# Investigations on the role of DNA methylation governing enzymes (TET1–3, DNMT1 and DNMT3A) for male fertility and ART treatment

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# CONTENTS

# 1. INTRODUCTION

## 1.1 Overview of human spermatogenesis

Human spermatogenesis represents a complex and multi-stage differentiation process starting with spermatogonia, which continuously divide and differentiate finally ending up in the permanent production of fertile sperm (Figure 1). Starting at puberty, spermatogenesis occurs at the surface of the somatic Sertoli cells within the seminiferous tubules of the testes and can be divided into three major periods: pre-meiotic (or mitotic), meiotic and post-meiotic (or spermiogenesis) (Krawetz 2005; Allais-Bonnet & Pailhoux 2014). Diploid spermatogonia located at the basal lamina undergo mitotic divisions and finally differentiate into diploid primary spermatocytes, which enter meiosis and generate haploid secondary spermatocytes (1st meiotic division) and haploid round spermatids (2nd meiotic division). The latter will not divide any more, but differentiate into elongated spermatids and within the epididymis into motile and mature spermatozoa with the capability to fertilize oocytes (Krawetz 2005; Allais-Bonnet & Pailhoux 2014).

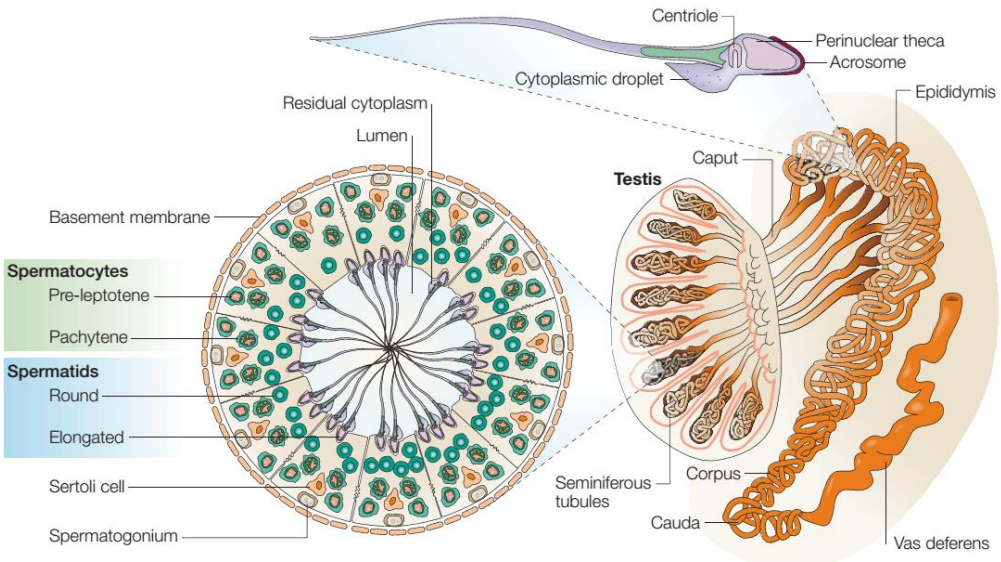



**Figure 1 Illustration of human spermatogenesis in the seminiferous tubules of the testes (Krawetz, 2005).**

## 1.2 Epigenetic processes accompanying human spermatogenesis

In the pre-meiotic period, it is important to safeguard the stem cell character during spermatogonial self-renewal, whereas the meiotic and post-meiotic periods are crucial for male germ cell haploidization and differentiation into fertile spermatozoa. These latter periods are known to be accompanied by a drastic epigenetic reprogramming including a genome-wide DNA methylation erasure of the somatic-like pattern and the re-establishment of sex-specific epigenetic pattern by *de novo* DNA methylation (Hackett *et al.* 2013; Molaro *et al.* 2014; Schagdarsurengin *et al.* 2016) (Figure 2). During spermiogenesis, round spermatids in addition undergo a global remodeling of their nuclei, which elongate and get compacted into the unique condensed nucleus structure of spermatozoa (Krawetz 2005; Gaucher *et al.* 2010). A drastic epigenetic modification of the chromatin structure takes place during this process as well including histone hyper-acetylation, maintenance of DNA methylation, chromatin remodeling and histone-to-protamine replacement (Moran-Crusio *et al.* 2011; Carrell 2012; Boissonnas *et al.* 2013; Schagdarsurengin *et al.* 2016) (Figure 2).

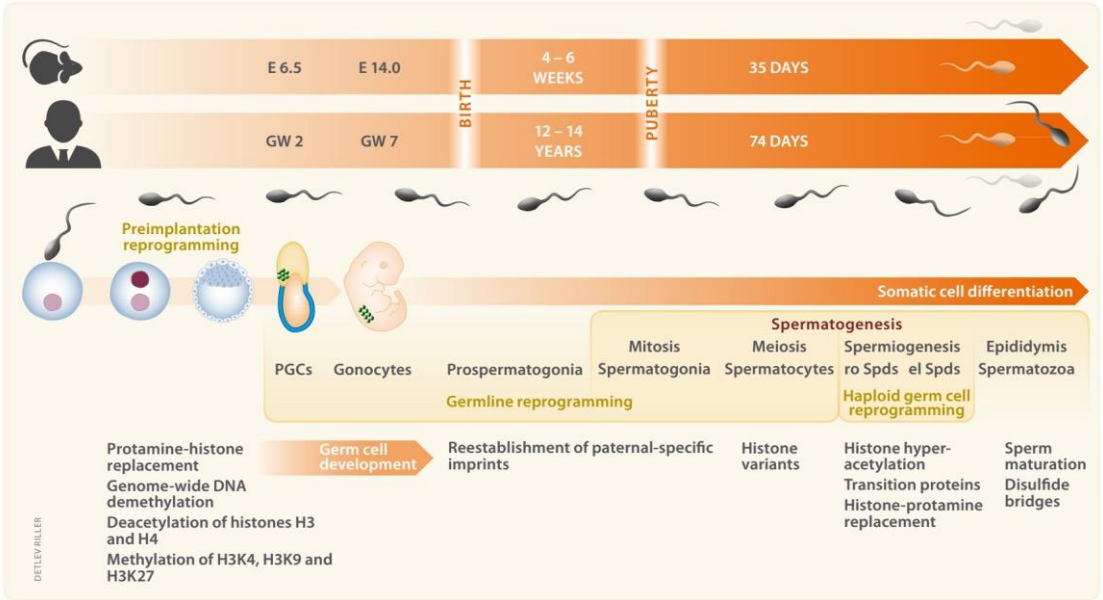



**Figure 2 Illustration of epigenetic processes occurring during spermatogenesis (Schagdarsurengin *et al.* 2016).** A process of genome-wide DNA demethylation and *de*

*novo* DNA methylation of paternal-specific imprints occurs in primordial germ cells (PGCs) before meiosis I. Then the DNA methylation patterns are maintained until fertilization. In post-meiotic maturation, male germ cell nuclei undergo a re-organization and a condensation of their genome including maintenance of DNA methylation, histone variants, histone hyper-acetylation, transition proteins and a replacement of histones by protamines. These epigenetic signatures may contribute to embryo genome activation and early embryonic development.

Accurate epigenetic modifications interact and interdepend on each other resulting in the proper generation of haploid spermatozoa with a highly-condensed heterochromatin and a paternal-specific epigenome (Wykes & Krawetz 2003). Spermatids will undergo a global remodeling of their nuclei with chromatin condensation, packing the majority of hyper-methylated paternal genome into protamine toroids (Aoki & Carrell 2003; Oliva 2006; Carrell 2012; Boissonnas *et al.* 2013). Therefore, the final differentiation step in human spermatogenesis is characterized by a genome-wide chromatin hypercondensation through nucleosome-to-protamine exchange. Nevertheless, the histone-to-protamine replacement is incomplete in all mammalian species analyzed so far, ranging from 1% in mouse (Balhorn *et al.* 1977) to over 50% in some marsupial species (Soon *et al.* 1997). In men, Samans et al. and Hammoud et al. recently reported that 2.9% and 4%, respectively, of the sperm genome is packed into remaining nucleosomes using micrococcal nuclease digestion and direct high-throughput sequencing of the 146-bp mononucleosomal DNA fraction (Hammoud *et al.* 2009; Samans *et al.* 2014). The incomplete replacement of histones by protamines in human sperm has shown the evidence of a residual histone code which is closely associated with developmental important genes (Arpanahi *et al.* 2009; Hammoud *et al.* 2009). Interestingly, the DNA hypomethylated promoters in mature sperm cells significantly overlapped with the developmental promoters bound by the self-renewal network transcription and signaling factors in human embryonic

stem cells (ESC) (Boyer *et al.* 2005; Hammoud *et al.* 2009; Carrell & Hammoud 2010). In addition, sperm centromere repeats, LINE1 and SINE are known to be hypomethylated, and a subset of genes has hypomethylated promoters enriched for factors relevant to human preimplantation development (Carone *et al.* 2014; Samans *et al.* 2014). However, it remains still unclear how these promoters escape the wave of *de novo* DNA methylation and maintain the hypomethylated status. Considering the proper network of epigenetic modifiers ensures the assembly of an intact and fully functional sperm epigenome during spermatogenesis, it provides a possible research direction for the elucidation of this phenomenon.

## 1.3 DNA methylation and demethylation processes in male germ cells

DNA methylation at the 5'-position of cytosine (5-methylcytosine; 5mC) is one of the key epigenetic signatures, which is sometimes called "the fifth nucleotide" and plays a crucial role in biological processes, such as X-chromosome inactivation, gene expression regulation, retrotransposon silencing and genomic imprinting (Jaenisch & Bird 2003; Robertson 2005; Reik 2007). Emerging evidences have suggested that mature sperm cells contribute much more than just the paternal genome to the zygote. The high impact of the sperm methylome was emphasized by the finding in zebrafish demonstrating that after fertilization the maternal genome is reprogrammed to match the paternal methylation, i.e. early embryo methylome is inherited from sperm cells but not oocytes (Jiang *et al.* 2013; Potok *et al.* 2013). DNA methylation in sperm identified as hypomethylated promoters is extensive, and correlated with developmental regulators contributing to totipotency, developmental decisions and imprinting patterns in embryogenesis (Arpanahi *et al.* 2009; Hammoud *et al.* 2009). Although sperm has a smaller cellular volume in comparison with oocyte carrying limited RNAs and proteins, sperm DNA

methylation may play a fundamental role for embryo and progeny on the development and inheritance of the epigenetic information. Mature spermatozoa exhibit a unique genome-wide CpG-methylation profile characterized by a paternal-specific methylation in imprinted genes, by a number of sperm-specific hypo- and hypermethylated gene promoters, and by global hypomethylation of centromere regions and some classes of retrotransposable repetitive DNA elements (Molaro *et al.* 2011; Krausz *et al.* 2012). Certain hypomethylated gene promoters in sperm could be associated with RNA- and protein-processing factors acting during pre-implantation development (Xue *et al.* 2013; Samans *et al.* 2014). On the other hand, abnormal DNA methylation levels of imprinted genes have been observed in infertile patients with oligozoospermia, azoospermia and idiopathic male infertility (Marques *et al.* 2008; Khazamipour *et al.* 2009; Poplinski *et al.* 2010; Minor *et al.* 2011). Aberrant sperm methylome at numerous sequences is closely associated with poor semen parameters (Houshdaran *et al.* 2007). Therefore, it could be supposed that sperm DNA methylation and demethylation processes are closely linked with male germ cell quality and human fertility.

DNA methylation is catalyzed by DNA methyltransferases (DNMTs) including DNMT1, DNMT3A, DNMT3B and the regulatory subunit DNMT3L, which have been identified and well characterized during mammalian spermatogenesis. DNMT1 ensures DNA methylation maintenance throughout spermatogenesis (Bestor 2000; Jaenisch & Bird 2003; Boissonnas *et al.* 2013). DNMT3A and DNMT3B have a catalytic activity for DNA methylation, whereas DNMT3L acts as a cofactor of DNMT3A and lacks catalytic activity (Okano *et al.* 1999; Chedin *et al.* 2002; Gowher *et al.* 2005). DNMT3A, together with its isoform DNMT3L, helps to re-establish DNA methylation during spermatogonial differentiation except for the Ras protein-specific guanine nucleotide-releasing factor 1 (Rasgrf1) locus, whose imprint is re-established

by DNMT3B (Chedin *et al.* 2002; Kaneda *et al.* 2004; Kato *et al.* 2007; Marques *et al.* 2011; Boissonnas *et al.* 2013). Although 5mC and DNMTs have been well characterized, little is known about the mechanisms regulating DNA methylation fidelity and preventing the accumulation of aberrant DNA methylation during mammalian spermatogenesis. Furthermore, two global DNA demethylation waves occur firstly on fertilization in the zygote, and secondly in primordial germ cells (PGCs) (Kohli & Zhang 2013). Regarding the maternal genome, the mechanisms of DNA demethylation in the zygote and in PGCs have been well analyzed and characterized (Dawlaty *et al.* 2011; Iqbal *et al.* 2011; Tan & Shi 2012; Dawlaty *et al.* 2013; Hackett *et al.* 2013; Huang *et al.* 2014). However, the mechanism of global demethylation in the paternal pronuclei remains still unclear.

## 1.4 TET family enzymes and their biological role

Recent studies have indicated that ten-eleven translocation (TET) proteins might act as a potential key to regulate DNA demethylation. Meanwhile, 5-hydroxymethylcytosine (5hmC) has been recognized as a stable intermediate of DNA demethylation with potential regulatory functions in the mammalian genome (Branco *et al.* 2012; Kohli & Zhang 2013; Song & He 2013). TET proteins, including TET1, TET2 and TET3, belong to the 2-oxoglutarate (2-OG) and the Fe(II)-dependent dioxygenase superfamily, which are a group of enzymes capable of converting 5mC into 5hmC, 5-formylcytosine (5fC) and 5-carboxycytosine (5caC) through consecutive oxidation reactions (Wu & Zhang 2011; Tan & Shi 2012; Williams *et al.* 2012; Kohli & Zhang 2013). TET proteins mainly contain three conserved domains including a N-terminal CXXC zinc finger that has a high affinity for clustered unmethylated CpG dinucleotides, a cysteine-rich (Cys-rich) region and a C-terminal catalytic domain that is typical of 2-OG and Fe(II)-dependent

dioxygenases (Tan & Shi 2012; Williams *et al.* 2012) (Figure 3). A CXXC domain can be detected in TET1 and TET3, but not in TET2, having the capability to identify target specific genomic regions for their action (Tan & Shi 2012; Williams *et al.* 2012; Boissonnas *et al.* 2013). A Cys-rich region and a double-stranded β-helix (DSBH) domain are detectable in all TET proteins, which possess a methylcytosine dioxygenase activity to convert 5mC to 5hmC, 5fC or 5caC with the help of the distinct co-factors, such as adenosine triphosphate (ATP) (Williams *et al.* 2012).


**Figure 3 Domain structure and function of human TET family proteins (L Tan *et al*., 2012).** All human TET proteins have a C-terminal CD domain with an ability of methylcytosine dioxygenase activity containing the cysteine (Cys)-rich region and double-stranded β-helix (DSBH) fold core oxygenase domain. TET1 and TET3, but not TET2, contain a CXXC domain, which mediates their direct DNA-binding ability.

Several studies indicated that TET proteins might function as DNA rate-limiting demethylation regulators, while 5hmC might act as an intermediate during DNA demethylation (Branco *et al.* 2012; Kohli & Zhang 2013; Song & He 2013). There are multiple pathways and mechanisms regulated by the TET protein family in order to convert 5hmC into cytosine, and further regulate the dynamics of DNA demethylation and gene transcription (Branco *et al.* 2012; Kohli & Zhang 2013) (Figure 4). Firstly, the deamination pathway involves activation-induced deaminase (AID) or APOBEC family deaminases to convert 5mC or 5hmC directly to thymidine or 5-hydroxymethyluracil (5hmU), respectively. Both deamination products could be further replaced by unmodified cytosine through the base-excision repair (BER) pathway-mediated demethylation (Gu *et al.* 2011), suggesting one route

to achieve TET protein-mediated active DNA demethylation. Secondly, the oxidation pathway involves further oxidation products of 5hmC to 5fC and 5caC by TET proteins, which can be further replaced by an unmodified cytosine through the BER pathway with the help of thymine DNA glycosylase (TDG) enzyme in vitro and in vivo (He *et al.* 2011; Ito *et al.* 2011; Pfaffeneder *et al.* 2011). A dominant mechanism of passive DNA demethylation can be observed during preimplantation development. Expression of 5hmC, 5fC and 5caC is closely associated with the paternal genome in the zygote, which is gradually reduced at this developmental stage (Inoue & Zhang 2011). In addition, 5fC and 5caC probably can be also catalyzed to become deformylated or decarboxylated resulting in the replacement by unmodified cytosine (Wu & Zhang 2011; Tan & Shi 2012; Williams *et al.* 2012; Kohli & Zhang 2013). Although current studies have proposed alternative routes mediated by TET proteins, active or passive DNA demethylation, the initial oxidation of 5mC to 5hmC is a prerequisite for the succeeding demethylation processes regulated by the Tet protein family to complete DNA demethylation (He *et al.* 2011; Ito *et al.* 2011; Maiti & Drohat 2011; Pfaffeneder *et al.* 2011), regardless of how these final steps were mediated (e.g. by deamination, BER or TDG action).

Although forms of TET-mediated DNA epigenetic modifications of 5mC into 5hmC in the genome have been studied for just several years, it has been demonstrated that the TET protein family is essential in a wide range of biological processes, such as the formations of hematologic malignancies and carcinoma, the function of stem cells, the development of embryo and the regulation of the adult nervous system in brain (Langemeijer *et al.* 2009; Moran-Crusio *et al.* 2011; Kunimoto *et al.* 2012; Tan & Shi 2012; Song *et al.* 2013; Yang *et al.* 2013; Zhang *et al.* 2013; Lee *et al.* 2014; Ko *et al.* 2015). Meanwhile, 5hmC is also regarded as the “sixth base” and deemed to have a potentially crucial role in a branch point in the DNA demethylation pathway

(Branco *et al.* 2012; Kohli & Zhang 2013; Song & He 2013). As our biochemical knowledge on TET proteins and its DNA modifying pathways increased, DNA demethylation seems to give us a new perspective to re-examine the various biological and pathological processes.

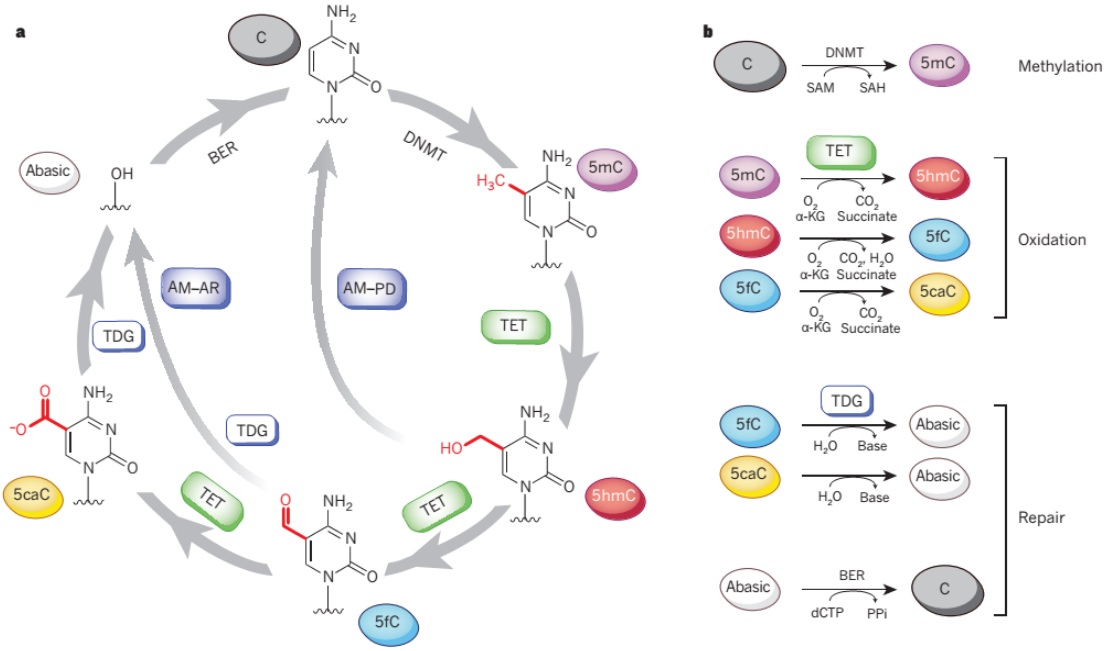



**Figure 4 The complete network for dynamic modifications of cytosine (C) (Kohli *et al*., 2013). (a)** A complete validated pathway for modification of C is indicated. Though DNA methyltransferase (DNMT) enzymes, C can be converted into 5mC bases. Then, TET protein family (TET1/2/3) can catalyze 5mC oxidation to 5hmC, and further into 5fC and 5caC. In the pathway of active modification (AM) followed by passive dilution (PD), 5hmC will be diluted in a replication-dependent manner and thereby complete DNA demethylation. In the pathway of AM followed by active restoration (AR), 5fC and 5caC can also be excised by thymine DNA glycosylase (TDG) generating an abasic site as part of the base-excision repair (BER) pathway to regenerate unmodified C. **(b)** The respective reactions of DNMT, TET and TDG proteins in the pathway are also shown with all reactants indicated. α-KG, α-ketoglutarate; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine, dCTP, deoxycytidine triphosphate; PPi, pyrophosphate.

Regarding the pre-implantation global DNA demethylation of the paternal genome in the zygote, the rapid and specific loss of 5mC expression, together with the level of demethylation products 5hmC, 5fC and 5caC, has been re-examined in light of the discovery of Tet proteins (Figure 5).

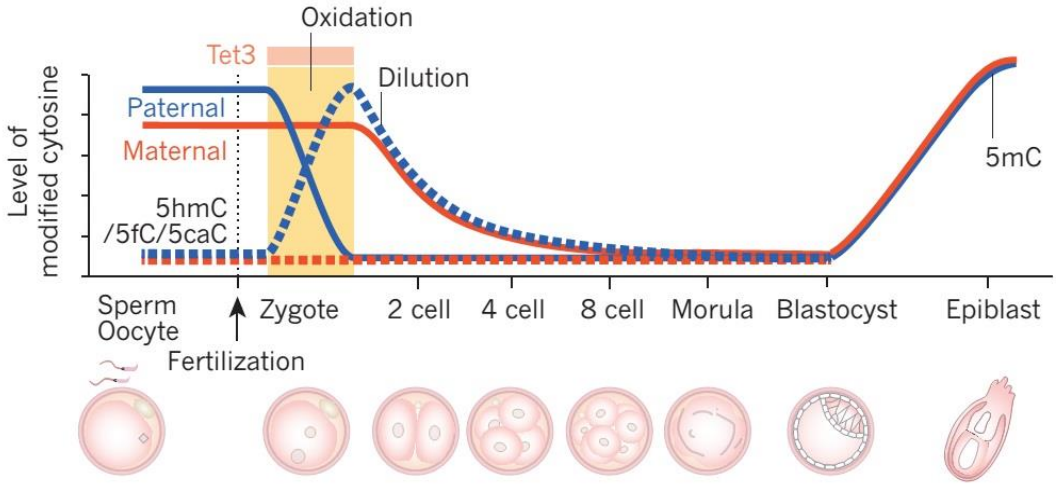



**Figure 5 Dynamics of 5mC, 5hmC/5fC/5caC and function of Tet3 in pre-implantation embryos (Kohli *et al*., 2013).** Maternal DNA is subjected to passive demethylation. However, the paternal genome will be demethylated in two steps. After fertilization, Tet3 converts 5mC into its oxidation products (5hmC/5fC/5caC) in the zygote, and the oxidation products are then diluted through a replication-dependent pathway. Subsequently, *de novo* methylation will be re-established by DNMTs from the blastocyst stage.

