Ménézo *et al*., 2010).

Other studies have demonstrated that an aberrant protamine ratio (P1/P2) is correlated to male infertility and it is caused by a decrease or an absence of protamine 2 (P2) expressions (Aoki *et al*., 2005, Hammadeh *et al*., 2010, Hamad *et al*., 2014; Moghbelinejad *et al*., 2015). In the present study, we have found that an aberrant protamine ratio correlated to a decrease in protamine 2, transition protein 2 and the testis-specific histone H2BFWT (**Table 28**). However, larger prospective studies are needed to confirm these correlations and to clarify the mechanism at the molecular level.

4.3.2. The relationship between the expression levels of the studied genes, sperm parameters, and ICSI results

In the non-smokers group, the semen parameters showed no correlation with the transcript level (ΔCt) of the PRM1, PRM2, TNP1, TNP2 and H2BFWT (**Table 21**). However, the protamine (P1–P2) mRNA ratio correlated significantly negatively with the semen volume (r=-0.463, p=0.002) (**Table 21**).

In the heavy-smokers group, the relative protamine 1 (PRM1) and transition protein 1 (TNP1) level correlated positively with the sperm concentration (r=0.335, r=0.391 respectively; $p<0.01$), total motility (r=0.203, r=0.238; $p<0.05$), progressive motility (r=0.337, r=0.361; $p<0.01$), and morphologically normal spermatozoa (r=0.214, p=0.035; r= 0.294, p=0.003 respectively). However, the PRM2 expression level correlated positivelywith the sperm concentration (r=0.329, p=0.001) and the progressive motility (PR) (r=0.338, p=0.001) (**Table 22**).

The relative expression level of TNP2 showed a highly positive correlation with sperm count (r=0.369, p=0.001), progressive motility (r=0.359, p=0.001) and the morphologically normal spermatozoa (r=0.303, p=0.004) (**Table 22**).

A significant positive correlation was also found between the relative amount of H2BFWT mRNA and progressive motility (r=0.230, p=0.027). Furthermore, the protamine mRNA ratio correlated positively significant with total motility (r=0.228, p=0.024) (**Table 22**).

These findings confirm the fact that the testis-specific histone-like H2BFWT, transition proteins, and protamines are essential for chromatin remodelling and compaction during spermatid differentiation to spermatozoa, which means that they actively participate in the spermatozoa morphology and motility.

The results of the present study are in accordance with the findingsof other studies that demonstrated a significant correlation between altered sperm parameters and abnormal protamine ratio (Mengual *et al*., 2003; Aoki *et al*., 2006; De Mateo *et al*., 2009; Simon *et al*., 2014; Hamad *et al*., 2014).

Moreover, these results are in agreement with others that found that sperm motility and protamine transcript correlated significantly and protamine mRNA ratio correlated positively with sperm concentration and motility (Depa-Martynow *et al*., 2012; Rogenhofer *et al*., 2013).

On the other hand, the outcomes of the present study are in contradiction to the results of Hamad *et al*. (2017), who found no significant association between protamine mRNA ratio and sperm motility and to the findings of others who demonstrated a negative correlation between progressive motility and protamine transcript level and a significant negative correlation between the protamine mRNA ratio and non-progressive motility (Rogenhofer*et al*., 2017).

Nevertheless, it has been found that knock-out mice for TNP1 and TNP2 genes had a reduction in the following seminal parameters: progressive motility and normal morphology (Adham *et al*. 2001; Shirley *et al*. 2004; Miyagawa *et al*., 2005; Savadi-Shiraz *et al*., 2015) and that the TNP2 gene mRNA level and its protein are important for normal sperm development (Liu *et al*., 2013) and the prevention of defects in sperm morphology (Tseden *et al*., 2007).

Taken together, there were no significant correlations between the gene expression levels mRNA and the clinical parameters after intracytoplasmic sperm injection ICSI was

observed. However, the relative amount of the TNP1 mRNA correlated negatively with the fertilization rate (r=0.401, p=0.008) in the non-smokers group (**Table 23**).