Specifically, Tet3 can be detected in the development of pre-implantation embryos in order to mediate 5mC expression, with the evidence that loss of 5mC just coincides with the appearance of 5hmC (Gu *et al.* 2011; Inoue & Zhang 2011; Iqbal *et al.* 2011; Wossidlo *et al.* 2011). Interestingly, the other two demethylation products 5fC and 5caC can be detected in the paternal pronucleus of late zygotic stages as well, whereas the significance of this finding is still elusive (Inoue *et al.* 2011). The maternal DNA is passively demethylated, while the paternal genome will be demethylated in two steps involving the Tet3 enzyme (Inoue *et al.* 2011; Smith *et al.* 2012). Using immunostaining and DNA sequencing, it has been revealed that Tet3 will first

oxidize 5mC in the paternal genome after fertilization with a dramatic increase of 5hmC, 5fC and 5caC, and a simultaneous significant decrease of 5mC before the 2-cell stage. Subsequently, the Tet3-generated oxidation products are gradually diluted through a replication-dependent process before the blastocyst stage. DNA methylation will then be re-established by DNMT enzymes with a significant increase of 5mC (Kohli & Zhang 2013). Therefore, Tet3 protein seems to be responsible for mediating active demethylation of the paternal genome and restore the unmodified cytosine though the passive dilution pathway.

After the establishment of the methylation patterns in the embryo, PGCs will go through a second epigenetic reprogramming process, an erasure of genome-wide DNA methylation patterns (Hackett *et al.* 2012) (Figure 6). Studies have revealed that both passive and active pathways contribute to this global loss of 5mC into 5hmC. Nevertheless, no dynamic changes of 5fC and 5caC could be observed (Seisenberger *et al.* 2012; Hackett *et al.* 2013; Kagiwada *et al.* 2013; Yamaguchi *et al.* 2013).

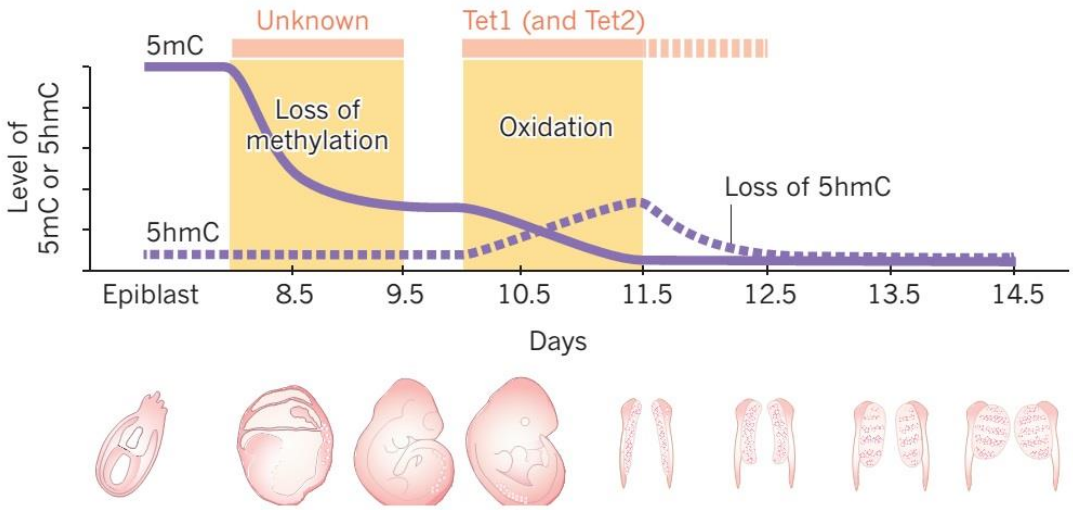



**Figure 6 Illustration of the dynamic expressions of 5mC and 5hmC, and the function of Tet1 (and Tet2) during the PGCs development (Kohli *et al*., 2013).** DNA demethylation in PGCs will go through three stages: loss of bulk DNA methylation in a

replication-dependent manner, oxidation of the remaining 5mC to 5hmC by Tet1 (and Tet2) protein in epiblast and loss of 5hmC through a replication-dependent passive dilution.

Specifically, this process of DNA demethylation in PGCs includes three stages: (1) loss of bulk DNA methylation in epiblast: a passive dilution of 5mC and 5hmC in an apparent replication-dependent (Tet-independent) manner (Seisenberger *et al.* 2012; Hackett *et al.* 2013; Kagiwada *et al.* 2013; Yamaguchi *et al.* 2013); (2) oxidation of the remaining 5mC to 5hmC by Tet1 and Tet2 proteins in epiblast: Tet1 and Tet2 are both expressed during PGCs reprogramming, however, only Tet1 is upregulated (Yamaguchi *et al.* 2012). Recent studies reported that loss of Tet1 function did affect locus-specific DNA demethylation, particularly at meiotic genes, although the targeted deletion and knockdown experiments revealed that Tet1 would not affect global DNA demethylation (Dawlaty *et al.* 2011; Yamaguchi *et al.* 2012; Vincent *et al.* 2013). Additionally, in *Tet1* and *Tet2* double knockout mice the demethylation patterns of some imprinted loci were greatly affected, whereas *Tet2* knockout alone did not affect PGC phenotype (Li *et al.* 2011; Quivoron *et al.* 2011); (3) loss of 5hmC through a replication-dependent passive dilution pathway (Kohli & Zhang 2013). Therefore, Tet1 and Tet2 proteins might regulate DNA demethylation during PGC reprogramming process, however further studies are necessary to explore the exact contributions of Tet1 and Tet2 in shaping the PGC methylome.

Substantial amount of 5hmC most likely is derived from enzymatic oxidation of 5mC by TET proteins in certain mammalian tissues (Jin *et al.* 2010). In general, the 5mC level maintains relatively constant but the 5hmC intensity varies significantly among tissues (Globisch *et al.* 2010; Jin *et al.* 2010). 5hmC is most abundant in specific cell types of the brain compared to other organs (Kriaucionis & Heintz 2009), and ESCs also have relatively high level of 5hmC (Tan & Shi 2012; Shen *et al.* 2013). For the distribution of 5hmC

in human spermatogenesis, Nettersheim and coworkers showed that levels of 5hmC, 5fC and 5caC were decreasing as spermatogenesis proceeded, while 5mC levels remained constant suggesting that active DNA demethylation becomes downregulated during human spermatogenesis (Nettersheim *et al.* 2013). Recently, Gan and colleagues have profiled the global 5hmC distribution in eight germ cell types during mouse spermatogenesis and found that 5hmC mapped differentially and changed dynamically in genomic regions related to the regulation of protein-coding genes, piRNA precursor genes, repetitive elements and various transcripts. It indicated that 5hmC in the mouse genome was crucial for the differentiation of spermatogenic cells (Gan *et al.* 2013). Moreover, they demonstrated that, for these three Tet members, mRNA expressions were well correlated with the total level of 5hmC during spermatogenesis, being much greater in diploid cells than in haploid cells (Gan *et al.* 2013).

## 1.5 Aims of the study

We hypothesize that enzymes involved in the erasure of DNA methylation (TETs), as well as the maintenance and establishment of *de novo* DNA methylation (DNMTs) are crucial for proper assembly of sperm-specific DNA methylation patterns, especially at imprinted genes, as well as sperm capability and the success of ART treatment.

Therefore, the five prominent aims of this study are:

1) to localize mRNA and protein expressions of the TET family members during human spermatogenesis by in-situ hybridization (ISH) and immunohistochemistry (IHC), respectively, as well as 5hmC using immunofluorescence (IF);

2) to demonstrate TET1–3 mRNA and protein presences in human mature spermatozoa applying reverse transcription-polymerase chain reaction (RT-PCR), Western-blot (WB) analysis and immunocytochemistry (ICC);

3) to analyze the possible associations of *TET1–3*, *DNMT1* and *DNMT3A* mRNA levels in mature sperm cells with semen parameters and the outcome of ART treatment using reverse transcription followed by quantitative PCR (RT-qPCR);

4) to detect the possible correlations of *TET1–3* mRNA expressions (RT-qPCR) in ejaculated sperm cells with DNA methylation status at *TET1–3* CpG-promoters measured by combined bisulfite restriction analysis (COBRA) and bisulfite pyrosequencing;

5) to identify the possible differences of DNA methylation at the imprinted gene *H19* in mature spermatozoa between fertile donors and subfertile patients applying COBRA, and to explore the possible associations of aberrant methylation patterns at the imprinted gene *H19* with DNA methylation governing enzymes using RT-qPCR (*TET1–3*, *DNMT1* and *DNMT3A*).

# 2. MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Human testicular tissue samples

Human testicular tissues were obtained from patients with obstructive azoospermia. Samples were fixed in Bouin's fixative and embedded in paraffin. For histological evaluation, 5µm paraffin sections were stained with hematoxylin-eosin, and scored according to Bergmann and Kliesch (Bergmann & Kliesch 1998). All testicular tissue specimens exhibiting histological normal spermatogenesis were used for IHC, ISH and IF analyses.

Written informed consent was obtained from each study participant. The study was approved by the Ethics Commission of the Medical Faculty of the Justus Liebig University Giessen (approval from 15 December 2010 in the frame of the Clinical Research Unit KFO181/Period 2 "Mechanisms of male factor infertility", confirmed on 17 December 2014).

### 2.1.2 Human semen samples

Human semen samples were obtained from healthy fertile donors at the Department of Urology, Pediatric Urology and Andrology Justus Liebig University Giessen, and from males of subfertile couples who underwent intracytoplasmic sperm injection (ICSI) treatment at the Division of Gynecological Endocrinology and Reproductive Medicine, Department of Gynecology and Obstetrics, Clinical Centre of the Ludwig Maximilians University Munich. In this study, the term "subfertile men" is preferred to the term "infertile men" describing a condition when a male is unable to father children through natural conception within one year despite regular unprotected intercourse, but still has an opportunity to father children via ART technology.

A total of 164 semen samples were analyzed for *TET1* mRNA levels (fertile donor, n = 70; subfertile patient, n = 94), 167 semen samples were analyzed for *TET2–3* mRNA levels (fertile donor, n = 75; subfertile patient, n = 92; hereof 149 samples overlapped with *TET1* analysis) and 96 semen samples were analyzed for *DNMT1* and *DNMT3A* mRNA levels (fertile donor, n = 48; subfertile couple, n = 48; all samples overlapped with *TET1–3* analyses). The non-overlapping samples were added in order to increase the cohorts. All semen specimens were obtained after sexual abstinence for 2–7 days, which were analyzed according to the recommendations of the 5th WHO (World Health Organization. 2010). For research purpose, all swim-up sperm cells from ICSI-patients were re-suspended in RNA later™ (Ambion, Heppenheim, Germany) and stored in liquid nitrogen tanks. Semen samples from healthy donors were analyzed by peroxidase staining in order to exclude the samples with a high number of peroxidase-positive white blood cells (WBCs: macrophages, granulocytes and T-lymphocytes). In this study, none of the control samples showed leukocytospermia (> $10^6$/ml): in 80% of control samples WBCs were not detectable, and 20% specimens contained a negligible quantity of WBCs ($10^5$/ml) when compared to sperm concentration (mean = 67.67 × $10^6$/ml). Ejaculated sperm pellets from healthy donors were washed twice in PBS buffer and stored in liquid nitrogen tanks as well (Rogenhofer *et al.* 2013). In order to confirm that the material from the controls (non-swim-up sperm cells, n = 63) was suitable in this study, we performed an additional experiment: fresh isolated RNAs of swim-up sperm cells from 12 donors were analyzed for *TET1–3, DNMT1* and *DNMT3A* mRNA levels by RT-qPCR. We did not find any differences between “swim-up” and “non-swim-up” samples regarding *TET1–3, DNMT1* and *DNMT3A* mRNAs (Supplementary information, Table 7) and thus, conclude that the material was suitable for this study.

The ICSI technology was composed of ovarian stimulation and fresh embryo transfer. The procedures of ICSI treatment were performed according to our previously described protocol (Rogenhofer *et al.* 2013). The cut-off for fertilization rate (FR) was set at 70% in concordance to the available literatures (van Golde *et al.* 2001; Tournaye *et al.* 2002; Palermo *et al.* 2009; Rogenhofer *et al.* 2013). On days 14–16 after ovulation induction, whole blood was collected from the females for the measurement of β–hCG to assess an early pregnancy outcome. Clinical pregnancy rates (fetal heart action per transferred patient; definition of pregnancy by ultrasound) as well as miscarriage rates (loss of entire pregnancy ≤ 24 weeks per transferred patient) were also assessed in this study.

All study participants gave their written informed consent. The study was approved by the Ethics Commission of the Medical Faculty of the Justus-Liebig-University Giessen (approval from 15 December 2010 in the frame of the Clinical Research Unit KFO181/Period 2 “Mechanisms of male factor infertility”, confirmed on 17 December 2014).

### 2.1.3 HeLa, PC3 and LNCaP cell lines

HeLa and PC3 cancer cell lines were used as positive control groups for TET1–3 protein expression (Zhang *et al.* 2014) in order to test the antibodies used in IHC, ICC and WB analyses, and LNCaP cancer cell line was used as a positive control group to check *TET1–2* CpG-promoters methylation by COBRA analysis.

All cell lines were cultured in appropriate culture media (DMEM and RPMI supplemented with 10% fetal calf serum and 1% penicillin/streptomycin, Life Technologies, Darmstadt, Germany) at 5% $CO_2$ and 37ºC, and introduced for immunostainings subsequently.

## 2.2 Methods

### 2.2.1 IHC and ICC analyses of TET1–3 proteins

Mature spermatozoa from fertile donors, formalin-fixed paraffin-embedded human testis tissues exhibiting histological normal spermatogenesis and HeLa cells as positive control (Zhang *et al.* 2014) were used for IHC and ICC analyses regarding TET1–3 protein staining.

HeLa cells were seeded in 8-well chamber slides and incubated at 37°C overnight. After three times washing with 2% Triton/PBS buffer, cells were fixed in 4% paraformaldehyde (PFA) for 10 min. Sperm cells were scratched out and dried on slides, incubated in 10 mM dithiothreitol (DTT) reagent for 15 min for decondensation (optimal time; > 15 min decondensation was critical and led to the bursting of the majority of sperm cells), treated with 10 mM lithium 3,5-diiodosalicylate/1 mM DTT solution for 2 h, and fixed in 4% PFA for 1 h. Human testis sections were cut from paraffin-embedded tissue blocks, soaked with 10% formaldehyde and fixed on slides. The slides were incubated at 42°C overnight and deparaffinized by soaking three times in xylol (each 10 min), twice in absolute ethanol (each 5 min), twice in 96% ethanol (each 5 min) and twice in 70% ethanol (each 5 min). The slides were rehydrated with Millipore water, placed in 10% citrate buffer, heated at 96°C for 30 min and finally recovered to room temperature (RT). Subsequently, all slides were rinsed with 2% Triton/PBS buffer for 5 min and blocked in 3% bovine serum albumin (BSA) buffer for 1 h. Primary antibodies were dropped to the slides (rabbit anti TET1: 1:400, GeneTex, San Antonio, USA; rabbit anti TET2: 1:400, Abcam, Cambridge, UK; rabbit anti TET3: 1:400, Novus, Littleton, USA), and the slides were incubated overnight at 4°C. For negative control, 3% BSA was used instead of antibodies. After washing with 2% Triton/PBS buffer, slides were incubated with the secondary antibody (goat anti rabbit IgG: 1:200, Dako,

Glostrup, Denmark) for 1 h at RT, and rinsed three times in PBS buffer for 5 min. For TET1 and TET3 staining, the slides were developed for 1 h using the VECTASTAIN Elite ABC-AP system (Vector, Peterborough, UK) and visualized with SIGMAFAST Fast Red (Sigma Aldrich, St. Louis, USA) for 10 min. For TET2 staining of human testis tissue slides, VECTASTAIN Elite ABC-Peroxidase (Vector, Peterborough, UK) and 3,3'-Diaminobenzidine (DAB) enhanced liquid substrate (Sigma-Aldrich, St. Louis, USA) systems were used. For TET2 staining in Hela cells and in human mature spermatozoa, ABC-AP system and Fast Red reagent were used as described above for TET1 and TET3 proteins. The reactions were terminated with distilled water, and the staining results were observed and documented using Leica ICC50 HD (Leica, Wetzlar, Germany).

### 2.2.2 IF detection of 5hmC

Slides with formalin-fixed paraffin-embedded human testis tissues were processed as described for IHC until the primary antibody step, and incubated over night at 4°C with the primary antibody (rat anti 5hmC: 1:300, Abcam, Cambridge, UK). After rinsing three times with Tris-HCl buffer, the slides were incubated with the secondary antibody (donkey anti rat IgG: 1:500, Abcam, Cambridge, UK) for 1 h and mounted with Vectashield® mounting medium with DAPI (Vector, Burlingame, USA). Images were analyzed and captured using Leica ICC50 HD (Leica, Wetzlar, Germany).

### 2.2.3 WB analysis of TET1–3 proteins

Mature spermatozoa from two healthy fertile donors and PC3 cells as positive control were used for WB analysis regarding TET1 (235 kDa), TET2 (224 kDa) and TET3 (179 kDa) proteins. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 37 kDa) was applied as a reference protein.

In the step of protein isolation, $2 \times 10^7$ sperm cells were washed twice in PBS buffer containing protease inhibitors, pelleted by centrifugation at 10,000 rpm (8940 × g), 4°C for 5 min, and re-suspended in 100 µl lysis buffer. PC3 cells were washed twice in PBS buffer and suspended in 100 µl lysis buffer. Sperm cells and PC3 cells were then lysed on ice for 30 min with extra extensive vortex for five cycles, each 1 min. Sperm cells were additionally treated with Ultra Turrax (IKA, Staufen, Germany) for five cycles, each 30 sec. After centrifugation at 15,000 rpm (20,120 × g), 4°C for 30 min, the protein extracts were in the supernatant. Protein concentration was measured by Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, USA), and all protein samples were stored at −80°C.

In total, 50 µg protein was mixed with 5 µl Laemmli loading buffer and denaturated at 95°C for 5 min. Samples were separated in a polyacryamid-gel (4% stacking gel and 7.5% separating gel) at 100 volt until the samples had gone through the stacking part, then turn up to 120 volt. Coomassie stain (ROTH, Karlsruhe, Germany) was used for visualization of proteins in gels. Semi-dry transfer method was used. PVDF-membranes were activated by methanol for 10 min. Three levels of Whatman filter paper were soaked in Cathode buffer and Anode buffer for 10 min, respectively. Cassette was prepared in the following order without bubbles: case (clear side), Whatman filter papers (Cathode buffer), gel, PVDF-membrane, Whatman filter papers (Anode buffer), case (black side), which was then placed in a semi-dry transfer cell (Bio-Rad, Munich, Germany) at 20 volt for 1 h. The PVDF-membrane was stained with 1 × Ponceau S for 10 min in order to check the transfer status.

After blocking in 5% non-fat milk in 1 × TBST buffer for 2 h at RT, membranes were incubated with primary antibodies diluted in blocking buffer overnight at 4°C (rabbit anti TET1: 1:2000, GeneTex, San Antonio, USA; rabbit anti TET2: 1:1000, GeneTex, San Antonio, USA; rabbit anti TET3: 1:1000, GeneTex, San Antonio, USA; rabbit anti GAPDH: 1:5000, Sigma-Aldrich, St.

Louis, USA), washed three times with 1 × TBST buffer for 10 min, incubated with the secondary antibody diluted in blocking buffer (Goat anti rabbit IgG-HRP: 1:5000, GeneTex, San Antonio, USA) for 1 h at 37°C, and washed three times with 1 × TBST for 10 min. Membranes were then developed by Pierce ECL Western blotting substrate (Thermo Scientific, Waltham, USA) according to the protocol, and images were captured with the chemiluminescence image analyzer Fusion FX7 advanced (Vilber Lourmat, Eberhardzell, Germany).

### 2.2.4 ISH analysis of *TET1–3* mRNA expression

Non-radioactive ISH was performed applying digoxygenin (DIG)-labelled cRNA probes (sense and anti-sense) to analyze *TET1–3* mRNA expression during human spermatogenesis. Formalin-fixed and paraffin-embedded testicular tissue sections from patients with obstructive azoospermia exhibiting histological normal spermatogenesis were used in this study.

The RNA template was isolated from HeLa cells with PeqGOLD TriFast (VWR, Erlangen, Germany), and reverse transcribed by M-MLV RT system (Promega, Mannheim, Germany). The cDNA was amplified for 40 cycles (initial denaturation 95°C for 5 min, cycling: 95°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec) with specific primer sets generated for *TET1–3* (Table 1) using Mastercycler (Eppendorf, Wesseling-Berzdorf, Germany). PCR products were confirmed by separating on a 2% agarose gel, and extracted using NucleoSpin Gel and PCR clean-up kit (Macherey Nagel, Düren, Germany), which were cloned into the pGEM-T Vector (Promega, Mannheim, Germany) and transformed into XL1-blue strain competent Escherichia coli cells (Stratagene, Heidelberg, Germany) according to the manufacturer’s protocols. White colonies were picked and cultured in 5 ml LB/Ampicillin medium under shaking at 37°C overnight. The recombinant plasmid DNA was isolated by QIAprep

Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The cloned inserts in the plasmid DNA were verified by sequencing with T7 and SP6 primers (T7 primer: 5'-TAATACGACTCACTATAGGG-3' and SP6 primer: 5'-ATTTAGGTGACACTATAGAA-3'). After confirming that the inserts were in accordance with the designed *TET1–3* PCR products (Figure 7), QIAfilter Plasmid Midi Kit (Qiagen, Hilden, Germany) was used to isolate the recombinant plasmid DNA from the positive E. coli cells. The isolated plasmid DNA was digested by NcoI and NotI restriction enzymes (Thermo Scientific, Waltham, USA). The cutting points of the enzymes are indicated in Figure 8. The DIG RNA Labeling Kit (SP6/T7) (Roche, Mannheim, Germany) was used according to the manufacturer's protocol in order to produce sense and anit-sense non-radioactive DIG-labelled *TET1–3* cRNA probes. The generated cRNA probes were stored at −80°C for ISH.

Human testicular tissue slides were deparaffinized by soaking three times in xylol (each 10 min), twice in ethanol absolute (each 5 min), twice in 96% ethanol (each 5 min) and twice in 70% ethanol (each 5 min). The slides were then rinsed in DEPC water for 5 min, soaked in 0.2 N HCl for 20 min and in 2 × SSC buffer at 70°C for 15 min, washed in PBSM buffer for 5 min, and treated with proteinase K (20 mg/ml) for 10 min at 37°C. After soaking in 0.2% glycin solution for 5 min, 20% acetic acid for 20 sec, washed in PBSM buffer for 5 min, sections were post-fixed in 4% PFA solution for 10 min and incubated in 20% glycerol for 1 h. Slides were then initially incubated at 70°C for 10 min with 1:200 dilution of DIG-labelled sense or anti-sense *TET1–3* cRNA probes in hybridization buffer, and then at 42°C in a humidified chamber overnight. In the post-hybridization step, the slides were washed four times with 4 × SSC buffer for 10 min at 42°C, with 2 × SSC buffer for 20 min at 60°C, 0.2 × SSC buffer for 20 min at 42°C, 0.1 × SSC buffer for 5 min at RT, 2 × SSC buffer for 5 min at RT and finally with 1 × TNMT buffer for 10 min at RT. After blocking with 3% BSA for 1 h, the sections were incubated with 1:500 dilution of the secondary

antibody (sheep anti Dig-AP, Roche, Mannheim, Germany) at 4°C overnight. Subsequently, sections were washed twice with 1 × TNMT buffer for 10 min at RT, 1 × NTB buffer for 5 min and 1 × NTB buffer containing 1 mM levamisole for 5 min. The staining was visualized by developing with nitroblue-tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP, KPL, Gaithersburg, USA) for approximate 6 h in a humidified chamber protected from light. Images were observed and captured using Leica ICC50 HD (Leica, Wetzlar, Germany).

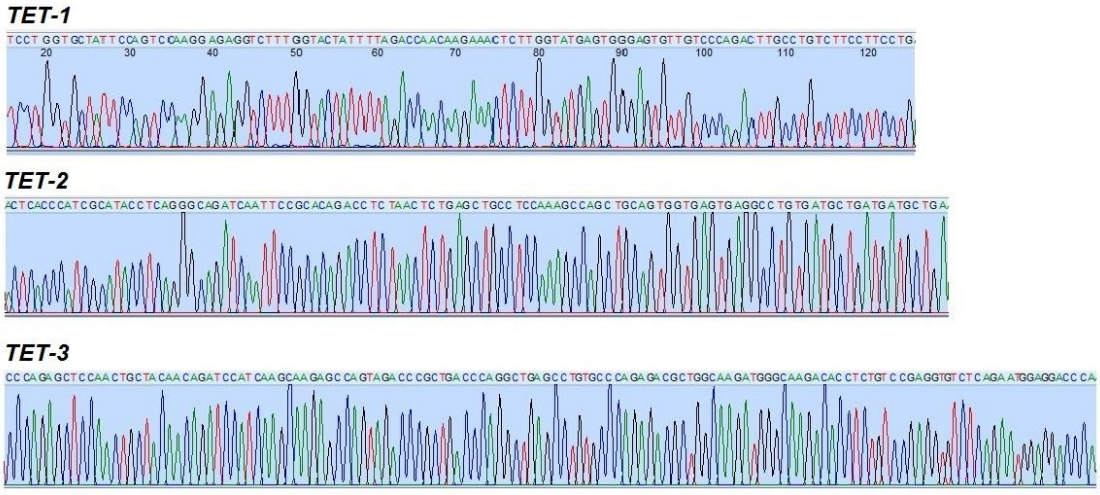



**Figure 7 The sequencing results of the cloned inserts in the plasmid DNA were accordance with the designed *TET1–3* PCR products.**

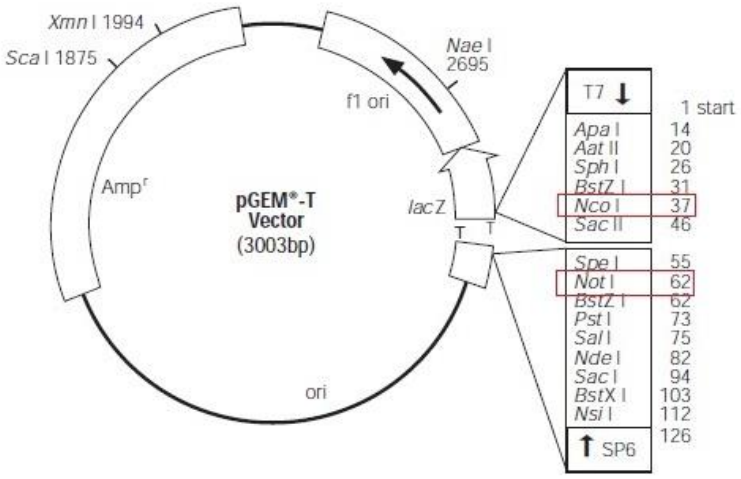



**Figure 8 The cutting points of *Nco* I and *Not* I restriction enzymes in the pGEM-T Vector circle map.**

### 2.2.5 RNA extraction, cDNA synthesis and real-time PCR

Patient RNAs were isolated from a minimum of 1.5 to a maximum of 70 million sperm cells, and control RNAs were extracted from a minimum of 30 to a maximum of 70 million sperm cells (in total). RNA extraction of sperm cells was preformed using RNeasy Plus Micro and Mini Kits (Qiagen, Hilden, Germany). Prior to cDNA synthesis, putative contamination of genomic DNA was eliminated by DNase I (RNase-free) (Thermo Scientific, Waltham, USA) treatment according to the manufacturer's protocol. After measuring RNA concentrations, the purified sperm RNA samples were stored at −80°C. First strand complementary DNA (cDNA) synthesis was performed using 100–300 ng RNA with iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, USA) according to the manufacturer's protocol. The generated cDNAs were then purified by QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). After measurement of the purified cDNA concentrations, all sperm cDNA samples were stored at −20°C.

Real-time PCRs were performed for *TET1*, *TET2* and *TET3* (Gene IDs: 80312, 54790 and 200424, respectively), *DNMT1* and *DNMT3A* (Gene IDs: 1786 and 1788), and the reference gene *GAPDH* (Gene ID: 2597) with appropriate designed primer sets (Table 1) using Rotor-Gene Q PCR cycler (Qiagen, Hilden, Germany). *GAPDH* was shown to be an appropriate reference gene for RNA analyses in human mature sperm cells (Aoki *et al.* 2006; Cavalcanti *et al.* 2011). For each sperm sample, 20–30 ng of cDNA were amplified using Rotor-Gene SYBR Green PCR Kit (Qiagen, Hilden, Germany) for 45 cycles (initial denaturation 95°C for 5 min, cycling: 95°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, and ended with incubation for 4 min at 72°C) according to the manufacturer's protocol, PCR products were confirmed by separating on a 2% agarose gel. All real-time PCR reactions were performed in duplicates.

Relative values of *TET1–3*, *DNMT1* and *DNMT3A* mRNAs were calculated using an intern calibrator in order to compare differences between groups. In order to select an appropriate calibrator RNA, we measured *TET1–3*, *DNMT1* and *DNMT3A* relative mRNA levels in sperm samples from three healthy donors. All three samples showed similar values for all *TET*s (*TET*1: 1, 1.29 and 0.83; *TET2*: 1, 0.98 and 1.16; *TET3*: 1, 0.99 and 1.09, respectively) and possessed well detectable amounts of *TET1–3* in sperm cells (confirmed by RT-PCR and Western blot), as well as *DNMT1* (1, 1.13 and 0.92) and *DNMT3A* (1, 0.87 and 1.05). The first RNA sample was selected as the calibrator RNA.

**Table 1 Primer sets used for quantification of *TET1–3*, *DNMT1*, *DNMT3A* and *GAPDH* transcripts in human mature spermatozoa**

| Gene | Primer sequence (5'-3') for RT-qPCR | PCR-product (bp) | *Tm* (°C) |
|---|---|---|---|
| *TET1* | F: TCCTGGTGCTATTCCAGTCC; | 110 | 58.80 |
| | R: AAAGCCTTCATACATCTCAAG | | 58.18 |
| *TET2* | F: ACTCACCCATCGCATACCTC; | 113 | 59.25 |
| | R: TCAGCATCATCAGCATCACA | | 57.30 |
| *TET3* | F: CCCAGAGCTCCAACTGCTAC; | 138 | 60.11 |
| | R: GAGAATACGAAGAAGTTCATCA | | 58.43 |
| *DNMT1* | F: GAACGGTGCTCATGCTTACAA; | 159 | 59.20 |
| | R: TCTCCATCGGACTTGCTCCT | | 60.32 |
| *DNMT3A* | F: CCTGCAGAAGCGGGTGAG; | 183 | 60.44 |
| | R: ATATGCGCAGGCTGCATC | | 58.34 |
| *GAPDH* | F: TGGAGAAGGCTGGGGCTCAT; | 176 | 63.14 |
| | R: GACCTTGGCCAGGGGTGCTA | | 64.04 |

RT-qPCR: reverse transcription of RNA followed by quantitative real-time PCR; F: forward primer; R: reverse primer; Tm: primer melting temperature; bp: base pair.

### 2.2.6 COBRA of *TET1–2* CpG-promoters and imprinted gene *H19*

In order to analyze the methylation status of *TET1–2* CpG-promoters in sperm cells, we selected subfertile patients with low mRNA level (*TET1*, n = 10; *TET2*, n = 10) and fertile donors with high mRNA expression (*TET1*, n=10; *TET2*, n = 11). Additionally, 18 subfertile patients and 20 fertile donors were chosen at random to check the methylation pattern of the imprinted gene *H19* in human mature spermatozoa.

In the step of sperm DNA isolation, $1 \times 10^7$ sperm cells were washed twice with PBS buffer at 3500 rpm (1160 × g) for 10 min. The sperm pellet was re-suspended in 1 ml lysis buffer and incubated overnight at 56°C. A volume of 1 ml phenol, 1ml phenol/chloroform and 1 ml chloroform was added separately, and the supernatant was collected each time with a centrifugation at 13,000 rpm (16,060 × g), 4°C for 10 min. In order to precipitate sperm DNA, the superntant was mixed with 100 µl 3 M sodium acetate (NaOAc), 5 µl glycogen (20 mg/ml) and 1 ml isopropanol. The mixture was placed at −20°C for 30 min. Sperm DNA was precipitated by centrifuging at 13,000 rpm (16,060 × g) for 30 min, washed with 70% ethanol and dissolved in 20 µl DEPC water. DNA concentrations were then measured, and all samples were stored at −20°C.

Bisulfite treatment of sperm DNAs from all participants was performed using EpiMark® Bisulfite Conversion Kit (New England Biolabs, Ipswich, USA) according to the manufacturer's protocol. COBRA PCR was performed using 20 ng bisulfite-treated sperm DNA as template. Specifically, the designed COBRA primers of *TET1–2* (Gene IDs: 80312, 54790) and the imprinted gene *H19* (Gene ID: 283120) (Table 2), together with MyTaq™ mix (Bioline, MA, USA), were used for PCR amplification in a thermal cycler (Bio-Rad, Munich, Germany) for 45 cycles (initial denaturation 95°C for 3 min, cycling: 95°C for 30 sec, 60°C (*TET1–2*) or 63°C (*H19*) for 30 sec, 72°C for 30 sec, and ended with incubation for 5 min at 72°C) according to the manufacturer's protocol. Based

on their PCR sequence, amplified products were digested with Bsh1236I (BstUI) restriction enzyme (Thermo Scientific, Waltham, USA). Mock digestion (no Bsh1236I enzyme) was included as negative controls. Bisulfite-treated DNA of LNCaP cells and M.SssI-treated (Thermo Scientific, Waltham, USA) sperm DNA were used as positive controls. The digested products were checked on a 2% agarose gel to identify the methylation status. Images were examined and captured by the gel documentation system BioDocAnalyze (Biometra, Göttingen, Germany).

**Table 2 Primer sets used for methylation analyses of *TET1–2* CpG-promoters and *H19* DMRs in human sperm cells**

| Gene | Primer sequence (5'-3') for COBRA | PCR-product (bp) | *Tm* (°C) | Restriction enzyme* |
|---|---|---|---|---|
| *TET1* | F: GTTTTGGGGAGATATTGTTGTTT;<br>R: CCCTcrAACAAACTTTCCAA | 333 | 55.63<br>60.08 | *Bsh1236I* |
| *TET2* | F: ATTTYgAAGTGGTGGTGGAG;<br>R: ACCCTCAcrCcrTACAATAA | 272 | 59.97<br>60.39 | *Bsh1236I* |
| *H19* | F: GGGGGTTTTTGTATAGTATATGGGTA;<br>R: CCCATAAATATCCTATTCCCAAATAAC | 239 | 58.01<br>56.17 | *Bsh1236I* |

COBRA: combined bisulfite restriction analysis; DMRs: differentially methylated regions; F: forward primer; R: reverse primer; Tm: primer melting temperature; bp: base pair. Restriction recognition sites (5'-3'): *Bsh1236I* (CG..CG)

### 2.2.7 Bisulfite pyrosequencing of *TET3* CpG-promoter

For the methylation status analysis at *TET3* CpG-promoter, we selected 10 subfertile patients with low *TET3*-mRNA level and 10 fertile donors with high *TET3*-mRNA expression.

The specific PCR primers of *TET3* (Gene ID: 200424) were from pre-designed PyroMark CpG assay of Qiagen (PM00103495, Qiagen, Hilden, Germany). The specific analyzed sequence for *TET3* included 6 CpG-sites (**Y**): "ATTG**Y**GTTTT**Y**GGGTGGAG**Y**GGAG**Y**GGAGTAGA**Y**GT**Y**GGGGGGGT". Bisulfite pyrosequencing PCR reactions of *TET3* were performed as *TET1–2* COBRA PCR program. Amplified PCR products (250 bp) were checked on a 2% agarose gel. Bisulfite pyrosequencing was performed using PyroMark Gold Q24 Reagents (Qiagen, Hilden, Germany) on a Pyromark Q24® instrument (Qiagen, Hilden, Germany) according to the manufacturer's guideline.

### 2.2.8 Statistical analysis

Data were analyzed using the SPSS 19.0 software (IBM, Chicago, IL, USA). Values are shown as mean and range (minimum to maximum) for semen parameters, and mean ± standard error of the mean (SEM) as well as ± standard deviation (SD) are given for mRNA analyses. SEM takes into account both the value of SD and ample size, and quantifies how precisely the mean of the studied population is. SD represents the degree of dispersion within the studied population. Data distributions were evaluated using the Kolmogorov-Smirnov test. Non-parametric variables were compared by the Mann–Whitney *U*-test. Receiver operating characteristic (ROC) curve analysis was used to assess the ability of *TET1–3*, *DNMT1* and *DNMT3A* mRNA level as a means of distinguishing fertile and subfertile men, low and high fertilization groups, and pregnant and non-pregnant couples concerning ICSI treatment. Correlations between variables were calculated using the Spearman's non-parametric method. Statistical significance was considered at $P < 0.05$ (moderately significant) and $P < 0.01$ (highly significant).

# 3. RESULTS

## 3.1 TET1–3 show a stage-specific expression during the human seminiferous epithelial cycle

Formalin-fixed and paraffin-embedded tissue sections of human testes from patients with obstructive azoospermia exhibiting histological normal spermatogenesis were used to analyze the expression of TET1–3 mRNA and protein during human spermatogenesis.

### 3.1.1 TET1

*TET1*-mRNA started to express in the cytoplasm of the early pachytene spermatocytes in stage I and was detectable up to the late pachytene spermatocytes in stage V (Figures 9B–C). In contrast to *TET1*-mRNA, TET1-protein was detectable after the differentiation of pachytene spermatocytes starting to express in the nuclei of step 1 round spermatids in stage I and remaining up to step 4 elongating spermatids in stage IV (Figures 10A–C).

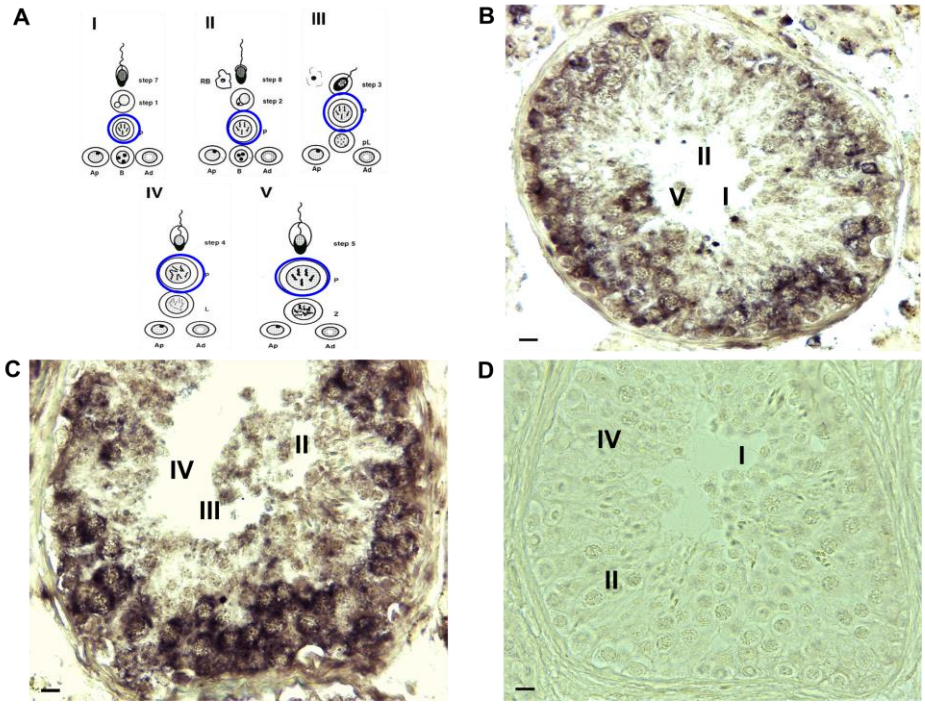



**Figure 9 In-situ hybridization for the detection of *TET1*-mRNA in human testicular tissue sections.** *TET1*-mRNA is present in the cytoplasm of pachytene spermatocytes

starting in stage I up to stage V. While cartoons summarize depicted *TET1*-mRNA in blue circles **(A)**, **B** and **C** show two exemplary tubules with different stages of the seminiferous epithelial cycle (Roman numbers). Negative staining **(D)** represents the serial section of **C**. Primary magnification (PM) × 400; scale bars (SB) 10 µm.

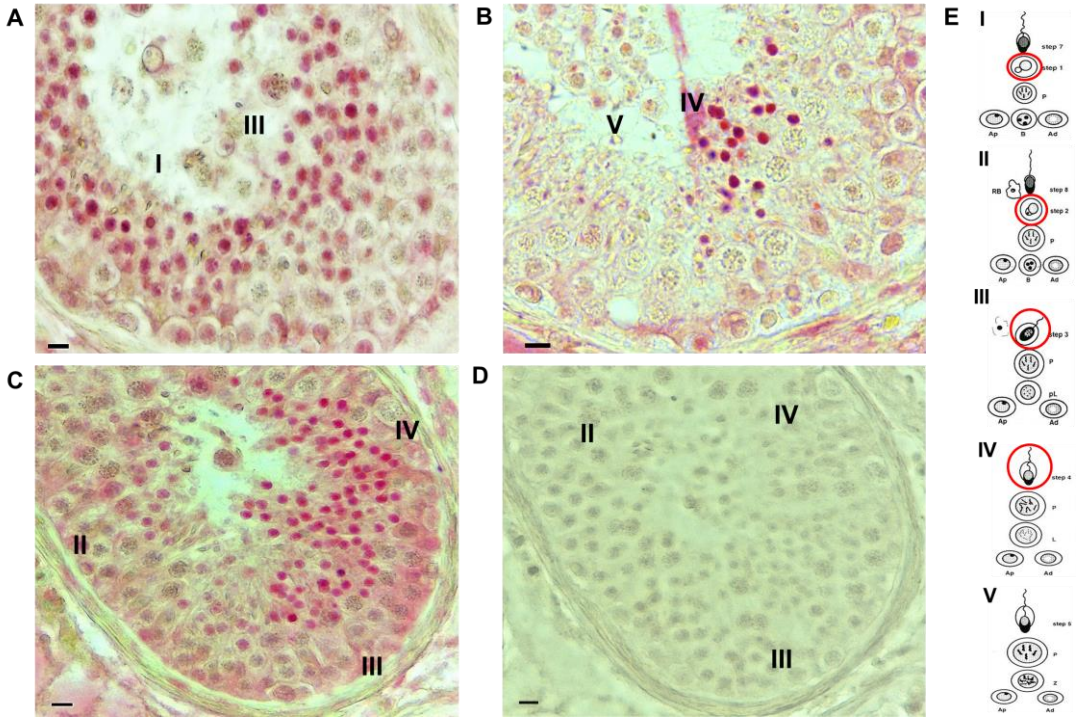



**Figure 10 Immunohistochemistry for the detection of TET1-protein in human testicular tissue sections.** TET1-protein starts to be expressed in the nuclei of step 1 round spermatids in stage I and is detectable until step 4 elongating spermatids in stage IV. **A–C** show three exemplary tubules with different stages of the seminiferous epithelial cycle (Roman numbers). Negative staining **(D)** represents the serial section of **C**. Cartoons summarize depicted TET1-protein in red circles **(E)**. PM × 400; SB 10 µm.

### 3.1.2 TET2

The expression pattern of *TET2*-mRNA was identical to that of *TET1*-mRNA starting in the cytoplasm of pachytene spermatocytes from stage I up to stage V (Figures 11B–C). TET2-protein, in contrast, occurred solely in the cytoplasm of the late pachytene spermatocytes of stage V (Figures 12B–C).

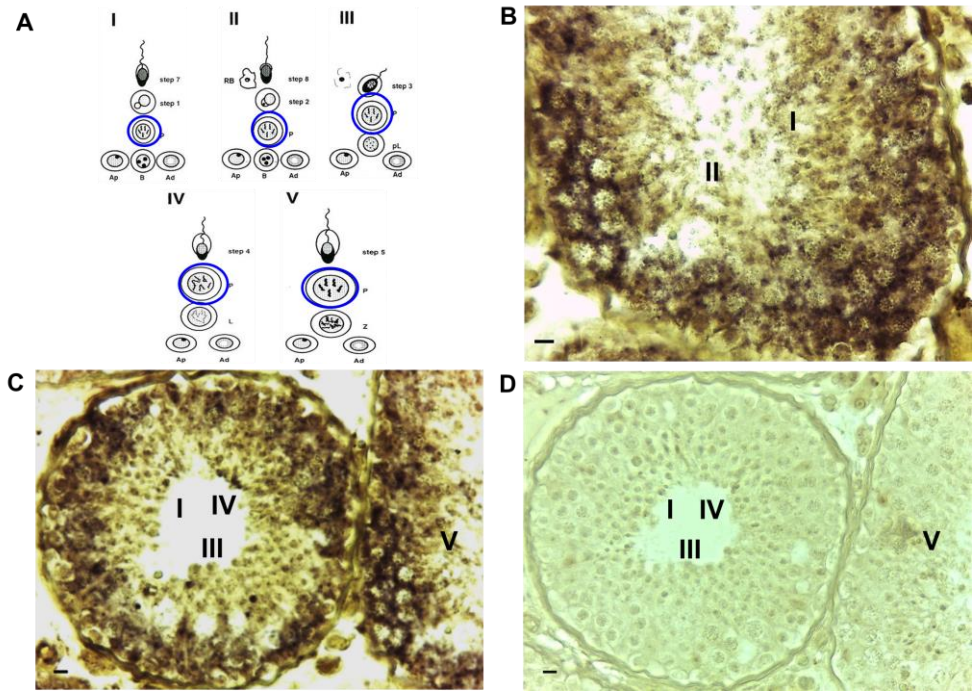



**Figure 11 In-situ hybridization for the detection of *TET2*-mRNA in human testicular tissue sections.** *TET2*-mRNA is present in the cytoplasm of pachytene spermatocytes starting in stage I up to stage V. While cartoons summarize depicted *TET2*-mRNA in blue circles **(A)**, **B** and **C** show two exemplary tubules with different stages of the seminiferous epithelial cycle (Roman numbers). Negative staining **(D)** represents the serial section of **C**. PM × 400; SB 10 µm.