It is also noteworthy that, although smoking affects the gene expression of spermatozoa no correlations were observed between the clinical parameters after ICSI (the fertilization rate, the number of cleaved embryos, the number of grade 1 embryos, the number of grade 2 embryos, the embryo grade score) and the relative expression levels of H2BFWT, TNP1, TNP2, PRM1, and PRM2 genes in the heavy–smokers group (**Table 24**).

In both groups too, the protamine mRNA ratio showed no correlation with the clinical parameters after ICSI (**Tables 23 & 24**).

These results are inconsistent with other studies that did not notice a difference in PRM1 and PRM2 mRNA levels (Depa-Martynow *et al*., 2007) and in the TNP2 mRNA level (Savadi-Shiraz *et al*., 2015) between a successful ICSI cycle and a failed one.

However, contradictory findings have been reported (Aoki *et al*., 2006; de Mateo *et al*., 2009; Simon *et al*., 2011; Rogenhofer *et al*., 2013), their data suggesting a correlation between an altered protamine (P1/P2) ratio and a lower fertilization rate, a lower embryo quality grade score, and a lower pregnancy score.

Rogenhofer *et al.* (2017) found that the protamines PRM1 and PRM2 transcript levels and the protamine mRNA ratio were notably different between spermatozoa from male partners whose women had more than 2 miscarriages and spermatozoa from both subfertile male partners of couples undergoing ART treatment (IVF/ICSI) and healthy males. From this they concluded that protamines mRNA in human spermatozoa is important for both successful fertilization and early embryo development.

The findings of this present study are in agreement with an earlier study (Shirley *et al*., 2004) that found that both the fertilization rate and the embryonic development were reduced in TNP1-and TNP2-null mice.

The current data also strongly suggest that these studied genes are expressed in a well-organized chronological manner and any alterations from an internal or external factor, such as smoking, may alter these mechanisms and thus result in altered spermiogenesis and sperm function.

4.4. Tobacco smoking and genetic alterations in H2BFWT, PRM1, PRM2

The genotoxic constituents of tobacco are responsible for some genetic damage to spermatozoa, namely, DNA adducts, single- or double-strand breaks, cross-links, aneuploidies, and chromosomal aberrations. If not correctly repaired, sperm DNA damage can lead to mutations or *de novo* mutations. It was found also that this mutation can be transmitted into the next generation and the types of mutations vary from insertions/deletions (indels) and single nucleotide variations (SNVs) to a greatrange of structural variation (Beal *et al*., 2017).

In fact, nicotine and Benzo[a] pyrene have been demonstrated to cause deleterious sperm DNA alterations which can be transmitted to offspring (Holloway *et al*., 2004; Mohamed *et al*., 2010).

Besides, recent studies demonstrated an association between genetic variations or polymorphisms in xenobiotic metabolism enzyme genes and male infertility in smokers like the variation 590G>A of N-acetyltransferase-2 gene (NAT2) (Yarosh *et al*., 2015), and 4621le/Val in cytochrome P450, family1, subfamily A polypeptide 1 gene (CYP1A1) (Yarosh*et al*., 2013).

In this current study, we looked for a possible connection between genetic variations in the H2BFWT gene, protamines genes (PRM1 and PRM2) and fertility alteration in heavy-smoker and non-smoker males.

For the H2BFWT (Xq22.2) a total of three homozygous single nucleotides polymorphism (SNPs): rs7885967, rs553509 and rs578953 were detected. The variant rs7885967 is located in the 5 prime untranslated regions. The variant rs553509 is a missense mutation (Arg/His) that was located in Exon 1 and rs578953 is an upstream gene variant. On testing the association between SNP alleles and each of the heavy-smokers and non-smokers groups, none of the SNPs were reported to be significant (**Table 30**).

One SNP has been identified in the PRM1 gene: rs737008. This SNP is a synonym variant that is located on Exon 2 (**Table 31**). Moreover, two SNPs were detected in the intronic region (rs2070923 and rs1646022) and one was located in the 3 prime untranslated regions

(rs424908) for the PRM2 gene (**Table 32**). Therewere also for these two investigated genes no significant differences in allele distribution among the heavy-smokers and non-smokers.