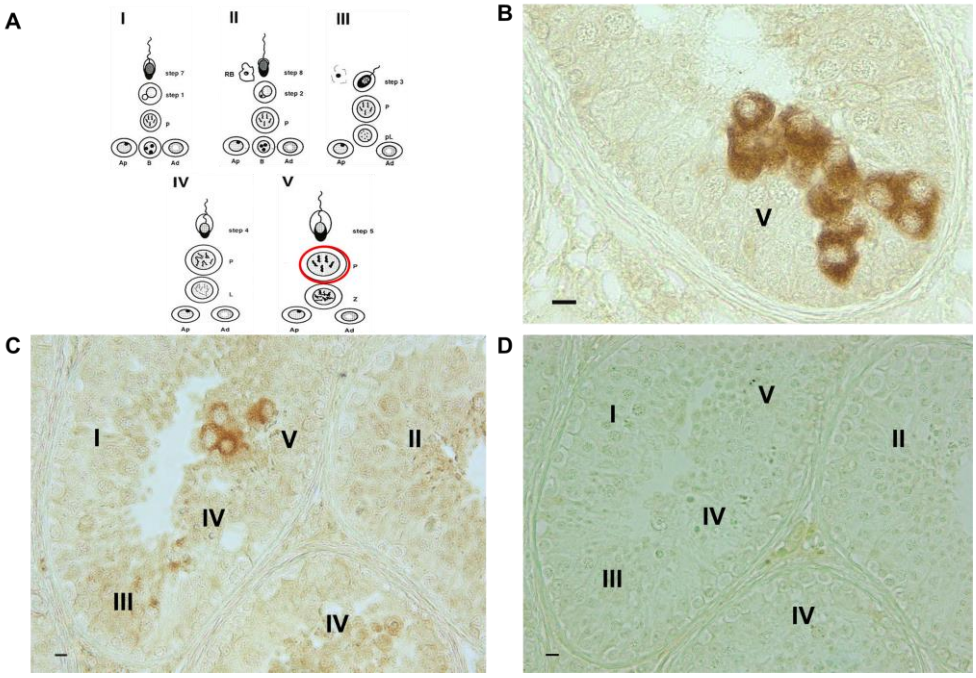



**Figure 12 Immunohistochemistry for the detection of TET2-protein in human**

**testicular tissue sections.** TET2-protein is detected only in the cytoplasm of late pachytene spermatocytes of stage V. While cartoons summarize depicted TET2-protein in a red circle **(A)**, **B** and **C** show two exemplary tubules with different stages of the seminiferous epithelial cycle (Roman numbers). Negative staining **(D)** represents the serial section of **C**. PM × 400; SB 10 µm.

### 3.1.3 TET3

Signals for *TET3*-mRNA were identical to that of *TET1*- and *TET2*-mRNAs, which could be observed in the cytoplasm of pachytene spermatocytes from stage I to V (Figures 13B–C). TET3-protein appeared in the nuclei of step 3 round spermatids of stage III and step 4 elongating spermatids of stage IV (Figures 14B–C).

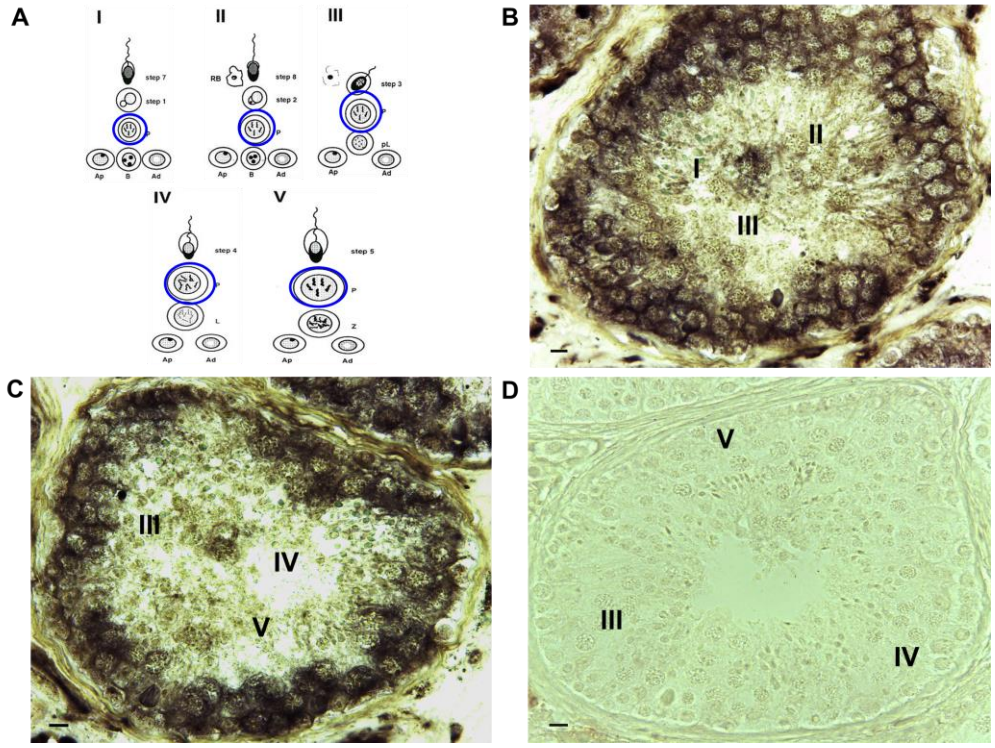



**Figure 13 In-situ hybridization for the detection of *TET3*-mRNA in human testicular tissue sections.** *TET3*-mRNA is present in the cytoplasm of pachytene spermatocytes from stage I up to stage V. While cartoons summarize depicted *TET3*-mRNA in blue

circles **(A)**, **B** and **C** show two exemplary tubules with different stages of the seminiferous epithelial cycle (Roman numbers). Negative staining was a tubule exhibiting stages III, IV and V **(D)**. PM × 400; SB 10 µm.

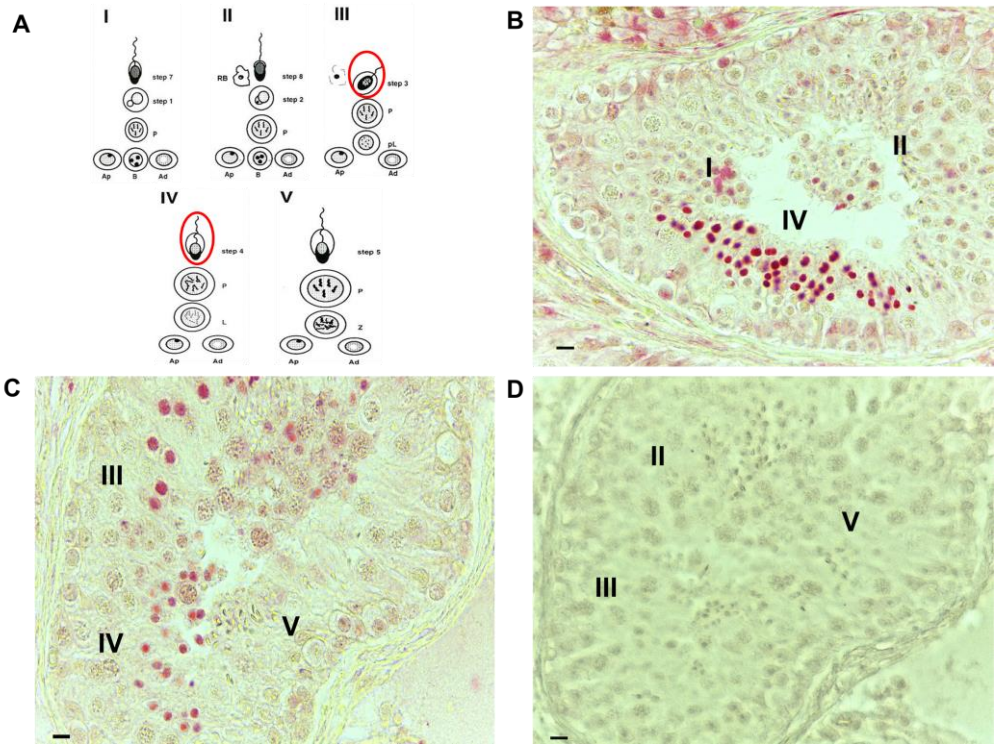



**Figure 14 Immunohistochemistry for the detection of TET3-protein in human testicular tissue sections.** TET3-protein is detectable in the nuclei of step 3 round spermatids in stage III and step 4 elongating spermatids in stage IV. While cartoons summarize depicted TET3-protein in red circles **(A)**, **B** and **C** show two exemplary tubules with different stages of the seminiferous epithelial cycle (Roman numbers). Negative staining **(D)** represents the serial section of **C**. PM × 400; SB 10 µm.

## 3.2 Detection of 5hmC during normal human spermatogenesis

All three members of the TET family possess methylcytosine dioxygenase activity and are important for DNA demethylation. Therefore, we also analyzed the occurrence of 5hmC during the human seminiferous cycle. Using IF staining, we could detect 5hmC only in elongated step 5 spermatids, whereas elongated step 7 and 8 spermatids lack 5hmC (Figure 15).

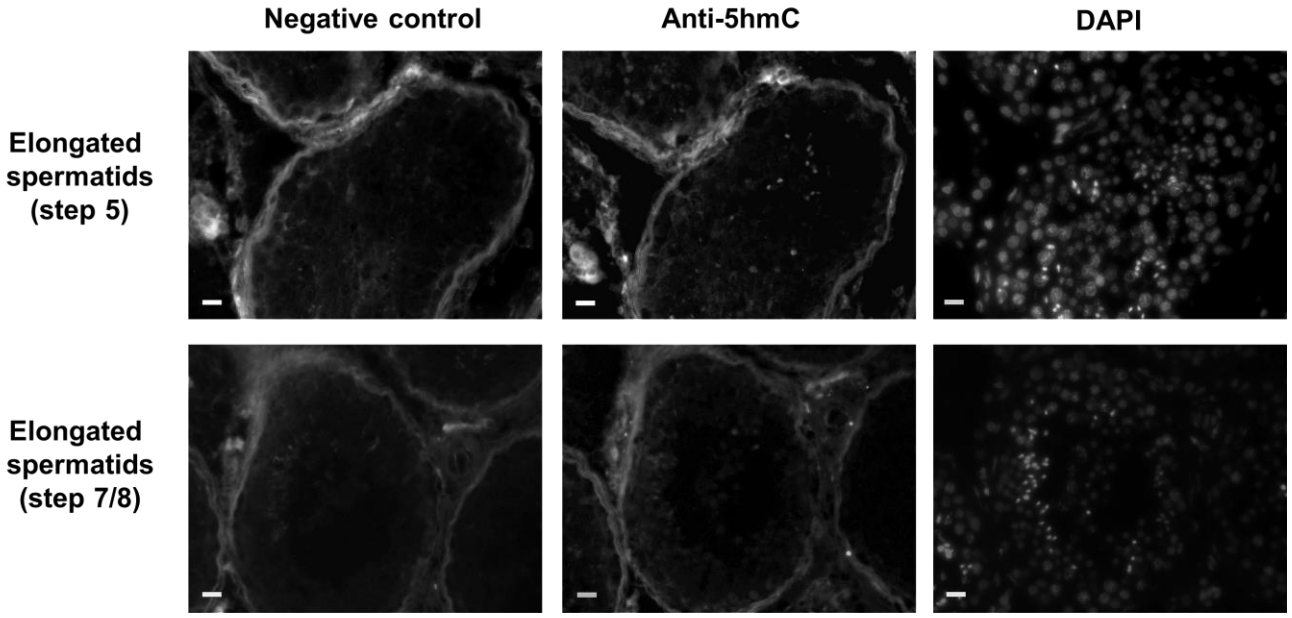



**Figure 15 Detection of 5hmC in human testis exhibiting histological normal spermatogenesis.** 5hmC could be detected by immunofluorescence in step 5 elongated spermatids of stage V, but not in steps 7 and 8 elongated spermatids (stage I and II, respectively). Negative control and DAPI (4′,6-diamidino-2-phenylindole) staining images are shown. PM × 400; SB 10 µm.

## 3.3 Summary of TET1–3 expression and 5hmC during normal human spermatogenesis

We found that *TET1–3* mRNA profiles were identical: all started to express in the cytoplasm of early pachytene spermatocytes of stage I and were detectable up to late pachytene spermatocytes of stage V. Interestingly, TET1–3 protein expressions occurred successively starting with TET2, which could be detected solely in the cytoplasm of the late pachytene spermatocytes of stage V, followed by TET1 starting to express in the nuclei of step 1 round spermatids of stage I and remaining up to step 4 elongating spermatids of stage IV. Finally, TET3 appeared in nuclei of step 3 round spermatids of stage III and was detectable until step 4 elongating spermatids

of stage IV. We could also detect 5hmC only in the elongated step 5 spermatids, whereas elongated steps 7 and 8 spermatids were free of 5hmC (Figure 16).

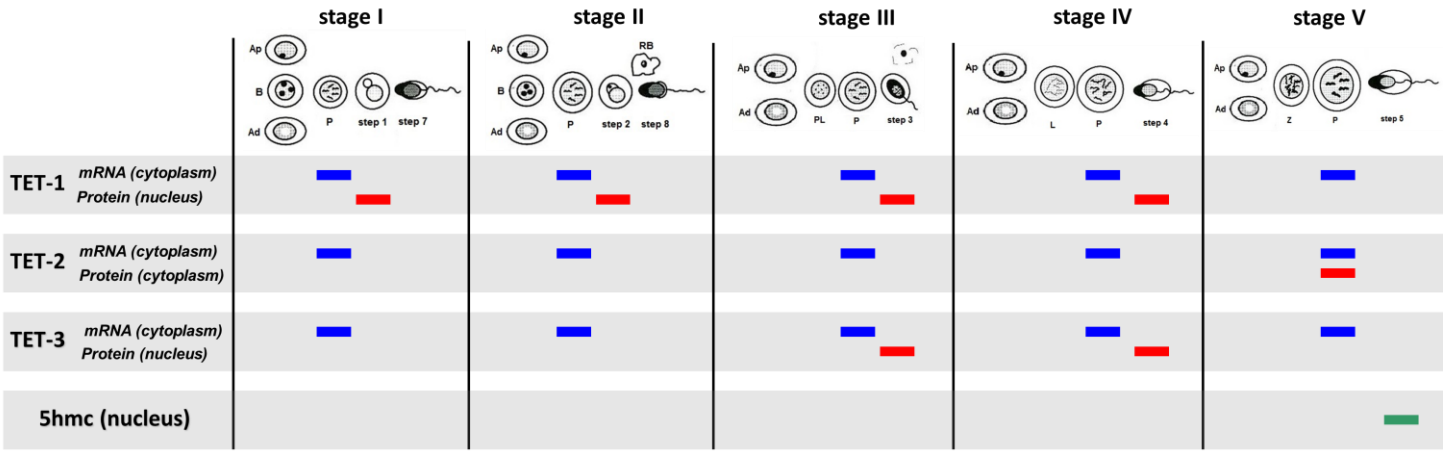



**Figure 16 Schematic overview demonstrates that TET1–3 and 5hmC are expressed at different stages during the human seminiferous epithelial cycle.** *TET1–3* mRNAs have the same expression profile, whereas the expressions of TET1–3 proteins are shifted. Blue lines: *TET1–3* mRNAs; red lines: TET1–3 proteins; green line: 5hmC.

## 3.4 Human ejaculated sperm cells contain considerable amounts of TET1–3 mRNA and protein

After isolating total sperm RNA from three healthy donors, RT-PCR was applied to confirm the presence of *TET1–3* mRNA in sperm cells. Specific bands of amplified RT-PCR products of *TETs (TET1* = 110 bp, *TET2* = 113 bp and *TET3* = 138 bp*)* and *GAPDH* (176 bp, served as a reference gene) were visible on a 2% agarose gel (Figure 17). WB and ICC analyses confirmed the presence of non-degraded TET1–3 protein in human mature spermatozoa. In addition, total protein extracts were isolated from sperm cells of two healthy donors and PC3 cells (positive control for applied antibodies). Specific bands of TETs (TET1 = 235 kDa, TET2 = 224 kDa and TET3 = 179 kDa) and GAPDH (37 kDa, served as a reference protein) could be detected in WB

(Figure 18A). ICC analysis confirmed our WB results showing positive immunostaining for TET1–3 proteins in the nuclei of decondensed mature human spermatozoa and HeLa cells (positive control for applied antibodies) (Figure 18B).

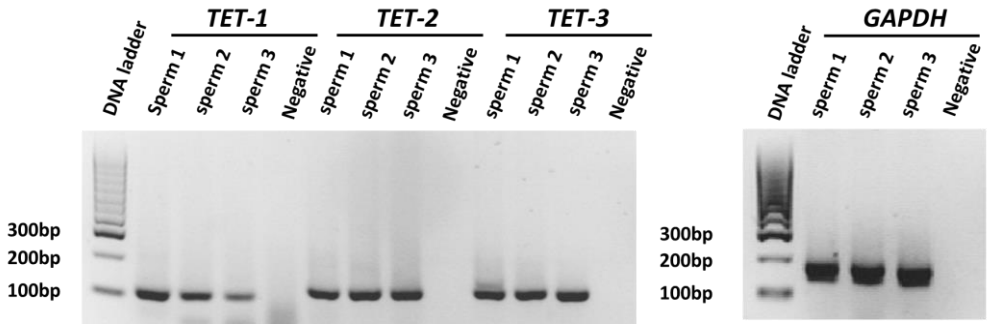



**Figure 17 *TET1–3* mRNA analyses in human mature spermatozoa.** mRNA analysis of *TET1* (110 bp), *TET2* (113 bp) and *TET3* (138 bp) using RT-PCR in sperm cells of fertile donors 1, 2 and 3. GAPDH was used as a reference gene (176 bp).

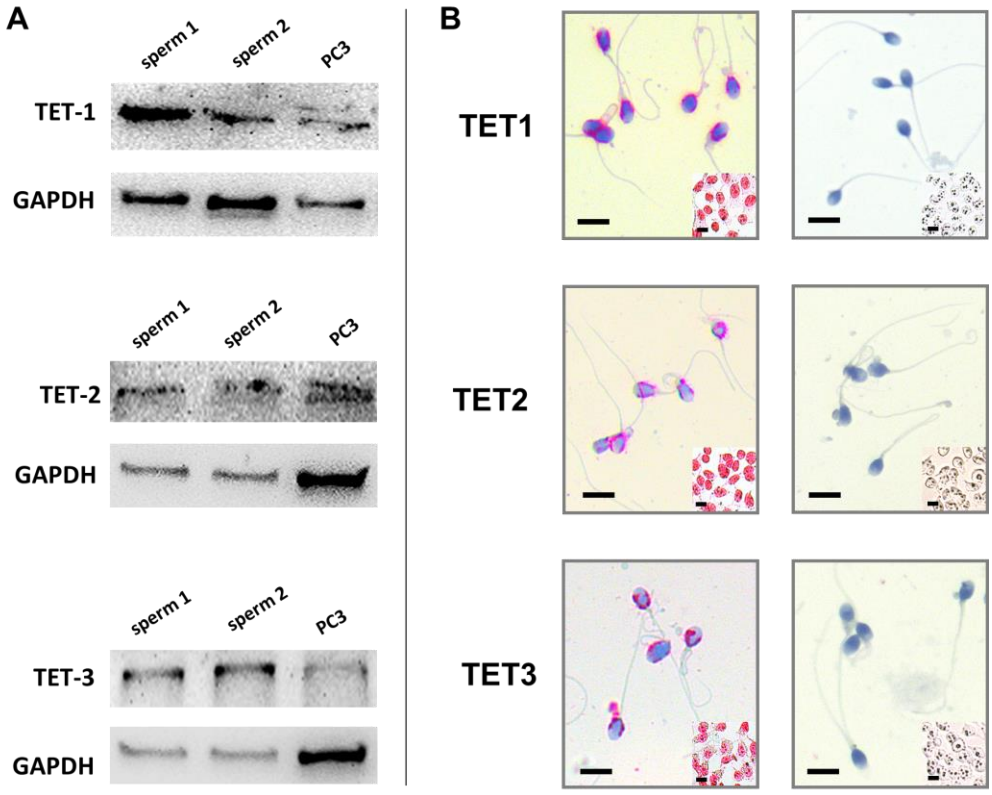



**Figure 18 Protein analyses of TET1–3 using WB and ICC in human mature spermatozoa. (A)** WB results of TET1 (235 kDa), TET2 (224 kDa), TET3 (179 kDa) and

GAPDH (reference protein, 37 kDa) in sperm cells from fertile donors 1 and 2, and in PC3 cells (positive control for the applied antibodies). **(B)** ICC staining of TET1**–**3 proteins in decondensed mature spermatozoa from two fertile donors (left: positive staining; right: negative staining) and in HeLa cells (positive control for the applied antibodies; inset). PM × 400; SB 10 µm.

## 3.5 *TET1–3*, *DNMT1* and *DNMT3A* mRNA levels in human sperm cells

### 3.5.1 *TET1–3* mRNA levels in heathy donors and subfertile patients

A general comparison of healthy donors and subfertile patients (Figure 19A), without differentiations concerning semen parameters, revealed that the relative *TET1* and *TET3* mRNA levels in sperm cells were much higher in healthy donors compared to subfertile patients (*TET1*: 1.45 ± 0.24 (± 1.97) versus 0.77 ± 0.07 (± 0.69), $P = 0.016$; *TET3*: 1.67 ± 0.18 (± 1.55) versus 1.09 ± 0.09 (± 0.91), $P = 0.015$; mean ± SEM (± SD), Mann–Whitney *U*-test). The *TET2* mRNA level did not differ significantly between these two groups (*TET2*: 0.91 ± 0.09 (± 0.79) versus 0.74 ± 0.07 (± 0.66), $P = 0.156$, Mann–Whitney *U*-test). Furthermore, ROC curve analyses of *TET1–3* mRNA levels were used to discriminate healthy donors and subfertile patients demonstrating that the AUC of *TET1* and *TET3* mRNAs were 0.611 ($P = 0.015$) and 0.610 ($P = 0.015$), respectively, with the best discriminative cut-off value of 0.430 (sensitivity = 42.55%, specificity = 74.29%) and 1.172 (sensitivity = 70.65%, specificity = 54.67%; Figure 20). However, the AUC of *TET2* mRNA was only 0.565 ($P = 0.156$). Comparisons of *TET1–3* mRNA levels among themselves revealed significant positive correlations ($P < 0.002$, Spearman's non-parametric correlation; Figures 19B–D). Semen parameters of controls and patients are given in Table 3.

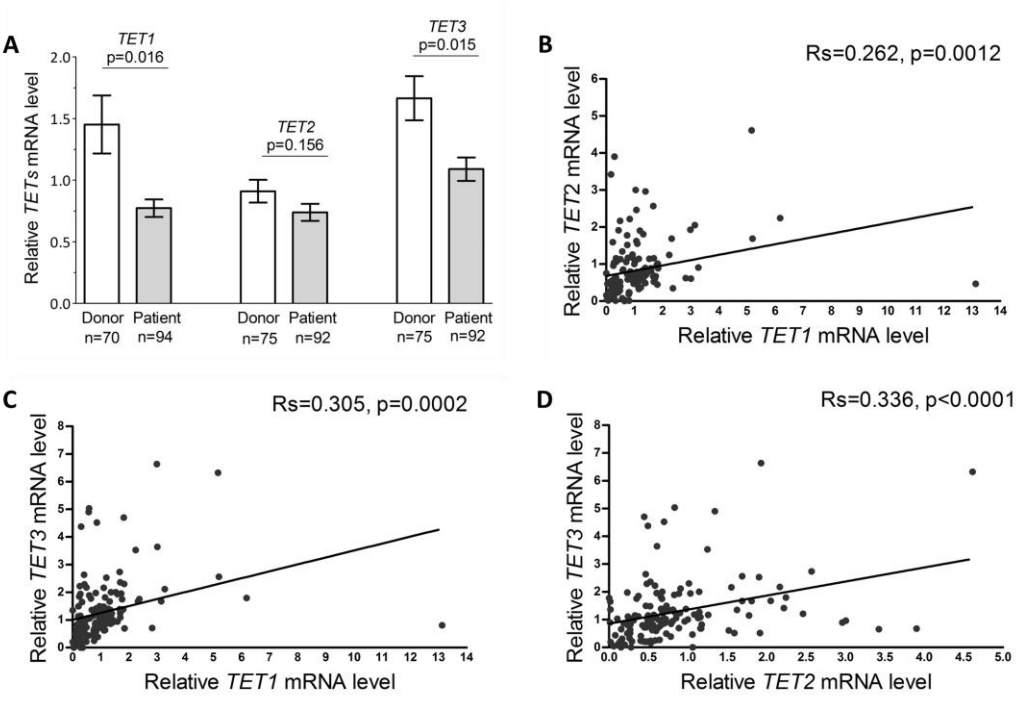
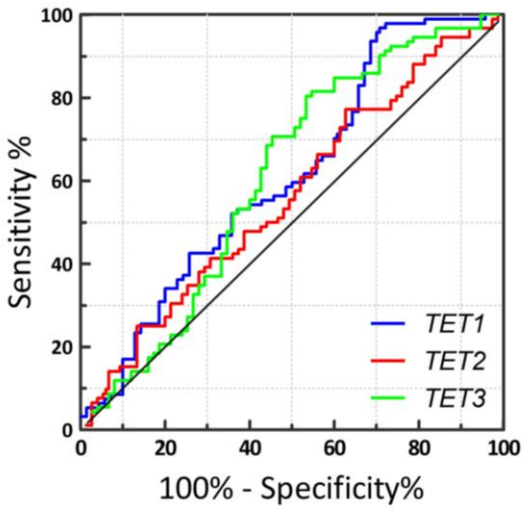



**Figure 19 RT-qPCR analyses of *TET1–3* mRNA in sperm cells of fertile donors and subfertile patients, who underwent ICSI procedures with their female partners. (A)** Comparison of *TET1–3* mRNA levels in healthy donors and subfertile patients (relative mean values ± SEM, *P*-values, Mann–Whitney *U*-test, and number of analyzed samples are given). **(B–D)** Correlations among *TET1–3* mRNA levels considering all sperm samples (Rs, *P*-values of Spearman's non-parametric correlation are given).




| | *TET1* | *TET2* | *TET3* |
|---|---|---|---|
| AUC | 0.611 | 0.564 | 0.610 |
| *P* value | 0.015 | 0.156 | 0.015 |
| Cutoff | 0.430 | 0.893 | 1.172 |
| Sensitivity | 42.55% | 77.17% | 70.65% |
| Specificity | 74.29% | 37.33% | 54.67% |

**Figure 20 ROC curve analyses for *TET1–3* mRNA levels in sperm cells.** *TET1* and *TET3* mRNA levels, but not *TET2*, could efficiently distinguish between fertile donors and subfertile patients (AUC, *P*-values, cutoff-values, sensitivity and specificity are given).