When the subjects of this study were arranged into patients without and with a variation or with nucleotide changes, no influences of the detected SNPs on the standard sperm parameters (Tables **33-35**), protamine deficiency (**Table 36**), sperm DNA fragmentation (**Table 37**), and ICSI outcomes (**Tables 38-43**) were observed.

However, this is the first study to report two variants rs424902 in PRM2 gene and rs57895 in H2BFWT in a population of patients undergoing ICSI therapy. The other variants were previously reported in a number of studies testing their association with male infertility.

However contradictory findings have been reported: some groups found an association between the two polymorphisms rs7885967 and rs553509 in the H2BFWT gene and male infertility (oligozoospermia and non-obstructive azoospermia) in the Korean population (Lee *et al*., 2009), in the Chinese population (Ying *et al*., 2012), and in the Iranian population (Rafatmanesh *et al*., 2018). In contrast, another study by Zargar *et al*. (2015) found no association.

For the PRM1 variant rs737008, some studies did not find a correlation to male infertility as was the case in our study (Tanaka *et al*., 2003; Aoki *et al*., 2006; Ravel *et al*., 2007; Imken *et al*., 2009; Jodar *et al*., 2011; Venkatesh *et al*., 2011) but in a meta-analysis conducted on subgroups, they found a significant association between this gene polymorphism and male infertility (Jiang *et al*., 2015).

Our results and those of others (Tanaka *et al*., 2003; Aoki *et al*., 2006; Imken *et al*., 2009; Jodar *et al*., 2011) demonstrated no association between the two variants rs1646022 and rs2070923 in PRM2 gene and infertility. However, in a meta-analysis, the rs1646022 in an Asian subgroup has been considered as a risk factor for male infertility (Jiang *et al*., 2015).

It can, therefore, be concluded that the absence of an association between SNP alleles and each of the classes of heavy-smokers and non-smokers indicates that smoking induces epigenetic changes that alter the gene expression but it seems unlikely that it alters the nucleotide sequence of the gene.

4.5. Conclusion

The outcomes of the current study demonstrate that tobacco smoking had a generally negative influence on standard semen parameters. Smoking was also associated with high levels of protamine deficiency, DNA fragmentation and a low pregnancy rate. Protamine deficiency leads to abnormal chromatin condensation and raises the sensitivity of sperm DNA to external stressors, causing an oxidative attack, leading to DNA damage and consequently impairment of the sperm quality.

Thus, CMA3 staining and TUNEL assay can be used for the evaluation of the negative effects of a variant of environmental risk factors, such as smoking, in a situation, for example, like idiopathic infertility where the standard measurement of semen parameters is not enough to provide a complete diagnostic.

Furthermore, tobacco smoking affects the expression of the following five genes, H2BFWT, TNP1, TNP2, PRM1, and PRM2, as well as the protamine mRNA ratio. Transcripts of these genes are therefore good biomarkers in the sperm of ICSI patients to evaluate sperm quality and its fertilizing capacity.

Moreover, no connection was found between the genetic variations found in the H2BFWT gene (rs7885967, rs553509 and rs578953), protamine genes PRM1 (rs737008) and PRM2 (rs2070923 and rs1646022) and fertility alteration in heavy-smoker and non-smoker males.

In conclusion, the present study has shown that smoking does indeed induce epigenetic changes which alter the gene expression but it seems unlikely that it alters the nucleotide sequence of the gene itself.

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6. APPENDICES

Supplementary Tables for Chapter **3.3.4. Association between SNPs and standard sperm parameters, protamine deficiency (CMA3 positivity), sperm DNA fragmentation, and clinical outcomes after ICSI therapy)**

Table 33: Association between the Single nucleotides polymorphisms (SNPs) and sperm concentration (10^6 per ml)