**Table 3 Semen parameters (*TET1–3* study) of healthy men (control group) and subfertile patients who underwent ICSI-procedure with female partners (patient group)**

| | Control group | Patient group | *P*-value* |
|---|---|---|---|
| ***TET1*:** | | | |
| Number | 70 | 94 | – |
| Mean age (min-max) | 29.23 (19–54) | 38.67 (28–55) | < 0.0001 |
| Partner age | Not available | 35.13 (25–45) | Not available |
| Concentration (× $10^6$/ml) | 71.49 (15.6–310) | 43.80 (2.2–243) | < 0.0001 |
| Total sperm count (× $10^6$) | 257.51 (43.2–930) | 161.55 (3.4–1021) | < 0.0001 |
| Progressive motility (%) | 63.06 (34–89) | 40.40 (6–64) | < 0.0001 |
| Total motility (%) | 71.40 (40–91) | 62.86 (25–82) | < 0.0001 |
| Normal morphology (%) | 15.74 (4–34) | 5.21 (0–13) | < 0.0001 |
| ***TET2* and *TET3*:** | | | |
| Number | 75 | 92 | – |
| Mean age (min-max) | 29.20 (19–54) | 38.21 (28–50) | < 0.0001 |
| Partner age | Not available | 34.97 (26–45) | Not available |
| Concentration (× $10^6$/ml) | 77.69 (15.6–310) | 43.70 (1–243) | < 0.0001 |
| Total sperm count (× $10^6$) | 289.94 (43.2–1384) | 154.07 (0.8–1021) | < 0.0001 |
| Progressive motility (%) | 63.00 (34–89) | 40.43 (6–64) | < 0.0001 |
| Total motility (%) | 71.20 (40–91) | 63.02 (25–83) | < 0.0001 |
| Normal morphology (%) | 15.65 (4–34) | 4.92 (1–13) | < 0.0001 |

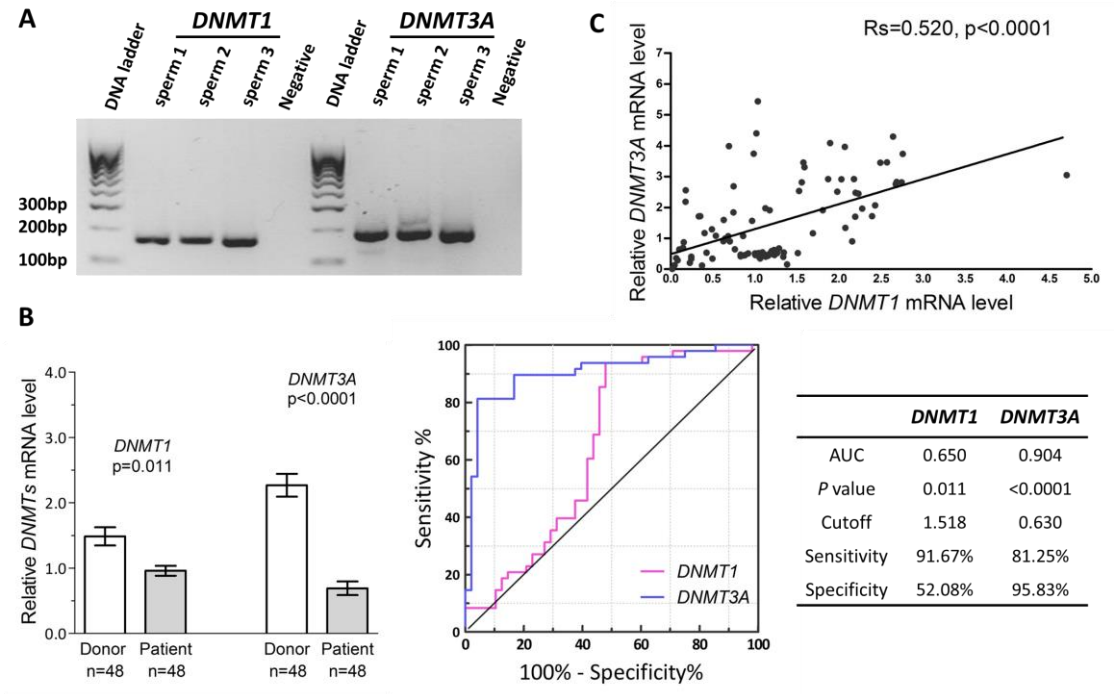
*P*-values are calculated by Mann-Whitney *U*-test.

### 3.5.2 *DNMT1* and *DNMT3A* mRNA levels in healthy donors and subfertile patients

After isolating the total sperm RNA from 48 healthy donors and 48 subfertile patients, RT-qPCR was used to measure relative *DNMT1 and*

*DNMT3A* mRNA levels with specific bands of 159 bp and 183 bp for amplified *DNMT1* and *DNMT3A* RT-PCR products, respectively (Figure 21A). Semen parameters of controls and patients are given in Table 4.

In general, our data demonstrated that *DNMT1 and DNMT3A* mRNA levels in sperm cells were much higher in healthy donors compared to the patients (*DNMT1*: 1.49 ± 0.14 (± 0.96) versus 0.96 ± 0.08 (± 0.53), $P = 0.011$; *DNMT3A*: 2.27 ± 0.17 (± 1.21) versus 0.69 ± 0.10 (± 0.72), $P < 0.0001$, Mann–Whitney *U*-test; Figure 21B). ROC curve analyses of *DNMT1 and DNMT3A* mRNAs were applied in order to discriminate healthy donors and subfertile patients (Figure 21B). The AUC of *DNMT1 and DNMT3A* mRNAs were 0.650 ($P = 0.011$) and 0.904 ($P < 0.0001$) respectively with the best cutoff value of 1.518 (sensitivity = 91.67%, specificity = 52.08%) and 0.630 (sensitivity = 81.25%, specificity = 95.83%). Furthermore, a significant positive correlation was found between *DNMT1 and DNMT3A* mRNAs (Rs = 0.520, $P < 0.0001$, Spearman's non-parametric correlation; Figure 21C).




| | DNMT1 | DNMT3A |
|---|---|---|
| AUC | 0.650 | 0.904 |
| *P* value | 0.011 | <0.0001 |
| Cutoff | 1.518 | 0.630 |
| Sensitivity | 91.67% | 81.25% |
| Specificity | 52.08% | 95.83% |

**Figure 21 RT-qPCR analyses of *DNMT1 and DNMT3A* mRNAs in sperm cells of fertile donors and subfertile patients, who underwent ICSI treatment with their**

**female partners. (A)** *DNMT1* (159 bp) *and DNMT3A* (183 bp) RT-PCR amplified products of mature human spermatozoa from fertile donors 1, 2 and 3. **(B)** Comparison of *DNMT1 and DNMT3A* mRNA levels in donor and patient groups (relative mean values ± SEM, *P*-values and number of analyzed samples are given; Mann–Whitney *U*-test). Furthermore, ROC curve analyses for *DNMT1 and DNMT3A* mRNAs in sperm distinguishing between fertile donors and subfertile patients (AUC, *P*-values, cutoff, sensitivity and specificity are given). **(C)** A significant positive correlation between *DNMT1 and DNMT3A* mRNA levels considering all sperm samples (Rs, *P*-values of Spearman's non-parametric correlation are given).

**Table 4 Semen parameters (*DNMT1* and *DNMT3A* study) of healthy men (control group) and subfertile patients who underwent ICSI-procedure with female partners (patient group)**

| | **Control group** | **Patient group** | ***P*-value*** |
|---|---|---|---|
| ***DNMT1 and DNMT3A*:** | | | |
| Number | 48 | 48 | – |
| Mean age (min-max) | 26.83 (19–46) | 38.98 (30–50) | < 0.0001 |
| Partner age | Not available | 35.33 (27–45) | Not available |
| Concentration ($\times 10^6$/ml) | 78.46 (16–256.5) | 34.93 (1.2–219) | < 0.0001 |
| Total sperm count ($\times 10^6$) | 247.32 (33.6–683) | 119.66 (5.1–491) | < 0.0001 |
| Progressive motility (%) | 63.10 (38–85) | 37.94 (9–62) | < 0.0001 |
| Total motility (%) | 71.06 (38–89) | 60.83 (25–83) | < 0.0001 |
| Normal morphology (%) | 19.42 (5–42) | 3.13 (0–6) | < 0.0001 |

* *P*-values are calculated by Mann-Whitney *U*-test.

### 3.5.3 *TET1–3, DNMT1* and *DNMT3A* mRNA levels in subfertile patients with oligo- and asthenozoospermia

We separated the subfertile patients suffering from oligozoospermia and/or asthenozoospermia in one “O + A” group (*TET1*: n = 39, *TET2–3*: n = 36, *DNMT1* and *DNMT3A*: n = 20), and compared them with the healthy donors and with the subfertile patients exhibiting normozoospermia “NZS” (*TET1*: n = 19, *TET2–3*: n = 17, *DNMT1* and *DNMT3A*: n = 28; Figure 22).

Our data demonstrated that *TET1*- (1.45 ± 0.24 (± 1.97), mean ± SEM (± SD)) and *DNMT3A*-mRNA (2.27 ± 0.17 (± 1.21)) levels in the healthy donors were significantly higher than whatever in the “O + A” or “NZS” subfertile patients (*TET1*: 0.59 ± 0.08 (± 0.48), $P = 0.002$, 0.39 ± 0.07 (± 0.30), $P = 0.0003$; *DNMT3A*: 0.96 ± 0.23 (± 1.03), $P < 0.0001$, 0.49 ± 0.05 (± 0.26), $P < 0.0001$, Mann–Whitney *U*-test), and the differences of *TET2*-, *TET3*-, *DNMT1*-mRNA levels were only observed between the healthy donors and “O + A” subfertile patients (*TET2:* 0.91 ± 0.09 (± 0.79) versus 0.75 ± 0.15 (± 0.93), $P = 0.028$; *TET3:* 1.67 ± 0.18 (± 1.55) versus 0.90 ± 0.11 (± 0.63), $P = 0.047$; *DNMT1*: 1.49 ± 0.14 (± 0.96) versus 0.92 ± 0.16 (± 0.73), $P = 0.023$, Mann–Whitney *U*-test; Figure 22). Overall, we found that the “O + A” subfertile patients revealed severely reduced *TET1–3, DNMT1* and *DNMT3A* mRNA levels in human sperm cells, and the “NZS” subfertile patients exhibited significant decreases of *TET1*- and *DNMT3A*-mRNA expressions. Semen parameters including sperm concentration, total sperm count and sperm progressive and total motility of the “O + A” and “NZS” groups are given in Table 5.

**Table 5 Semen parameters of analyzed healthy men (controls), subfertile men suffering from oligozoospermia and/or asthenozoospermia (O + A patients), and subfertile men exhibiting normoozoospermia (NZS patients)**

| | Controls | O+A Patients[a] | NZS Patients |
|---|---|---|---|
| ***TET1*:** | | | |
| Number | 70 | 39 | 19 |
| Concentration (× $10^6$/ml) | 71.49 (15.6–310) | 16.09 (2.2–86)* | 82.14 (15–219)** |
| Total sperm count (× $10^6$) | 257.51 (43.2–930) | 43.34 (3.4–240.8)* | 294.20 (46.5–706)** |
| Progressive motility (%) | 63.06 (34–89) | 29.54 (6–55)* | 51.74 (37–60)** |
| Total motility (%) | 71.40 (40–91) | 50.18 (25–82)* | 75.11 (65–82)** |
| ***TET2* and *TET3*:** | | | |
| Number | 75 | 36 | 17 |
| Concentration (× $10^6$/ml) | 77.69 (15.6–310) | 14.43 (1–86)* | 75.60 (15–137)** |
| Total sperm count (× $10^6$) | 289.94 (43.2–1384) | 40.22 (0.8–240.8)* | 258.89 (46.5–511)** |
| Progressive motility (%) | 63.00 (34–89) | 28.72 (6–54)* | 51.12 (37–60)** |
| Total motility (%) | 71.20 (40–91) | 49.25 (25–82)* | 74.82 (65–82)** |
| ***DNMT1 and DNMT3A*** | | | |
| Number | 48 | 20 | 28 |
| Concentration (× $10^6$/ml) | 78.46 (16–256.5) | 12.22 (1.2–57)* | 51.14 (15–219)** |
| Total sperm count (× $10^6$) | 247.32 (33.6–683) | 35.49 (5.1–136.8)* | 179.78 (37–491)** |
| Progressive motility (%) | 63.10 (38–85) | 26.50 (9–54)* | 46.11 (32–62)** |
| Total motility (%) | 71.06 (38–89) | 47.15 (30–66)* | 70.61 (53–83)** |

[a] Description of (O + A) group: in total 10 oligo-, 21 oligoastheno- and 8 asthenozoospermia patients were available in our study. One asthenozoospermia patient exhibited in total 240 million sperm cells and had a 27% progressive motility; mean values (minimum to maximum) are given.

* $P < 0.01$ (O + A vs. controls; Mann-Whitney *U*-test); ** $P < 0.01$ (O + A patients vs. NZS patients; Mann-Whitney *U*-test).

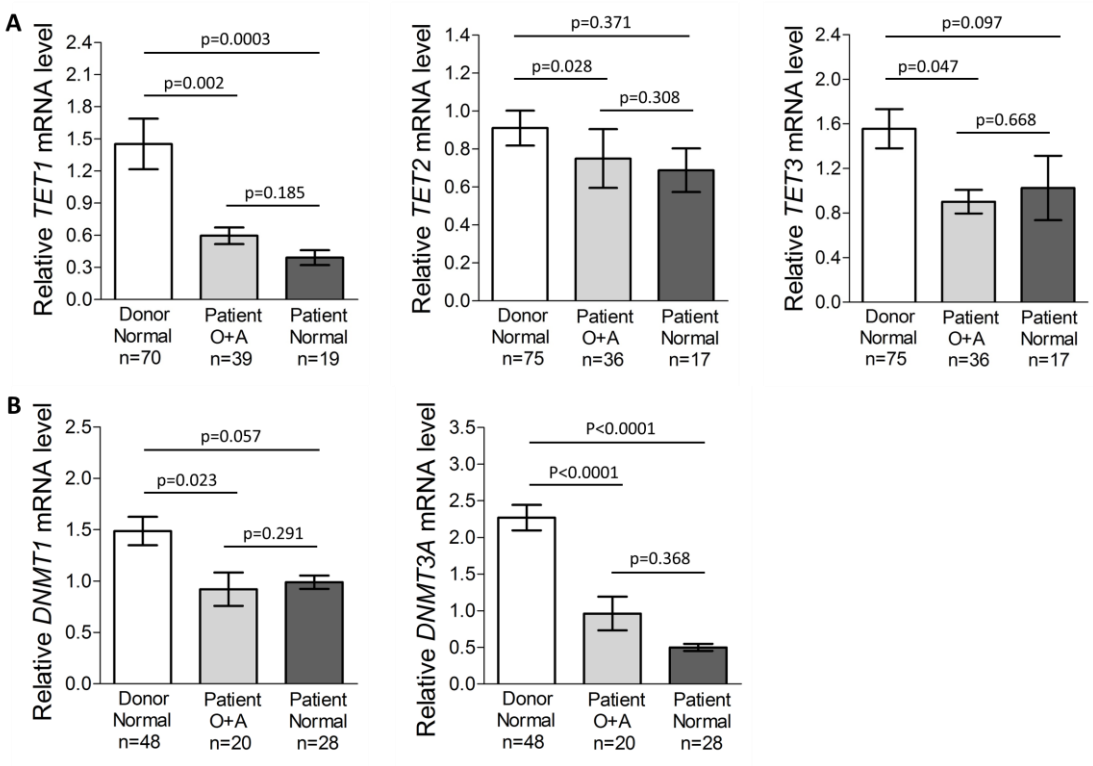



**Figure 22 *TET1–3* (A), *DNMT1* and *DNMT3A* (B) mRNA levels in mature sperm cells of subfertile patients suffering oligozoospermia and/or asthenozoospermia (O + A) in comparison with normozoospermic (Normal) patients and healthy donors.** Relative mean values ± SEM, *P*-values, Mann–Whitney *U*-test, and number of analyzed samples are indicated.

### 3.5.4 Associations of *TET1–3*, *DNMT1* and *DNMT3A* mRNA levels with male age and seminal parameters

We performed a descriptive analysis by comparing values of *TET1–3, DNMT1 and DNMT3A* relative mRNA levels to different parameters including age and semen quality parameters (Table 6). Here, we considered the whole study collective with the respective data for each person without separating into the control and patient groups.

Highly significant negative correlations were observed between age and

the levels of *TET1* (Rs = – 0.209, *P* = 0.007), *TET3* (Rs = – 0.193, *P* = 0.013), *DNMT1* (Rs = – 0.334, *P* = 0.0009) and *DNMT3A* (Rs = – 0.387, *P* < 0.0001) mRNAs in human mature spermatozoa (Spearman's non-parametric correlation). Moreover, the correlations of semen parameters with *TET1–3*, *DNMT1* and *DNMT3A* mRNA levels were described: (1) significant positive correlations were found between sperm concentration and *TET1* (Rs = 0.182, *P* = 0.020), *TET2* (Rs = 0.223, *P* = 0.004), *TET3* (Rs = 0.169, *P* = 0.029) and *DNMT3A* (Rs = 0.270, *P* = 0.008) mRNA levels; (2) positive correlations were indicated between sperm progressive motility and *TET1* (Rs = 0.261, *P* = 0.0008), *TET3* (Rs = 0.189, *P* = 0.014) and *DNMT3A* (Rs = 0.293, *P* = 0.004) mRNA levels; (3) a positive correlation of sperm total motility was observed with *TET1* mRNA level (Rs = 0.210, *P* = 0.007); and (4) positive correlations of normal morphology were indicated with *TET1* (Rs = 0.210, *P* = 0.043), *DNMT1* (Rs = 0.365, *P* = 0.015) and *DNMT3A* (Rs = 0.388, *P* = 0.009) mRNA levels (Spearman's non-parametric correlation).

**Table 6 Associations of *TET1–3*, *DNMT1* and *DNMT3A* mRNA levels in mature human spermatozoa with male age and seminal parameters**

| Characteristics | | Age (Year) | Sperm concentration (Mil/ml) | Progressive motility (%) | Total motility (%) | Normal morphology (%) |
|---|---|---|---|---|---|---|
| *TET1* | *Rs* | ***–0.209*** | ***0.182*** | ***0.261*** | ***0.210*** | ***0.210*** |
| | *p value* | ***0.007*** | ***0.020*** | ***0.0008*** | ***0.007*** | ***0.043*** |
| *TET2* | *Rs* | –0.055 | ***0.223*** | 0.091 | 0.031 | 0.169 |
| | *p value* | 0.482 | ***0.004*** | 0.243 | 0.689 | 0.100 |
| *TET3* | *Rs* | ***–0.193*** | ***0.169*** | ***0.189*** | 0.124 | 0.185 |
| | *p value* | ***0.013*** | ***0.029*** | ***0.014*** | 0.110 | 0.071 |
| *DNMT1* | *Rs* | ***–0.334*** | 0.049 | 0.110 | 0.057 | ***0.365*** |
| | *p value* | ***0.0009*** | 0.637 | 0.284 | 0.579 | ***0.015*** |
| *DNMT3A* | *Rs* | ***–0.387*** | ***0.270*** | ***0.293*** | 0.048 | ***0.388*** |
| | *p value* | ***<0.0001*** | ***0.008*** | ***0.004*** | 0.961 | ***0.009*** |

Rs, *P*-values of Spearman's non-parametric correlation are given.

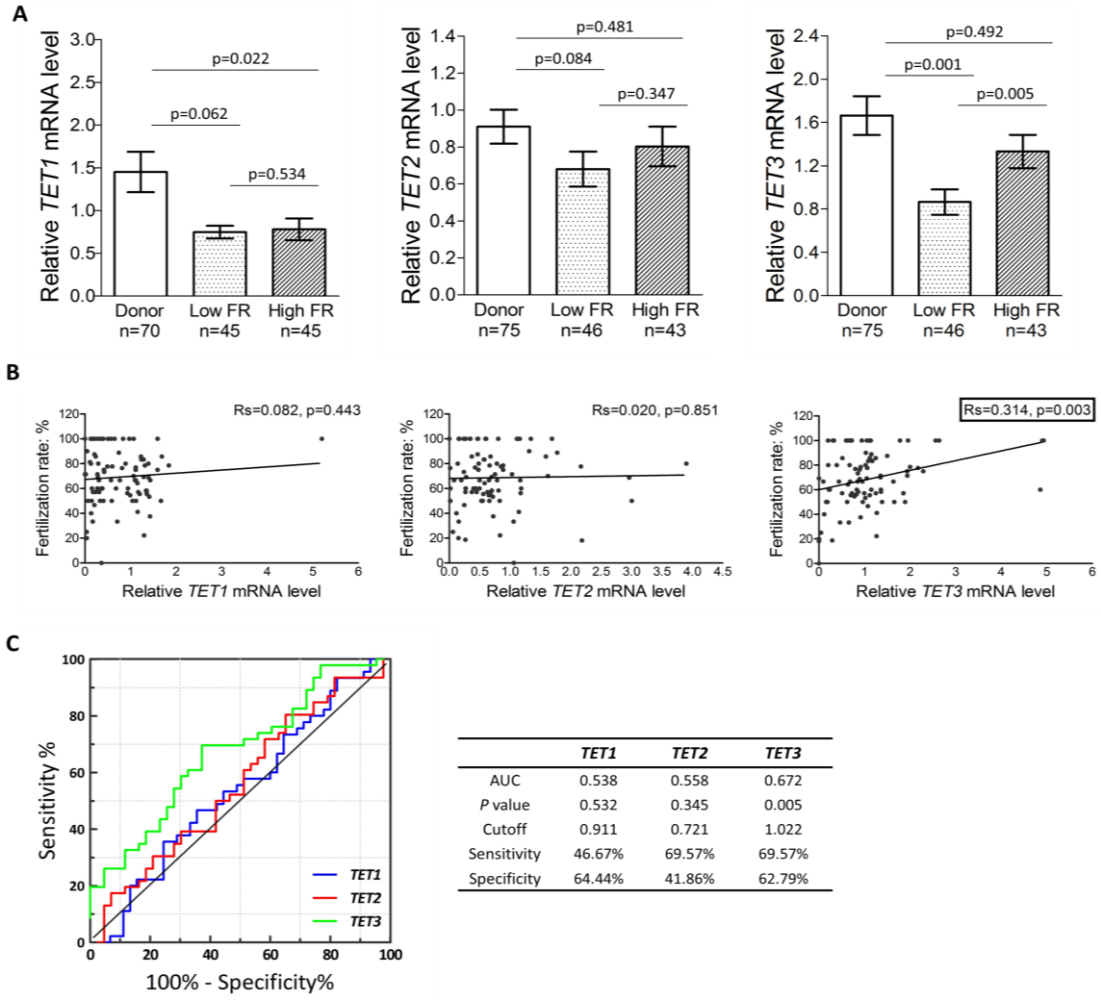
### 3.5.5 Correlations of *TET1–3*, *DNMT1* and *DNMT3A* mRNA levels with fertilization rate after ICSI treatment

Based on the threshold of fertilization rate (FR) as 70% after ICSI treatment (van Golde *et al.* 2001; Tournaye *et al.* 2002; Palermo *et al.* 2009; Rogenhofer *et al.* 2013), the subfertile patients were divided into two groups “low FR” (< 70%) and “high FR” (≥ 70%), and compared concerning *TET1–3*, *DNMT1* and *DNMT3A* mRNA levels in sperm cells.