SNPs	**Test**	**p-value**
rs424908	{0/0} vs. {0/1. 1/1}	-
	{0/0. 0/1} vs. {1/1}	0.9933746147277847
rs2070923	{0/0} vs. {0/1. 1/1}	0.9389749144130906
	{0/0. 0/1} vs. {1/1}	0.4162086134220676
rs1646022	{0/0} vs. {0/1. 1/1}	0.865806699149712
	{0/0. 0/1} vs. {1/1}	0.743865463175486
rs737008	{0/0} vs. {0/1. 1/1}	0.7470590680767566
	{0/0. 0/1} vs. {1/1}	0.4208094054177579
rs553509	{0/0} vs. {1/1}	0.013075074553835884
rs7885967	{0/0} vs. {1/1}	0.0624252968856986
rs578953	{0/0} vs. {1/1}	0.49508868402949857

0: reference allele; 1: alternative allele

Table 34: Association between the Single nucleotides polymorphisms (SNPs) and progressive motility (PR.%)

SNPs	Test	p-value
rs424908	{0/0} vs. {0/1. 1/1}	-
	{0/0. 0/1} vs. {1/1}	0.2803684675318613
rs2070923	{0/0} vs. {0/1. 1/1}	0.8732777094259344
	{0/0. 0/1} vs. {1/1}	0.3345142031373297
rs1646022	{0/0} vs. {0/1. 1/1}	0.3360119664696074
	{0/0. 0/1} vs. {1/1}	0.48973067293663575
rs737008	{0/0} vs. {0/1. 1/1}	0.8685910748187006
	{0/0. 0/1} vs. {1/1}	0.9982207458581356
rs553509	{0/0} vs. {1/1}	0.31528758179812033
rs7885967	{0/0} vs. {1/1}	0.753394761339557
rs578953	{0/0} vs. {1/1}	0.27954292428841765

0: reference allele; 1: alternative allele

Table 35: Association between the Single nucleotides polymorphisms (SNPs)and morphologically normal spermatozoa (%)

SNPs	Test	p-value
rs424908	{0/0} vs. {0/1. 1/1}	-
	{0/0. 0/1} vs. {1/1}	0.28783437373616927
rs2070923	{0/0} vs. {0/1. 1/1}	0.5304279142863653
	{0/0. 0/1} vs. {1/1}	0.04737686873584445
rs1646022	{0/0} vs. {0/1. 1/1}	0.14925943059995164
	{0/0. 0/1} vs. {1/1}	0.6240573525042739
rs737008	{0/0} vs. {0/1. 1/1}	0.2468074490858797
	{0/0. 0/1} vs. {1/1}	0.16005734623194479
rs553509	{0/0} vs. {1/1}	0.5169346030783695
rs7885967	{0/0} vs. {1/1}	0.6269185540134425
rs578953	{0/0} vs. {1/1}	0.5146999222652533

0: reference allele; 1: alternative allele

Table 36: Association between the Single nucleotides polymorphisms (SNPs) and protamine deficiency (CMA3 positivity) (%)

SNPs	Test	p-value
rs424908	{0/0} vs. {0/1. 1/1}	-
	{0/0. 0/1} vs. {1/1}	0.3755043873403856
rs2070923	{0/0} vs. {0/1. 1/1}	0.37823394618353245
	{0/0. 0/1} vs. {1/1}	0.621939276796292
rs1646022	{0/0} vs. {0/1. 1/1}	0.6640026536625103
	{0/0. 0/1} vs. {1/1}	0.10469665725189653
rs737008	{0/0} vs. {0/1. 1/1}	0.5983378737757894
	{0/0. 0/1} vs. {1/1}	0.17098512863304105
rs553509	{0/0} vs. {1/1}	0.42983991504241614
rs7885967	{0/0} vs. {1/1}	0.9174186222372497
rs578953	{0/0} vs. {1/1}	0.05393230321624033

0: reference allele; 1: alternative allele

Table 37: Association between the Single nucleotides polymorphisms (SNPs) and sperm DNA fragmentation (sDF)(%)

SNPs	Test	p-value
rs424908	{0/0} vs. {0/1. 1/1}	-
	{0/0. 0/1} vs. {1/1}	0.8803792333541915
rs2070923	{0/0} vs. {0/1. 1/1}	0.744517217394393
	{0/0. 0/1} vs. {1/1}	0.18095775178707674
rs1646022	{0/0} vs. {0/1. 1/1}	0.07503701808365268
	{0/0. 0/1} vs. {1/1}	0.9080644191000308
rs737008	{0/0} vs. {0/1. 1/1}	0.34997829639914957
	{0/0. 0/1} vs. {1/1}	0.8785043586856714
rs553509	{0/0} vs. {1/1}	0.4604095358234265
rs7885967	{0/0} vs. {1/1}	0.1776852063461899
rs578953	{0/0} vs. {1/1}	0.6776226698224479

0: reference allele; 1: alternative allele

Table 38: Association between the Single nucleotides polymorphisms (SNPs) and number of fertilized oocytes

SNPs	**Test**	**p-value**
rs424908	{0/0} vs. {0/1. 1/1}	-
	{0/0. 0/1} vs. {1/1}	0.3271506094332418
rs2070923	{0/0} vs. {0/1. 1/1}	0.2593886335850649
	{0/0. 0/1} vs. {1/1}	0.6550926034477063
rs1646022	{0/0} vs. {0/1. 1/1}	0.48339423927838554
	{0/0. 0/1} vs. {1/1}	0.03730154990781587
rs737008	{0/0} vs. {0/1. 1/1}	0.13138212802869292
	{0/0. 0/1} vs. {1/1}	0.7769820145473822
rs553509	{0/0} vs. {1/1}	0.7863717431014478
rs7885967	{0/0} vs. {1/1}	0.6409038069961046
rs578953	{0/0} vs. {1/1}	0.3496003973275277

0: reference allele; 1: alternative allele

Table 39: Association between the Single nucleotides polymorphisms (SNPs) and number of cleaved embryos

SNPs	**Test**	**p-value**
rs424908	{0/0} vs. {0/1. 1/1}	-
	{0/0. 0/1} vs. {1/1}	0.26706279458557747
rs2070923	{0/0} vs. {0/1. 1/1}	0.2631388274185684
	{0/0. 0/1} vs. {1/1}	0.49233956686094926
rs1646022	{0/0} vs. {0/1. 1/1}	0.45379632292278616
	{0/0. 0/1} vs. {1/1}	0.038779287881035324
rs737008	{0/0} vs. {0/1. 1/1}	0.18149180857202296
	{0/0. 0/1} vs. {1/1}	0.743795165131018
rs553509	{0/0} vs. {1/1}	0.9173305750089161
rs7885967	{0/0} vs. {1/1}	0.40467844353878535
rs578953	{0/0} vs. {1/1}	0.22642945601731423

0: reference allele; 1: alternative allele

Table 40: Association between the single nucleotides polymorphisms (SNPs) and fertilization rate (%)

SNPs	**Test**	**p-value**
rs424908	{0/0} vs. {0/1. 1/1}	-
	{0/0. 0/1} vs. {1/1}	0.8103550592026044
rs2070923	{0/0} vs. {0/1. 1/1}	0.03205097462877848
	{0/0. 0/1} vs. {1/1}	0.23903206039691183
rs1646022	{0/0} vs. {0/1. 1/1}	0.6151144251975146
	{0/0. 0/1} vs. {1/1}	0.03483077162368566
rs737008	{0/0} vs. {0/1. 1/1}	0.23470723921223913
	{0/0. 0/1} vs. {1/1}	0.025423491541043317
rs553509	{0/0} vs. {1/1}	0.014718771454381614
rs7885967	{0/0} vs. {1/1}	0.04174337049411485
rs578953	{0/0} vs. {1/1}	0.7763816892409101

0: reference allele; 1: alternative allele

Table 41: Association between the single nucleotides polymorphisms (SNPs) and number of grade 1 embryos (G1)

SNPs	**Test**	**p-value**
rs424908	{0/0} vs. {0/1. 1/1}	-
	{0/0. 0/1} vs. {1/1}	0.8103550592026044
rs2070923	{0/0} vs. {0/1. 1/1}	0.05339003970229222
	{0/0. 0/1} vs. {1/1}	0.48886995903603236
rs1646022	{0/0} vs. {0/1. 1/1}	0.3085498910762364
	{0/0. 0/1} vs. {1/1}	0.003794939181221096
rs737008	{0/0} vs. {0/1. 1/1}	0.7507765647101192
	{0/0. 0/1} vs. {1/1}	0.31470590161892176
rs553509	{0/0} vs. {1/1}	0.9173305750089161
rs7885967	{0/0} vs. {1/1}	0.3362025017457013
rs578953	{0/0} vs. {1/1}	0.3496003973275277