In general, considering *TET1–3* mRNA levels, the healthy controls showed the highest *TET1–3* mRNA values in comparison with both “low FR” and “high FR” subfertile patients. A remarkable difference could be observed among patients for *TET3*-mRNA: “low FR” men had significantly reduced *TET3*-mRNA expression in sperm cells in comparison with “high FR” (0.87 ± 0.12 (± 0.79) versus 1.33 ± 0.15 (± 1.01), $P = 0.005$), and with healthy donors (1.67 ± 0.18 (± 1.55), $P = 0.001$, Mann–Whitney *U*-test; Figure 23A). The best cut-off value of *TET3*-mRNA for differentiation between “low FR” and “high FR” groups was calculated at 1.022 (AUC = 0.672, $P = 0.005$, sensitivity = 69.57% and specificity = 62.79%), and the calculated correlation coefficient was 0.314 ($P = 0.003$, Spearman’s non-parametric correlation; Figures 23B–C). In contrast, no correlations of *TET1–2* mRNA levels were detected between “low FR” and “high FR” groups (*TET1*: 0.75 ± 0.07 (± 0.50) versus 0.78 ± 0.13 (± 0.85), $P = 0.534$; *TET2*: 0.68 ± 0.09 (± 0.64) versus 0.80 ± 0.11 (± 0.70), $P = 0.347$; Mann–Whitney *U*-test; Figure 23A).

On the other hand, regarding *DNMT1* and *DNMT3A* mRNA levels, in general the healthy donors indicated the highest *DNMT1* (1.49 ± 0.14 (± 0.96)) and *DNMT3A* (2.27 ± 0.17 (± 1.21)) mRNA values compared to both “low FR” and “high FR” men (Figure 24A). However, no differences could be found between “low FR” and “high FR” patients for *DNMT1* (0.93 ± 0.13 (± 0.63) versus 0.95 ± 0.11 (± 0.48), $P = 0.618$) and *DNMT3A* (0.77 ± 0.20 (± 0.96)

versus 0.66 ± 0.10 (± 0.45), *P* = 0.401) mRNA levels (Mann–Whitney *U*-test; Figure 24A). There were no correlations between fertilization rate and the mRNA levels of *DNMT1* (Rs = – 0.145, *P* = 0.421) and *DNMT3A* (Rs = – 0.108, *P* = 0.481, Spearman's non-parametric correlation; Figure 24B).




| | *TET1* | *TET2* | *TET3* |
|---|---|---|---|
| AUC | 0.538 | 0.558 | 0.672 |
| *P* value | 0.532 | 0.345 | 0.005 |
| Cutoff | 0.911 | 0.721 | 1.022 |
| Sensitivity | 46.67% | 69.57% | 69.57% |
| Specificity | 64.44% | 41.86% | 62.79% |

**Figure 23 Associations of *TET1–3* mRNA levels in sperm cells with fertilization rates (FRs) after ICSI treatment. (A)** Patients were divided into two subgroups (high FR ≥ 70% and low FR < 70%), and compared to the healthy donors concerning *TET1–3* mRNA (relative mean values ± SEM, *P*-values of Mann–Whitney *U*-test, and number of analyzed samples are given). **(B)** Correlations of *TET1–3* mRNA with FRs (Rs- and *P*-values, Spearman's non-parametric correlation). **(C)** ROC curve analyses for *TET1–3* mRNA distinguishing between subfertile patients with low and high FRs (AUC, *P*-values, cut-off, sensitivity and specificity are given).

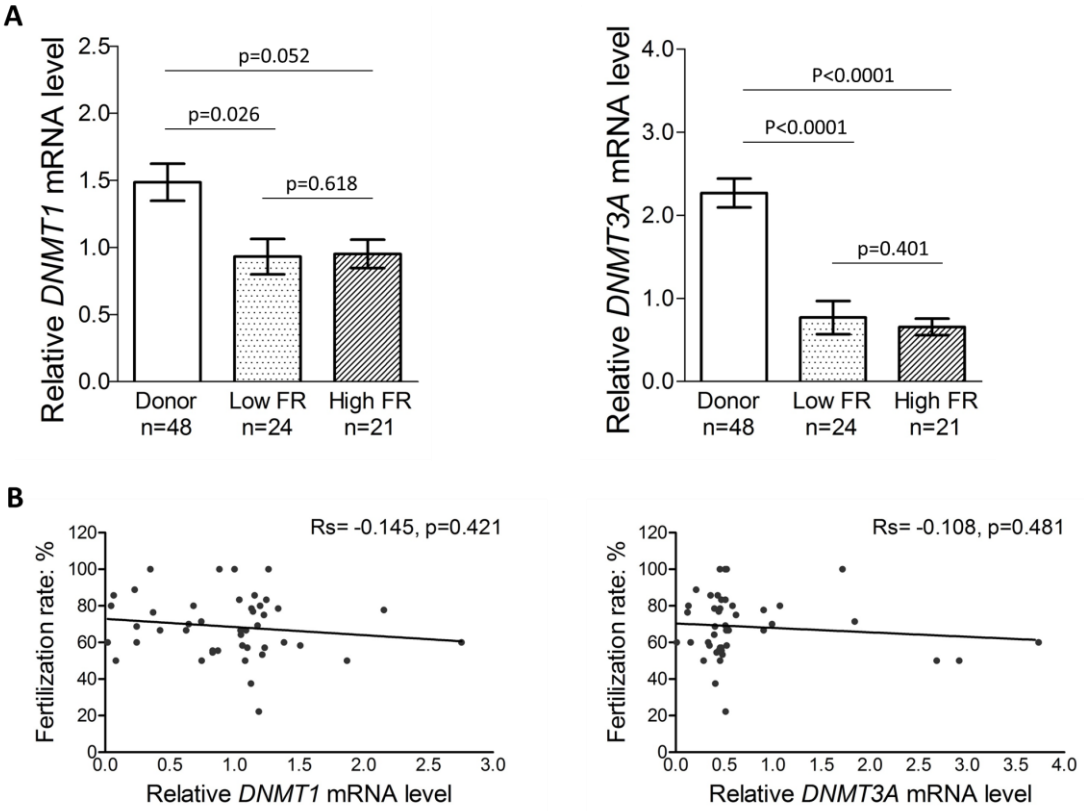


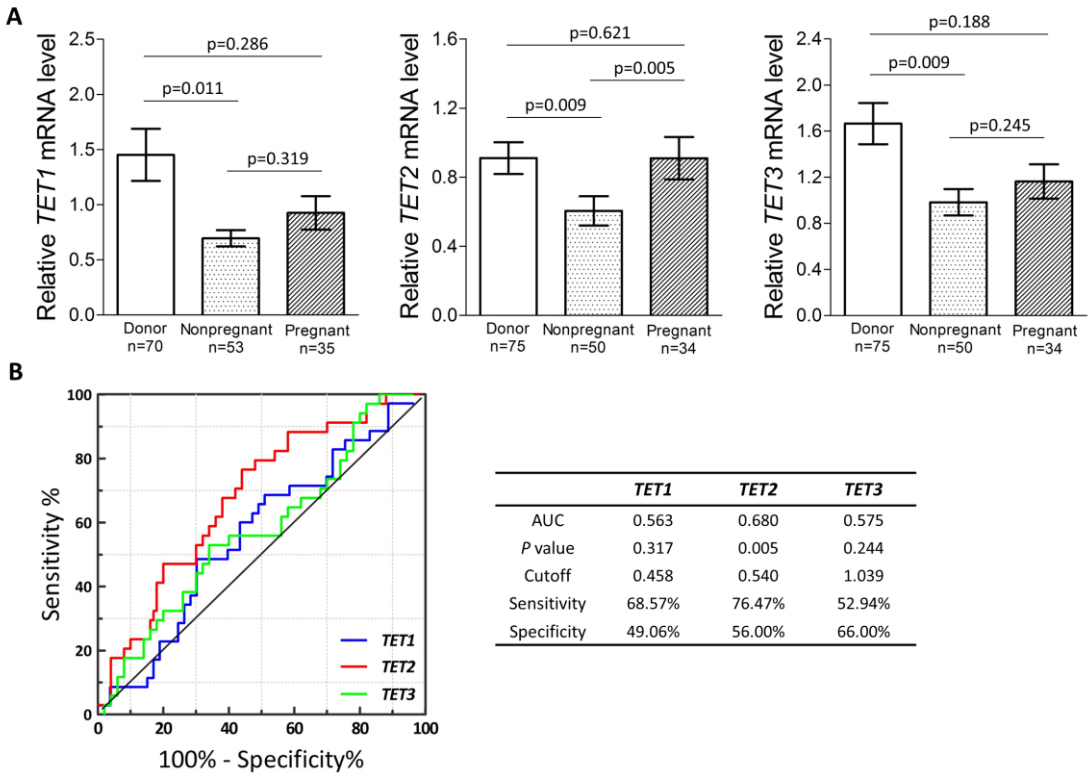

**Figure 24 Association studies of *DNMT1* and *DNMT3A* relative mRNA levels in sperm cells with fertilization rates (FRs) after ICSI treatment. (A)** The comparisons of *DNMT1* and *DNMT3A* mRNA levels between patients with low FR (< 70%) and high FR (≥ 70%), and the comparisons with healthy donors (relative mean values ± SEM, *P*-values of Mann–Whitney *U*-test, and number of analyzed samples are given). **(B)** Correlations of *DNMT1 and DNMT3A* mRNA levels with FRs (Rs- and *P*-values, Spearman's non-parametric correlation).

### 3.5.6 Correlations of *TET1–3*, *DNMT1* and *DNMT3A* mRNA levels with pregnancy status after ICSI treatment

Considering the pregnancy status after ICSI (pregnant versus non-pregnant), the overall achieved pregnancy rates for couples analyzed in this study were 39.77% in the *TET1* study (pregnant, n = 35; non-pregnant, n

= 53), 40.48% in the *TET2–3* study (pregnant, n = 34; non-pregnant, n = 50) and 52.08% in the *DNMT1* and *DNMT3A* study (pregnant, n = 25; non-pregnant, n = 23). In this part we aimed to investigate the correlations of *TET1–3*, *DNMT1* and *DNMT3A* mRNA levels with the pregnancy status and the final outcome of the ICSI treatment.

In general, in terms of *TET1–3* mRNA levels, the men in the "non-pregnant" group showed the lowest *TET1–3* mRNA levels in comparison with controls (*TET1*: 1.45 ± 0.24 (± 1.97) versus 0.70 ± 0.07 (± 0.54), *P* = 0.011; *TET2*: 0.91 ± 0.09 (± 0.79) versus 0.60 ± 0.09 (± 0.60), *P* = 0.009; *TET3*: 1.67 ± 0.18 (± 1.55) versus 0.98 ± 0.11 (± 0.81), *P* = 0.009; mean ± SEM (± SD), Mann–Whitney *U*-test) and with men in the "pregnant" group (Figure 25A). Interestingly, by comparing "non-pregnant" and "pregnant" groups, we found a highly significant association between high level of *TET2* mRNA in sperm cells and pregnancy status after ICSI treatment (0.91 ± 0.12 (± 0.72) versus 0.60 ± 0.09 (± 0.60), *P* = 0.005, Mann–Whitney U-test). The best cut-off value of *TET2*-mRNA for differentiation between "non-pregnant" and "pregnant" groups was calculated at 0.540 (AUC = 0.680, *P* = 0.005, sensitivity = 76.47% and specificity = 56.00%; Figure 25B). Nevertheless, no differences in *TET1* and *TET3* mRNA levels were detected between "non-pregnant" and "pregnant" groups (*TET1*: 0.70 ± 0.07 (± 0.54) versus 0.93 ± 0.15 (± 0.89), *P* = 0.319; *TET3*: 0.98 ± 0.11 (± 0.81) versus 1.16 ± 0.15 (± 0.87), *P* = 0.245; Mann–Whitney *U*-test; Figure 25A). On the other hand, the highest level of *DNMT1* (1.49 ± 0.14 (± 0.96)) and *DNMT3A* (2.27 ± 0.17 (± 1.21)) mRNAs occurred in the healthy donors compared to both "non-pregnant" and "pregnant" men (Figure 26). Nevertheless, no differences were detected between "non-pregnant" and "pregnant" patients for *DNMT1* (0.99 ± 0.13 (± 0.61) versus 0.93 ± 0.09 (± 0.44), *P* = 0.642) and *DNMT3A* (0.72 ± 0.18 (± 0.88) versus 0.66 ± 0.11 (± 0.55), *P* = 0.235) mRNA levels (Mann–Whitney *U*-test; Figure 26).




| | *TET1* | *TET2* | *TET3* |
|---|---|---|---|
| AUC | 0.563 | 0.680 | 0.575 |
| *P* value | 0.317 | 0.005 | 0.244 |
| Cutoff | 0.458 | 0.540 | 1.039 |
| Sensitivity | 68.57% | 76.47% | 52.94% |
| Specificity | 49.06% | 56.00% | 66.00% |

**Figure 25 Associations of *TET1–3* mRNA levels in sperm cells with pregnancy status after ICSI treatment. (A)** Subfertile patients were divided into "pregnant" and "non-pregnant" groups, and compared with healthy donors concerning *TET1–3* mRNA expressions in sperm cells (relative mean values ± SEM, *P*-values of Mann–Whitney *U*-test, and number of analyzed samples are given). **(B)** ROC curve analyses for *TET1–3* mRNA levels distinguishing between "pregnant" and "non-pregnant" groups after ICSI treatment (AUC, *P*-values, cut-off, sensitivity and specificity are given).

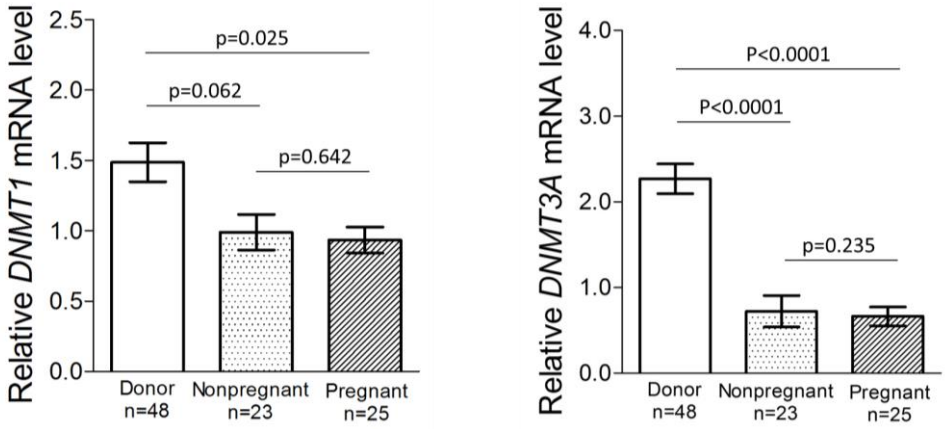



**Figure 26 Associations of *DNMT1* and *DNMT3A* relative mRNA levels in sperm cells with pregnancy status after ICSI treatment.** The comparison of *DNMT1* and

*DNMT3A* mRNA levels between “pregnant” and “non-pregnant” groups, and the comparisons with healthy donors as well (relative mean values ± SEM, *P*-values of Mann–Whitney *U*-test, and number of analyzed samples are given).

### 3.6 *TET1–3* CpG-promoter methylation analyses in human sperm cells

Previous results from this study found that low *TET1–3* mRNA expressions in sperm cells are associated with male fertility and affected the outcome of ICSI treatment. Since DNA methylation at CpG-promoters may affect mRNA transcription, we aimed to investigate *TET1–3* CpG-promoter methylation status in human sperm cells.

#### 3.6.1 *TET1* CpG-promoter methylation status

Based on the former results of *TET1*-mRNA level measured by RT-qPCR, 10 semen samples with low *TET1*-mRNA expression from subfertile patients and an additional 10 semen samples with high *TET1*-mRNA expression from healthy donors were used to analyze the methylation status by COBRA analysis (0.55 ± 0.10 (± 0.33) versus 1.59 ± 0.14 (± 0.44), $P < 0.0001$, Mann–Whitney *U*-test; Figure 27A). Specific designed COBRA primers of *TET1* CpG-promoter were used for PCR amplification (333 bp) products with 6 restriction recognition sits (CG..CG) digested by *Bsh1236I* restriction enzyme (Figure 27B). Bisulfite-treated DNAs from LNCaP cells and M.SssI-treated sperm DNA were used as positive controls in order to ensure the accuracy of *TET1*-COBRA primers and *Bsh1236I* restriction enzyme (Figure 27D). Our results showed that *TET1* CpG-promoters in sperm cells, in general, were unmethylated regardless of healthy donors or subfertile patients, while partially methylated and hypermethylated promoters were observed in LNCaP cells and M.SssI-treated sperm DNA (M.SssI), respectively (Figures 27C–D).

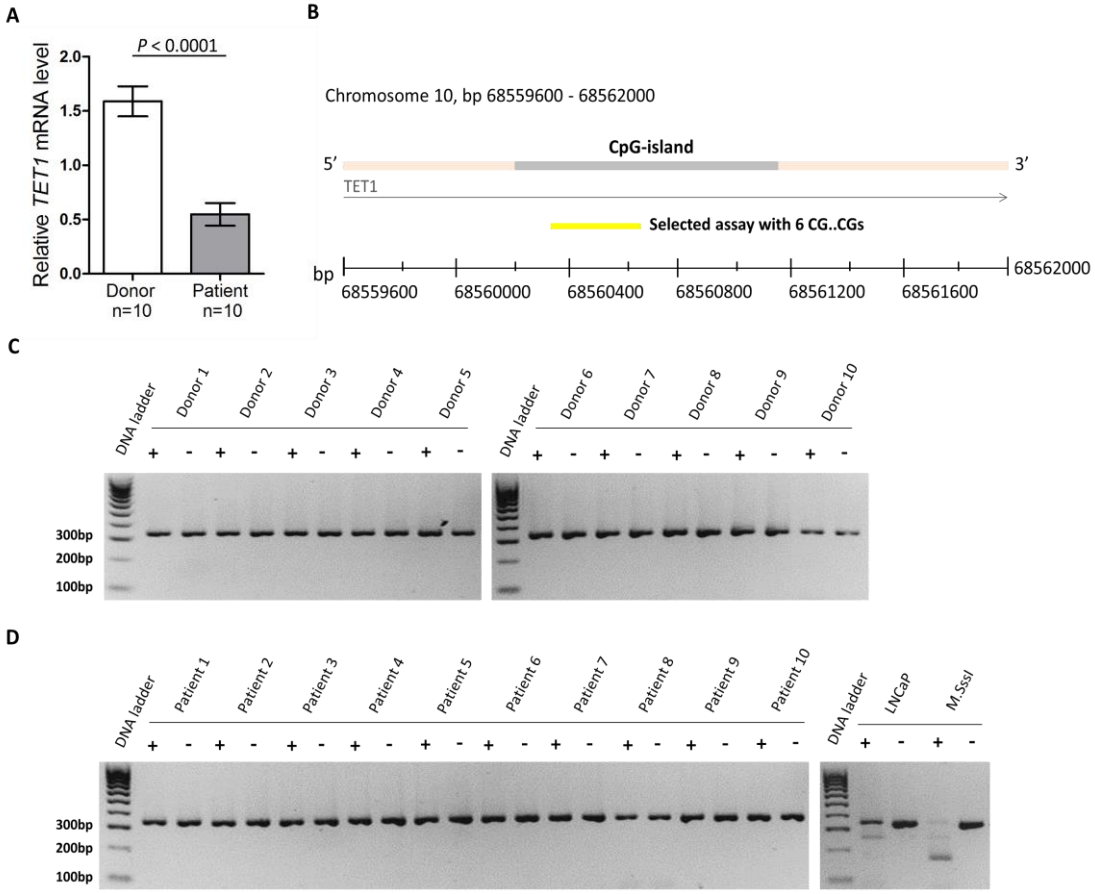



**Figure 27 COBRA analysis of sperm *TET1* CpG-promoter methylation in men with low (subfertile patients, n = 10) and high (healthy donors, n = 10) *TET1*-mRNA expression. (A)** Comparison of sperm *TET1* relative mRNA level in the selected donors and patients (relative mean values ± SEM, *P*-values of Mann–Whitney *U*-test, and number of analyzed samples are given). **(B)** A schematic overview of *TET1* CpG-promoter and the selected assay containing 6 CG..CG restriction recognition sites (brown line: CpG-island in the nucleotide strand, yellow line: selected assay for COBRA analysis). **(C–D)** Sperm *TET1* CpG-promoters in both donor and patient groups were unmethylated, while the CpG-promoters of positive control groups including LNCaP cells and M.SssI-treated sperm DNA (M.SssI) were partially methylated and hypermethylated, respectively ("+" means digestion with *Bsh1236I* restriction enzyme, "-" means mock digestion).

### 3.6.2 *TET2* CpG-promoter methylation status

Using COBRA analysis, we investigated the methylation status in men with low (n = 11, subfertile patients) and high (n = 10, healthy donors) *TET2*-mRNA levels (0.35 ± 0.10 (± 0.31) versus 1.30 ± 0.14 (± 0.48), *P* = 0.0002, Mann–Whitney *U*-test; Figure 28A). The *Bsh1236I* restriction enzyme was used to digest *TET2* CpG-promoter COBRA PCR amplification (272 bp) products with 4 CG..CG sites (Figure 28B). We found that *TET2* CpG-promoters in sperm cells were unmethylated in all selected participants no matter healthy donors and subfertile patients, while LNCaP cells and M.SssI-treated sperm DNA (M.SssI) exhibited hypermethylated CpG-promoters (Figures 28C–D).

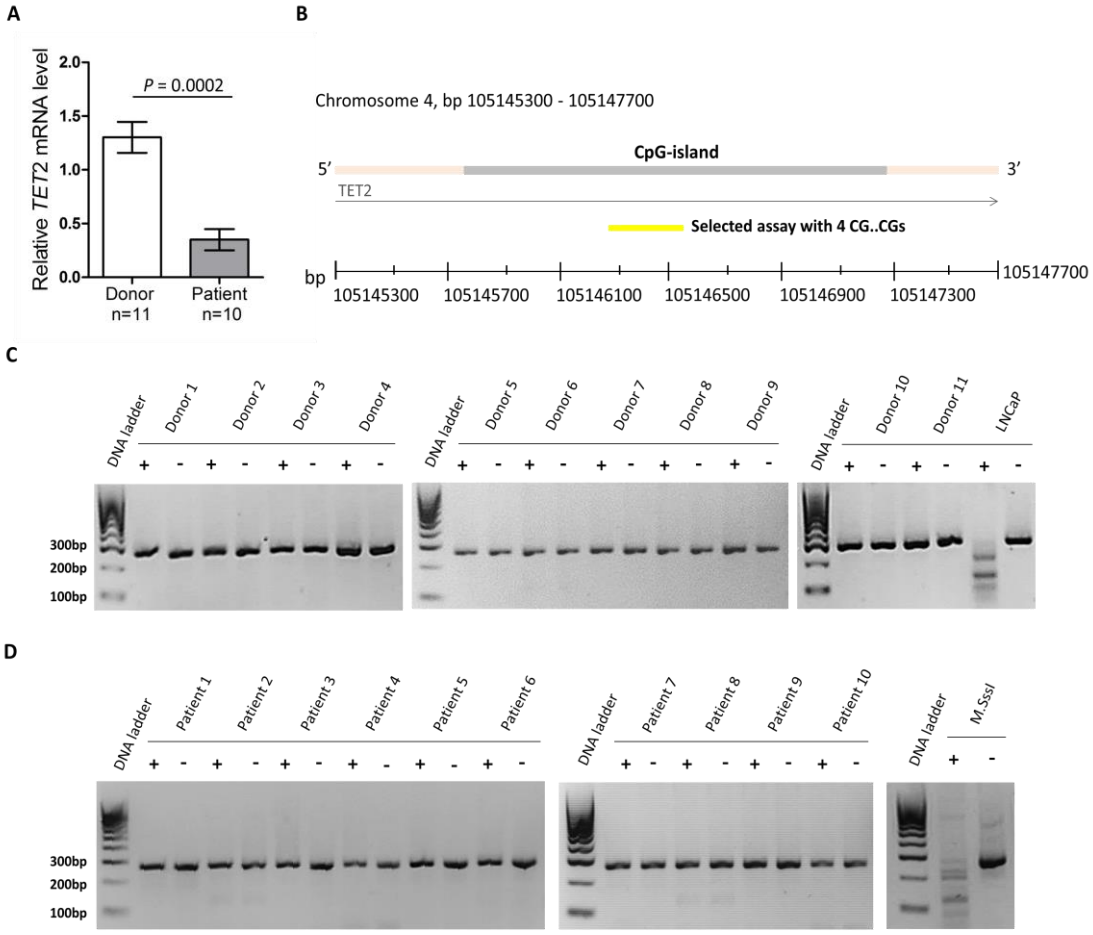
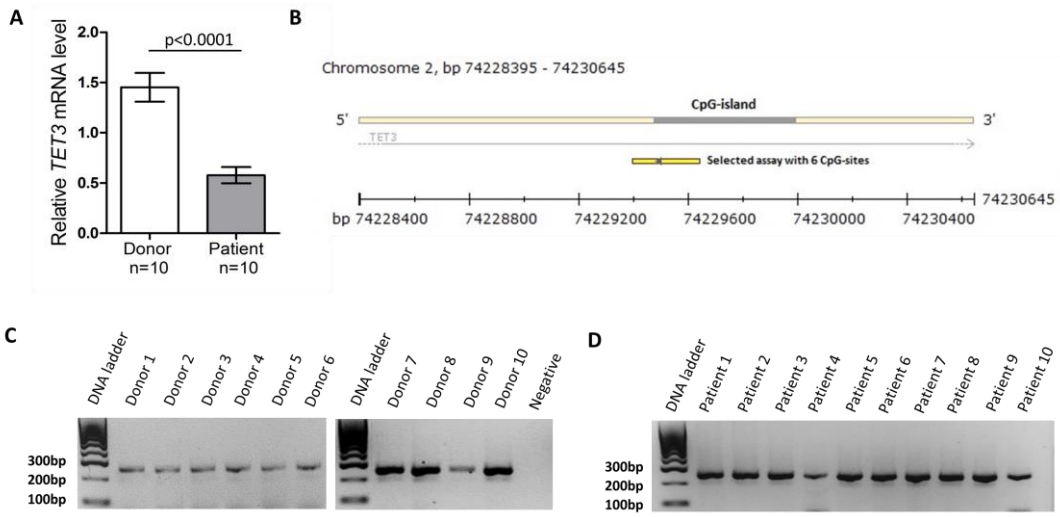



**Figure 28 Sperm *TET2* CpG-promoter methylation status was analyzed in men with low (subfertile patients, n = 10) and high (healthy donors, n = 11) *TET2*-mRNA**

**expression by COBRA analysis. (A)** Comparison of sperm *TET2*-mRNA level in the selected healthy donors and subfertile patients (relative mean values ± SEM, *P*-values of Mann–Whitney *U*-test, and number of analyzed samples are given). **(B)** A schematic overview of *TET2* CpG-promoter and the selected assay containing 4 CG..CG restriction recognition sites (brown line: CpG-island in the nucleotide strand, yellow line: selected assay for COBRA). **(C–D)** Sperm *TET2* CpG-promoters in the donor and patient groups were both unmethylated, while promoters of positive control groups, LNCaP cells and M.SssI-treated sperm DNA (M.SssI), were both hypermethylated ("+" means digestion with *Bsh1236I* restriction enzyme, "-" means mock digestion).

### 3.6.3 *TET3* CpG-promoter methylation status

Bisulfite pyrosequencing was applied to check the methylation pattern of *TET3* CpG-promoters in men with low (n = 10, subfertile patients) and high (n = 10, healthy donors) *TET3* mRNA levels (0.58 ± 0.08 (± 0.26) versus 1.41 ± 0.12 (± 0.39), $P < 0.0002$, Mann–Whitney *U*-test; Figure 29A). After amplifying bisulfite-treated sperm DNA, PCR products (250 bp) were used to examine the methylation status of 6 CpG-positions at *TET3* CpG-promoters (Figures 29B–D). We found that sperm *TET3* CpG-promoters in the healthy donor group exhibiting high *TET3* mRNA level, in general, were hypomethylated with mean methylation values of 6 CpG-sites ranging from 1.83% to 9.33% (Figure 29E). However, the DNA methylation degrees at *TET3* CpG-promoters in two subfertile patients were much higher up to 37.00% and 35.50%, respectively, accompanying low *TET3* mRNA expression (0.220 and 0.439), while the other eight patients presented hypomethylated *TET3* CpG-promoters in sperm cells with mean methylation percentages ranging from 5.33% to 6.17% (Figure 29E).




**E**

| *Characteristics* | CpG-P1 | CpG-P2 | CpG-P3 | CpG-P4 | CpG-P5 | CpG-P6 | Mean |
|---|---|---|---|---|---|---|---|
| Donor 1 | 2% | 4% | 3% | 1% | 1% | 2% | 2.17% |
| Donor 2 | 1% | 4% | 3% | 2% | 1% | 3% | 2.33% |
| Donor 3 | 1% | 5% | 4% | 1% | 1% | 1% | 2.17% |
| Donor 4 | 3% | 3% | 3% | 2% | 0% | 0% | 1.83% |
| Donor 5 | 3% | 3% | 13% | 13% | 3% | 3% | 6.33% |
| Donor 6 | 4% | 3% | 8% | 10% | 4% | 4% | 5.50% |
| Donor 7 | 3% | 6% | 10% | 14% | 3% | 4% | 6.67% |
| Donor 8 | 6% | 6% | 16% | 20% | 4% | 4% | 9.33% |
| Donor 9 | 4% | 7% | 13% | 16% | 0% | 8% | 8.00% |
| Donor 10 | 4% | 3% | 13% | 14% | 3% | 6% | 7.17% |
| Patient 1 | 1% | 2% | 11% | 12% | 3% | 4% | 5.50% |
| Patient 2 | 1% | 2% | 12% | 13% | 3% | 3% | 5.67% |
| Patient 3 | 41% | 32% | 47% | 46% | 17% | 39% | 37.00% |
| Patient 4 | 39% | 31% | 46% | 45% | 16% | 36% | 35.50% |
| Patient 5 | 1% | 3% | 11% | 11% | 3% | 3% | 5.33% |
| Patient 6 | 1% | 2% | 12% | 13% | 2% | 4% | 5.67% |
| Patient 7 | 0% | 2% | 11% | 12% | 3% | 4% | 5.33% |
| Patient 8 | 1% | 2% | 12% | 14% | 3% | 2% | 5.67% |
| Patient 9 | 1% | 2% | 11% | 13% | 3% | 3% | 5.50% |
| Patient 10 | 2% | 3% | 13% | 13% | 3% | 3% | 6.17% |

**Figure 29 Sperm *TET3* CpG-promoter methylation status was measured in men with low (n = 10, subfertile patients) and high (n = 10, healthy donors) *TET3* mRNA expression using bisulfite pyrosequencing. (A)** Comparison of *TET3*-mRNA level in sperm cells between the selected healthy donor group and subfertile patient group (relative mean values ± SEM, *P*-values of Mann–Whitney *U*-test, and number of analyzed samples are given). **(B)** A schematic overview of *TET3* CpG-promoter and the

selected assay containing 6 CpG-sites (brown line: CpG-island in the nucleotide strand, yellow line: selected assay for bisulfite pyrosequencing). **(C–D)** Amplified PCR products (250 bp) of bisulfite-treated sperm DNA from the selected healthy donors and subfertile patients were shown on a 2% agarose gel. **(E)** Results of 6 CpG-positions (CpG-P1 to CpG-P6) methylation in sperm *TET*3 CpG-promoter of the selected donors and patients, *TET3* CpG-promoters in donor group were generally hypomethylated, while the mean methylation values in two subfertile patients were much higher up to 37.00% and 35.50% presenting low *TET3*-mRNA expression (0.220 and 0.439).

## 3.7 Associations of *TET1–3*, *DNMT1* and *DNMT3A* mRNA expression with the methylation status of the imprinted gene *H19*

In this part, we aimed to underscore the impact of disturbed spermatogenesis on the risk of producing gametes with genomic imprinting defects, and investigate the influences of *TET1–3*, *DNMT1* and *DNMT3A* mRNA expressions on the methylation pattern of the paternal imprinted gene *H19*. The imprinted gene *H19* was selected, which was completely methylated in the paternal allele and unmethylated in the maternal allele. The imprinting signatures of sperm from 18 healthy donors and 20 subfertile patients were studied using bisulfite modification and COBRA analysis.

After amplifying bisulfite-treated sperm DNA, PCR products (239 bp) were used to check the methylation status of the imprinted gene *H19* with 3 restriction recognition sits (CG..CG) digested by *Bsh1236I* restriction enzyme (Figure 30A). Applying COBRA, we found that only one healthy donor (1/18 = 5.56%) showed aberrant methylation status at the *H19* gene (Don-Ab-Me-*H19*, partially methylation), while the other 17 donors presented normal methylation status at the *H19* gene (Don-N-Me-*H19*, fully methylation; Figure 30B and Figure 31). On the other hand, we separated subfertile men

suffering from oligozoospermia and/or asthenozoospermia in the “O + A” group (n = 16) and patients exhibiting normozoospermia in the “NZS” group (n = 4). Our data indicated that subfertile patients with the aberrant methylation of the *H19* gene (Pat-Ab-Me-*H19;* 7/20 = 35%) included one “NZS” patient (1/4 = 25%), two “O + A” patients with a sperm count above 10 Mil/ml (2/8 = 25%) and four “O + A” patients with a sperm count below 10 Mil/ml (4/8 = 50%; Figure 30C and Figure 31). Among these seven subfertile patients carrying aberrant methylation at the imprinted gene *H19*, five of them (5/7 = 71.43%) suffered from low FR (< 70%), whereas four female partners (4/7 = 57.14%) were pregnant after ICSI treatment.

Considering *TET1–3*, *DNMT1* and *DNMT3A* mRNA expression, the only single donor with aberrant methylation of the *H19* gene had normal *TET1–3* mRNA levels, but *DNMT1* (0.630 versus 0.950) and *DNMT3A* (1.597 versus 2.669) were much lower in comparison with the mean of relative mRNA levels in the Don-N-Me-*H19* group (Figure 31). Furthermore, regarding the patient group, although mean of *TET1–3* and *DNMT1* mRNA levels was the lowest in the Pat-Ab-Me-*H19* group, no statistical differences were found compared to the men of Pat-N-Me-*H19* (*TET1*: 0.62 ± 0.15 (± 0.40) versus 0.66 ± 0.12 (± 0.44), *P* = 1.000; *TET2*: 0.99 ± 0.43 (± 1.13) versus 1.13 ± 0.28 (± 1.01), *P* = 0.579; *TET3*: 0.85 ± 0.19 (± 0.52) versus 1.00 ± 0.12 (± 0.45), *P* = 0.526 and *DNMT1*: 0.72 ± 0.29 (± 0.78) versus 0.84 ± 0.17 (± 0.60), *P* = 0.322; Mann–Whitney *U*-test; Figures 32A–B). Interestingly, relative *DNMT3A* mRNA level was the lowest in the Pat-Ab-Me-*H19* group compared to the men of Pat-N-Me-*H19* (0.50 ± 0.13 (± 0.34) versus 1.38 ± 0.31 (± 1.13), *P* = 0.047) and Don-N-Me-*H19* (0.50 ± 0.13 (± 0.34) versus 2.73 ± 0.48 (± 1.99), *P* = 0.002, Mann–Whitney *U*-test; Figure 32B). Finally, the changes of mRNA levels were shown by a color gradient intensity scale. It seemed that men in the Pat-Ab-Me-*H19* group would more frequently exhibit low *TET1–3*, *DNMT1* and *DNMT3A* mRNA levels simultaneously (Figure 31).

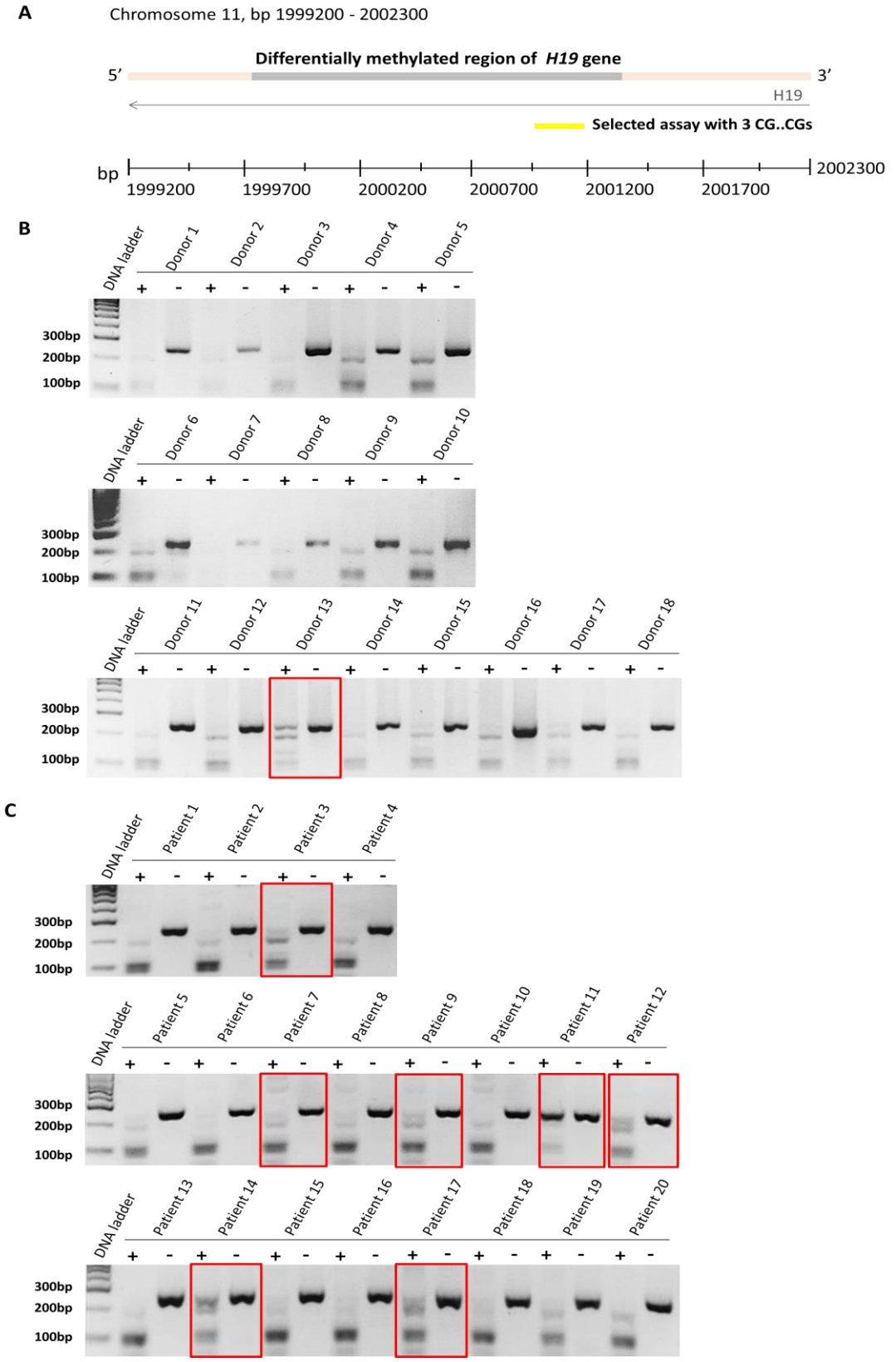



**Figure 30 Methylation status of the imprinted gene *H19* in sperm cells from healthy donors (n = 18) and subfertile patients (n = 20) using bisulfite modification and COBRA analysis. (A)** A schematic overview of the methylation pattern of the *H19* gene

and the selected assay containing 3 CG..CG restriction recognition sites (brown line: CpG-island in the nucleotide strand, yellow line: selected assay for bisulfite pyrosequencing). **(B)** Only one healthy donor had aberrant methylation of the *H19* gene highlighted by a red box (1/18, partially methylation), while the other 17 donors presented normal methylation (fully methylation). **(C)** The imprinted gene *H19* was aberrantly methylated in 7 subfertile patients highlighted by red boxes ("+" means digestion with *Bsh1236I* restriction enzyme, "-" means mock digestion).

| ***Characteristics*** | ***TET1*** | ***TET2*** | ***TET3*** | ***DNMT1*** | ***DNMT3A*** |
|---|---|---|---|---|---|
| **Don-N-Me-*H19* (Mean, n=17)** | 1.488 | 1.083 | 2.755 | 0.950 | 2.669 |
| **Don-Ab-Me-*H19* (n=1)** | 2.237 | 1.249 | 3.531 | 0.630 | 1.597 |
| **Pat-N-Me-*H19* (n=13)** | | | | | |
| 1. NZS | 0.744 | 1.776 | 1.152 | 0.228 | 0.210 |
| 2. NZS | 0.503 | 1.132 | 1.056 | 0.350 | 1.717 |
| 3. NZS | 0.767 | 1.169 | 0.686 | 0.425 | 0.532 |
| 4. O+A (≥ 10 Mil/ml) | 0.683 | 0.017 | 1.361 | 0.243 | 0.335 |
| 5. O+A (≥ 10 Mil/ml) | 1.070 | 1.624 | 1.353 | 0.646 | 0.992 |
| 6. O+A (≥ 10 Mil/ml) | 1.668 | 0.660 | 1.387 | 0.747 | 2.687 |
| 7. O+A (≥ 10 Mil/ml) | 0.236 | 1.591 | 0.515 | 0.681 | 3.784 |
| 8. O+A (≥ 10 Mil/ml) | 0.292 | 3.901 | 0.675 | 0.683 | 1.072 |
| 9. O+A (≥ 10 Mil/ml) | 0.276 | 1.032 | 1.940 | 0.744 | 1.843 |
| 10. O+A (< 10 Mil/ml) | 1.098 | 0.787 | 0.849 | 1.090 | 0.546 |
| 11. O+A (< 10 Mil/ml) | 0.252 | 0.726 | 0.232 | 1.871 | 2.919 |
| 12. O+A (< 10 Mil/ml) | 0.816 | 0.013 | 0.897 | 2.228 | 0.736 |
| 13. O+A (< 10 Mil/ml) | 0.154 | 0.271 | 0.833 | 0.959 | 0.577 |
| **Pat-Ab-Me-*H19* (n=7)** | | | | | |
| 1. NZS | 0.891 | 0.783 | 0.390 | 0.046 | 0.130 |
| 2. O+A (≥ 10 Mil/ml) | 0.273 | 0.496 | 1.149 | 1.383 | 0.154 |
| 3. O+A (≥ 10 Mil/ml) | 1.397 | 2.961 | 0.895 | 0.243 | 0.404 |
| 4. O+A (< 10 Mil/ml) | 0.493 | 2.166 | 1.175 | 2.156 | 0.904 |
| 5. O+A (< 10 Mil/ml) | 0.360 | 0.436 | 0.220 | 0.534 | 0.723 |
| 6. O+A (< 10 Mil/ml) | 0.573 | 0.080 | 0.439 | 0.626 | 0.905 |
| 7. O+A (< 10 Mil/ml) | 0.331 | 0.015 | 1.653 | 0.082 | 0.287 |

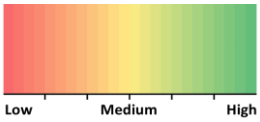



**Figure 31 Overview of *TET1–3*, *DNMT1* and *DNMT3A* mRNA expressions in donors (n = 18) and patients (n = 20) with normal and aberrant methylation status at the**

**imprinted gene *H19.*** One donor with aberrant methylation of the *H19* gene (Don-Ab-Me-*H19*, n = 1) presented high *TET1–3* mRNA levels, but lower *DNMT1* and *DNMT3A* compared to the donors with normal methylation status (Don-N-Me-*H19*, n = 17). Patients with aberrant methylation at the imprinted gene *H19* (Pat-Ab-Me-*H19*, n = 7) would more frequently exhibit low *TET1–3*, *DNMT1* and *DNMT3A* mRNA levels. The changes of mRNA expression were demonstrated by a color gradient intensity scale, as shown at the bottom. The deepest red color indicates a maximal decrease and the deepest green color indicates a maximal increase in gene expression.