0: reference allele; 1: alternative allele

Table 42: Association between the single nucleotides polymorphisms (SNPs) and number of grade 2 embryos (G2)

SNPs	**Test**	**p-value**
rs424908	{0/0} vs. {0/1. 1/1}	-
	{0/0. 0/1} vs. {1/1}	0.7932402626860902
rs2070923	{0/0} vs. {0/1. 1/1}	0.8143293315952094
	{0/0. 0/1} vs. {1/1}	0.01588018924527925
rs1646022	{0/0} vs. {0/1. 1/1}	0.9222363838165708
	{0/0. 0/1} vs. {1/1}	0.3019768269072909
rs737008	{0/0} vs. {0/1. 1/1}	0.012829806626289617
	{0/0. 0/1} vs. {1/1}	0.3962068790552832
rs553509	{0/0} vs. {1/1}	0.6185276634748336
rs7885967	{0/0} vs. {1/1}	0.5852618660581088
rs578953	{0/0} vs. {1/1}	0.8978186803918672

0: reference allele; 1: alternative allele

Table 43: Association between the single nucleotides polymorphisms (SNPs) and embryo grade score

SNPs	Test	p-value
rs424908	{0/0} vs. {0/1. 1/1}	-
	{0/0. 0/1} vs. {1/1}	0.3134077545844637
rs2070923	{0/0} vs. {0/1. 1/1}	0.9952562583457744
	{0/0. 0/1} vs. {1/1}	0.27991438124204193
rs1646022	{0/0} vs. {0/1. 1/1}	0.9610138754251201
	{0/0. 0/1} vs. {1/1}	0.9072766392858082
rs737008	{0/0} vs. {0/1. 1/1}	0.12465617088794856
	{0/0. 0/1} vs. {1/1}	0.818568695774981
rs553509	{0/0} vs. {1/1}	0.9953105824678025
rs7885967	{0/0} vs. {1/1}	0.9110792158872906
rs578953	{0/0} vs. {1/1}	0.8356635486803874

0: reference allele; 1: alternative allele

ACKNOWLEDGMENT

For most, I yould like to expressmy sincere gartitude to my supervisor **Prof. Dr. Dr. Mohamad Eid Hammadeh**, manager of Reproductive Laboratory in department of Obstetrics, Gynecology and Reproductive Medicine, at Saarland University Clinic. His office door was always open whenever we needed advice and guidance in research or writing. His unlimit support, immense knowledge and motivation push me award throuh this work.

I am also thankful to **Prof. Dr. med. Erich-Franz Solomayer**, director of the department of Obstetrics, Gynecology and Reproductive Medicine at at Saarland University Clinic, for his financial support and believe in our work.

Besides, I would like to express my immense gratitude to all the members and collegues in the Reproductive Laboratory for their support and help during this journey.

I woul like also to thank **Prof. Dr. Tobias Marschall** and **Ms. Jana Ebler** in the Center of Bioinformatics at Saarland University for their passionate participation and help in analyzing the Sanger sequencing data.

I am also very grateful to**Univ. - Prof. Dr. Stefan Wagenpfeil,** director of the Institute of Medical Biometry, Epidemiology and Medical Informatics (IMBEI) at Saarland University Clinic, for his advice and guidance in the statisical analysis of the present work.

I would like to express my thanks to **Prof. Charles Godall**, for his linguistic revision.

And last but not least, I would like to dedicate this humble work to my family members, especially my mother for her unlimited support and sacrifices, my brother and sisters as well. To my little son and little daughter, you both mean the world to me.

Thanks to all patients enrolled in this PhD work.

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„Aus datenschutzrechtlichen Gründen wird der Lebenslauf in der elektronischen Fassung der Dissertation nicht veröffentlicht.“