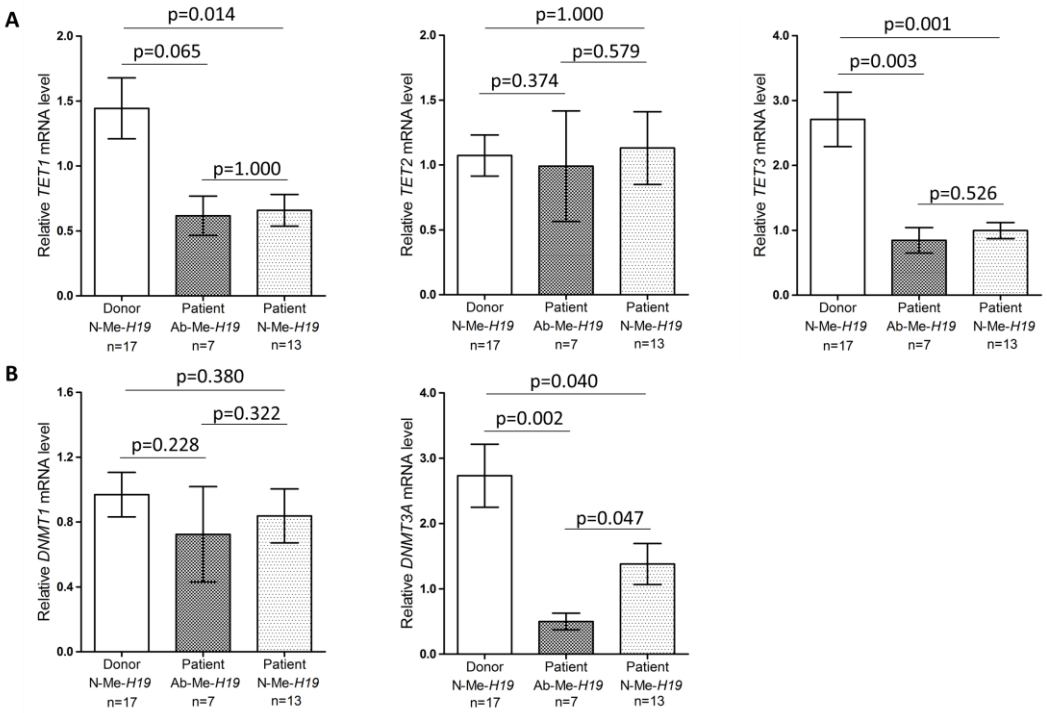



**Figure 32 Comparisons of *TET1–3*, *DNMT1* and *DNMT3A* mRNA levels among the donors with normal methylation pattern at the imprinted gene *H19*, and the patients exhibiting aberrant and normal methylation status. (A)** Regarding *TET1–3* mRNA levels, no statistical differences were found in the patients with aberrantly methylated *H19* gene (Pat-Ab-Me-*H19,* n = 7) compared to the patients with normal methylation status (Pat-N-Me-*H19,* n = 13). **(B)** The lowest *DNMT3A* mRNA level was found in the Pat-Ab-Me-*H19* group (n = 7) compared to the men in Pat-N-Me-*H19* (n = 13) and Don-N-Me-*H19* (n = 17) groups. However, no differences of *DNMT1* mRNA level could be observed among groups (relative mean values ± SEM, *P*-values of Mann–Whitney *U*-test, and number of analyzed samples are given).

# 4. DISCUSSION

## 4.1 Expression of TET1–3 and 5hmC in human male germ cells

Dynamic epigenetic changes occur during mammalian germ-cell development, which are regulated by a number of epigenetic modifiers including TETs (involved in DNA demethylation), DNMTs (responsible for DNA methylation), histone-modification enzymes and their regulatory factors. It is known that the male germ line includes two waves of global DNA demethylation: the first occurs in the paternal pronucleus of the pre-implantation embryo shortly after fertilization, and the second takes place during the differentiation and migration of the PGCs (Iqbal *et al.* 2011; Wu & Zhang 2011; Kohli & Zhang 2013; Gkountela *et al.* 2015). Previous results indicated that once spermatogenesis is initiated paternal imprints should have been already established (Hackett *et al.* 2013; Molaro *et al.* 2014; Tang *et al.* 2015). DNA methylation during mammalian germ cell differentiation and the expression of the involved DNMTs have already been described in detail (Tucker *et al.* 1996; Kierszenbaum 2002; Yaman & Grandjean 2006; Takashima *et al.* 2009; Marques *et al.* 2011; Yao *et al.* 2015), whereas the impact of TETs and DNA demethylation is only sparsely documented, especially on human spermatogenesis and male fertility. Concerning DNA demethylation during spermatogenesis, studies in mice and human have demonstrated that male gametogenesis occurs without significant changes in 5mC, but involves a dynamic variation in 5hmC (Gan *et al.* 2013; Nettersheim *et al.* 2013; Hammoud *et al.* 2014), and emphasized herewith the impact of TETs on the establishment of a feature-complete sperm DNA methylation. In order to get more insights into this issue, in the present study, we analyzed the stage specific expression pattern of all TET members (TET1–3) at both

mRNA and protein levels, together with 5hmC, in human testis tissues exhibiting normal spermatogenesis, and in mature spermatozoa.

So far, only two studies investigated TET expression during spermatogenesis, and both were performed solely at the mRNA level (Gan *et al.* 2013; Nettersheim *et al.* 2013). In one study, cDNA microarray analysis was used to investigate *TET1–3* mRNA expression in human normal testicular tissues (NTTs) obtained from patients with prostate carcinoma and in germ cell cancer tissues (GCCs) (Nettersheim *et al.* 2013). Based on data of this study, *TET1* mRNA expression was significantly elevated in seminomas and embryonal carcinoma tissues, whereas low expression of *TET1* was detected in NTTs, carcinoma-in situ (CIS), teratoma and mixed GCCs. *TET2* expression was elevated only in CIS, and both *TET2 and TET3* mRNA expressions were low in NTTs as well as in GCCs. Interestingly, a strong signal of 5hmC was found in spermatogonia of NTTs, and a gradual reduction of the 5hmC signal was observed towards germ cells located near the lumen of the seminiferous tubules. Moreover, all CIS tissues and the majority of seminomas were detected to be hypohydroxymethylated in comparison with non-seminomas (Nettersheim *et al.* 2013). Another genome-wide transcriptome study in eight separated consecutive types of germ cells in murine spermatogenesis showed that *Tet1–3* mRNA expression presented at much higher level in the diploid germ cells compared to the haploid cell types, with *Tet3* mRNA showing much more abundant than *Tet1* and *Tet2* mRNA*s* (Gan *et al.* 2013). Authors concluded that Tet3 may represent a key enzyme for the production of 5hmC from 5mC in male germ cells, as is the case in mouse oocytes and zygotes (Gu *et al.* 2011; Sakashita *et al.* 2014). Using high-throughput sequencing, they also profiled a genome-wide 5hmC distribution in eight continuous germ cell types and observed elevated 5hmC level in spermatogonia, which was then decreased

towards round spermatids and increased again in elongating spermatids and mature spermatozoa (Gan *et al.* 2013).

In this study, we purposed to reveal the comprehensive profiles of TET1–3 mRNA and protein expression in normal human spermatogenesis through ISH and IHC. Our results indicated that TET1–3 expressions follow a specific pattern. Specifically, the three *TET*-mRNAs simultaneously start to appear in early pachytene spermatocytes in stage I, and are still detectable in late pachytene spermatocytes in stage V. Interestingly, TET1–3 proteins could be successively detected at different stages. TET2-protein could be first observed in the cytoplasm of the late pachytene spermatocytes exclusively in stage V, followed by TET1 and TET3 beginning in the nuclei of steps 1 and 3 round spermatids, respectively. Expression of both ended in step 4 elongating spermatids. On the other hand, 5hmC was detected exclusively in step 5 elongated spermatids of stage V. This finding suggests that an active DNA demethylation pathway may occur with the involvement of the TET enzymes during human spermiogenesis. We did not observe any 5hmC signal in spermatogonia as previously has been reported in human testis tissue (Nettersheim *et al.* 2013). Since we did not detect TET1–3 expression in spermatogonia as well, we supposed a passive DNA demethylation. Nevertheless, technical differences (e.g. tissue fixation procedures, used antibodies), but also the various amount of 5hmC during passive and active DNA demethylation, might lead to different observations. The localization of TET2 protein in the cytoplasm of pachytene spermatocytes was surprising as TET2 protein is normally regarded as a nuclear protein with the ability of DNA demethylation in, e.g. embryonic stem cells, hematopoietic stem cells and lymphomagenesis (Ko *et al.* 2011; Koh *et al.* 2011; Li *et al.* 2011; Quivoron *et al.* 2011; Vincent *et al.* 2013). However, as has been demonstrated in recent studies, TET2 protein may also show a cytoplasmic localization, e.g. in neurons involved in the regulation of cell survival (Mi *et al.* 2015), in colorectal

cancer cells in association with metastasis (Huang *et al.* 2016), and in human embryonic kidney 293T (HEK293T) cells (Chang *et al.* 2014; Fu *et al.* 2014). Multiple studies recently reported the existence of 5hmC in both mammalian DNA and RNA, and Tet enzymes also possess the activity of catalyzing the formation of 5hmC in RNA (Fu *et al.* 2014; Delatte *et al.* 2016; Zhang *et al.* 2016). Furthermore, Delatte and coworkers found that RNA hydroxymethylation can affect mRNA translation, and Tet-deficient fruitflies suffer from impaired brain development accompanied with highly decreased RNA hydroxymethylation (Delatte *et al.* 2016). These results let us hypothesize that the cytoplasmic TET2 protein in human pachytene spermatocytes probably has the ability of regulating mRNA translation. Our IHC results revealed a negative staining for TET1–3 protein in elongated spermatids, which might be due to an antigen masking since these final germ cell stages are known to exhibit highly condensed chromatin. For that reason, it was barely possible to determine the exact translational stop points for TET proteins in human spermatogenesis. However, our WB and ICC analyses demonstrated that TET1–3 proteins were all present in human mature spermatozoa, while the localization of TET1–3 proteins could be attributed to the nuclei, when mature spermatozoa were treated with chromatin decondensation buffer.

## 4.2 *TET1–3*, *DNMT1* and *DNMT3A* mRNA levels in mature spermatozoa are significantly reduced in subfertile men

The establishment of methylation in germ cells is a unique process and is essential for proper sperm production. Using both single-gene and genome-wide analyses, several studies reported close associations of abnormal DNA methylation with certain aberrant repetitive elements and imprinted genes, especially in sperm cells from subfertile patients with poor

semen quality (Houshdaran *et al.* 2007; Kobayashi *et al.* 2007; Marques *et al.* 2008; Khazamipour *et al.* 2009; Hammoud *et al.* 2010; Minor *et al.* 2011; Li *et al.* 2013; Urdinguio *et al.* 2015). Furthermore, the outcome of ART treatment was generally poor when using the sperm presenting aberrant DNA methylation patterns (Kobayashi *et al.* 2007; Marques *et al.* 2008).

Concerning DNA methylation, DNMT1 mainly ensures DNA methylation maintenance, while DNMT3A, 3B, and 3L are specifically responsible for the process of *de novo* DNA methylation (Carrell & Hammoud 2010; Carrell 2012; Boissonnas *et al.* 2013). The direct evidence using *Dnmt3a* and *Dnmt3L* knock-out mice demonstrated that lack of these enzymes could result in multiple serious spermatogenic defects including arrest at pachytene spermatocytes, imprinting failure, derepression of retrotransposons and even impaired chromosome synapsis (Bourc'his & Bestor 2004; Kaneda *et al.* 2004; Webster *et al.* 2005; Hata *et al.* 2006). A recent study characterized DNMTs expression in human normal spermatogenesis by isolating all kinds of spermatogenic cells from testicular biopsies through micromanipulation (Marques *et al.* 2011). They found that *DNMT1*, *DNMT3A* and *DNMT3B* mRNAs were constantly detectable at a high level from spermatogonia to round spermatids using RT-qPCR. Nevertheless, *DNMT3L* mRNA could not be detected in any germ cell stages but in whole testicular tissue probably due to low mRNA synthesis (Marques *et al.* 2011). Interestingly, in purified mature spermatozoa *DNMT3B* mRNA expression was absent but *DNMT1* and *DNMT3A* mRNAs were highly expressed probably revealing a post-testicular upregulation of *DNMT1* and *DNMT3A*, which were consistent with our results. In contrast, immunostaining showed that DNMT1, DNMT3A and DNMT3B proteins were all detectable in different germ cell stages (Marques *et al.* 2011). Another research group applying ISH and IHC methods got a similar result that *DNMT1* mRNA was expressed in spermatogonia, pachytene spermatocytes and round spermatids in human

normal spermatogenesis, whereas DNMT1 protein was absent in pachytene spermatocytes with an appearance in the nuclei of spermatogonia and in the cytoplasm of round spermatids (Omisanjo *et al.* 2007). Interestingly, in impaired spermatogenesis exhibiting round spermatids maturation arrest, it showed an absence of DNMT1 protein in round spermatids but a precocious appearance of DNMT1 protein was detected in the nuclei of pachytene spermatocytes, which might contribute to the failure of round spermatids to differentiate into elongating spermatids (Omisanjo *et al.* 2007).

On the other hand, regarding DNA demethylation in male germ cells, apart from our findings that TET enzymes and 5hmC are successively expressed during human spermatogenesis, several functional studies using knock-out mice and murine ESCs also revealed a close association of *Tet1* and *Tet2* depletion with an abnormal 5hmC level both genome-wide and at specific genome regions, such as gene promoters, imprinting loci and repetitive sequences, (Dawlaty *et al.* 2011; Dawlaty *et al.* 2013; Vincent *et al.* 2013; Dawlaty *et al.* 2014; Huang *et al.* 2014). In contrast to Tet1 and Tet2 proteins, Tet3 enzyme is mainly expressed in various types of male and female germ cells (Dawlaty *et al.* 2013; Gan *et al.* 2013; Gkountela *et al.* 2015). Recently, 5hmC was also shown to mark the sites of DNA damage and promote the stability of genome (Kafer et al., 2016). Mature spermatozoa are transcriptionally inactive cells. Nevertheless, they might still contain non-degraded mRNAs from the spermatogenesis program, and thereby could provide the evidences of transcriptional levels of *DNMTs* and *TETs* during mammalian spermatogenesis.

Based on the previous findings that *DNMT1*, *DNMT3A* and *TET1–3* mRNAs were closely related to human spermatogenesis, and were all expressed in purified ejaculated sperm cells, we aimed to perform several descriptive analyses by measuring and comparing the mRNA levels of these

cytosine modifying enzymes in ejaculated spermatozoa of fertile donors and subfertile patients who underwent ICSI-treatment with their female partners. In general, we found that the relative levels of *DNMT1*, *DNMT3A*, *TET1* and *TET3* mRNAs in mature sperm cells of fertile men were markedly higher than in subfertile patients, and they were negatively correlated with male age. Age-associated sperm DNA methylation alterations have been recently reviewed in the context of the whole sperm epigenome (Jenkins *et al.* 2014; Jenkins *et al.* 2015). Our data is consistent with this finding and indicates that the mRNA expression of these vital DNA methylation regulating enzymes is also dramatically decreased as male age is increasing. Concerning the correlations with routine semen parameters, they seem to be also linked with sperm production and semen quality, with close associations with sperm concentration (*TET1–3* and *DNMT3A*), progressive motility (*TET1, TET3* and *DNMT3A*), total motility (*TET1*) and normal morphology (*TET1*, *DNMT1* and *DNMT3A*). As the coefficients of correlation in the present study are relatively low, further studies on a large cohort are necessary to strengthen these findings. Finally, we found that patients suffering from oligo- and asthenozoospermia, and also normoozoospermic subfertile patients, showed significantly decreased *DNMT1*, *DNMT3A* and *TET1–3* mRNA expressions in mature spermatozoa. Through a genome-wide or a single-gene manner, several studies have already identified multiple aberrant alterations of sperm DNA methylation not only at broad specific loci, but also at certain imprinted genes and repetitive sequences in patients with idiopathic male infertility and normoozoospermic patients with unexplained male infertility (Poplinski *et al.* 2010; Wu *et al.* 2010; Urdinguio *et al.* 2015). Therefore, our findings indicated that aberrant transcription of DNA-methylation governing enzymes (*DNMT1*, *DNMT3A* and *TET1–3*) could probably provide a research direction to elucidate the aberrant DNA methylation patterns of ejaculated sperm cells in men with idiopathic and unexplained male infertility.

## 4.3 *TET2* and *TET3* mRNAs in mature sperm cells are significantly associated with the ICSI outcome

Global dynamic modification of cytosine, namely DNA methylation and demethylation, has been observed during both pre- and post-implantation embryonic development (Okano *et al.* 1999; Hirasawa *et al.* 2008; Gu *et al.* 2011; Wossidlo *et al.* 2011; Saitou *et al.* 2012; Kohli & Zhang 2013). Therefore, understanding the distinctive epigenetic modifications, which are adjusted by reprogramming during embryo development, has important implications for the outcome of ART treatment. It is now generally accepted that sperm contributes not only the paternal genome, but also epigenetic signatures to the oocytes. Although the oocyte is able to repair aberrant sperm DNA methylation, its capacity is limited (Wossidlo *et al.* 2010). Using immunostaining for DNA methylation, one study linked the global status of sperm DNA methylation to the pregnancy rate, associating methylation defects with embryo development and infertility (Benchaib *et al.* 2005). Sperm cells from oligozoospermic patients have more chances of carrying aberrant DNA methylation patterns and, therefore, a higher risk of transmitting incorrect primary imprints to their offspring. Furthermore, the outcome of ART was generally poor (Kobayashi *et al.* 2007; Marques *et al.* 2008). We therefore purposed to analyze the associations of mRNA expression of DNA methylation governing enzymes including *DNMT1*, *DNMT3A* and *TET1–3*, which were detected and measured by RT-qPCR in ejaculated sperm cells, with ICSI outcome including fertilization rate (FR) and pregnancy status.

It is known that DNMT1 and DNMT3A enzymes are essential for DNA methylation maintenance and re-establishment of *de novo* methylation during embryonic development, respectively (Okano *et al.* 1999; Howell *et al.* 2001; Kaneda *et al.* 2004; Hirasawa *et al.* 2008). Conditional knockout of maternal and zygotic *Dnmt1* will lead to a complete DNA demethylation at imprinted genes in mouse blastocyst (Hirasawa *et al.* 2008). Several studies showed

that Dnmt1 protein was expressed in the cortical cytoplasm using immunostaining (Howell *et al.* 2001; Ratnam *et al.* 2002; Hirasawa *et al.* 2008; Gu *et al.* 2011). However, Dnmt1 protein could also be detected in the nuclei of pre-implantation embryos, which was at a low level, but sufficient to maintain DNA methylation of imprinted genes (Hirasawa *et al.* 2008). A recent study also demonstrated that human DNMT1 protein was expressed in both oocytes and zygotes, which was localized in the cytoplasm and in the pronuclei as well (Petrussa *et al.* 2014). However, concerning the paternal effect, our data indicated that decreased level of *DNMT1* in human mature sperm cells had no influence on fertilization rate and pregnancy, probably because maternal (oocyte) DNMT1 protein was already enough for the maintenance of DNA methylation during pre-implantation development. In comparison with Dnmt1, Dnmt3a was mainly observed in male and female pronuclei of mouse zygotes showing dispensable for the maintenance of DNA methylation in the pre-implantation stages (Hirasawa *et al.* 2008), but indispensable for *de novo* methylation after implantation (Okano *et al.* 1999; Kaneda *et al.* 2004). Interestingly, *Dnmt3a* knockout mouse embryos can develop to term, but die shortly after birth, with loss of genomic imprinting signatures in germ cells, indicating that absence of Dnmt3a enzyme will not impede embryonic development (Okano *et al.* 1999; Kaneda *et al.* 2004). Consistent with these findings, our data showed *DNMT3A* mRNA level did not differ in terms of fertilization rate and pregnancy status. Furthermore, in human zygotes, different to mouse Dnmt3a protein expression, DNMT3A was not detectable until the morula stage (Petrussa *et al.* 2014). This finding leads to the assumption that paternal-contributed DNMT3A protein might be degraded in human oocytes after fertilization.

In terms of DNA demethylation in pre-implantation embryo, compared to *Tet1* and *Tet2, Tet3* shows exclusively high levels of mRNA expression confined to oocytes and zygotes (Gu *et al.* 2011; Iqbal *et al.* 2011; Wossidlo

*et al.* 2011). It has been shown that maternally derived Tet3 enzyme is responsible for hydroxymethylation of the epigenetic reprogramming on the zygotic paternal genome already in the pronucleus stage (Gu *et al.* 2011; Iqbal *et al.* 2011). Conditional knockout of zygotic *Tet3* would result in a failed conversion of 5mC into 5hmC and constantly elevated 5mC level in the paternal genome (Gu *et al.* 2011). Female mice depleted of *Tet3* in the germ line exhibited severely reduced fecundity, and their Tet3-deficient oocytes had a reduced ability of regulating DNA hydroxymethylation to reprogram the injected nuclei from somatic cells (Gu *et al.* 2011). However, loss of maternal Tet3 alone would not block differentiation and embryonic development with the evidence that homozygous mutant *Tet3* mice can develop to term but die shortly after birth (Gu *et al.* 2011), probably due to the compensational function of paternally derived Tet3. Our data revealed that *TET3*-mRNA expression in human sperm cells was closely related with FR. When defining the threshold of fertilization rate as 70% (van Golde *et al.* 2001; Tournaye *et al.* 2002; Palermo *et al.* 2009; Rogenhofer *et al.* 2013), subfertile patients whose sperm achieved high-FRs showed significantly increased *TET3* in comparison with those with low-FRs. Therefore, this finding indirectly demonstrated that TET3 in sperm cells might also have the contribution of 5hmC in fertilization. Nevertheless, *TET3*-mRNA showed no differences between the pregnancy and the non-pregnancy group in our study. Animal studies demonstrated that *Tet3* was nearly absent in 2-cell embryos, ESCs and mouse embryonic fibroblasts (MEFs) (Iqbal *et al.* 2011; Wossidlo *et al.* 2011), and germ cell-specific *Tet3* depletion in mouse would not significantly affect DNA hydroxymethylation in post-implantation stages and early embryogenesis (Gu *et al.* 2011).

In mice, Tet1 and Tet2 are especially involved in mediating the expression of pluripotency transcription factors, such as Nanog, Esrrb (estrogen-related receptor β) and Prdm14 (PR domain containing 14) (Ito *et*

*al.* 2010; Ficz *et al.* 2011; Koh *et al.* 2011; Williams *et al.* 2011). Conditional knockout of *Tet1* or/and *Tet2* in mouse ESCs, they remained pluripotent, but impaired differentiation might occur with a dramatic reduction of 5hmC, and lead to a development towards the extra-embryonic lineages (Ito *et al.* 2010; Ficz *et al.* 2011; Koh *et al.* 2011; Williams *et al.* 2011). Furthermore, *Tet2* is extensively expressed in the mouse oocytes and embryonic development, such as zygotes, 2-cell embryos, ESCs and MEFs (Ito *et al.* 2010; Iqbal *et al.* 2011; Koh *et al.* 2011; Wossidlo *et al.* 2011). Our results also demonstrated that men of couples, whose female partners achieved pregnancy, had a significantly higher level of *TET2* in their sperm compared to men in the non-pregnant group. This finding indicated that TET2 enzyme in human sperm probably regulated special certain pluripotency transcription genes in zygotes, which might affect post-implantation embryonic development. However, *TET2* mRNA level showed no correlation with FR in this study, probably due to the complementary contribution of TET3, which plays a key function to generate 5hmC in the mammalian zygote (Gu *et al.* 2011; Iqbal *et al.* 2011). On the other hand, *Tet1* is exclusively detected at a high level in ESCs, whereas it is barely detected in oocytes and zygotes (Dawlaty *et al.* 2011; Ficz *et al.* 2011; Iqbal *et al.* 2011; Koh *et al.* 2011; Williams *et al.* 2011; Wossidlo *et al.* 2011; Dawlaty *et al.* 2013). Our data revealed that the *TET1*-mRNA level in human sperm cells was not associated with FR and pregnancy. Although a fraction of the mutant embryos exhibited midgestation abnormalities with perinatal lethality, *Tet1* knockout mice of both sexes were viable and fertile with a reduced size (Dawlaty *et al.* 2011; Dawlaty *et al.* 2013). However, these mutant mice revealed aberrant methylation at various imprinted loci with failed conversion of 5mC into 5hmC (Dawlaty *et al.* 2011; Dawlaty *et al.* 2013). Overall, given the fact that we also found considerable amounts of non-degraded TET proteins in human sperm, we could speculate about the developmental impact of paternally contributed TET2 and TET3 enzymes.

## 4.4 Aberrant methylation of *TET3* CpG-promoter might down-regulate *TET3*-mRNA transcription in sperm cells

In mammalian genomes, many genes exhibit CpG islands in their promoters that are associated with gene transcription. In the human genome, approximate 70% of gene promoters reveal a high CpG content in or near the promoters (Weber *et al.* 2007; Deaton & Bird 2011). In most instances, unmethylated CpG islands are associated with gene expression, while methylated CpG sites result in the repression of gene expression. Therefore, in many diseases, hypomethylation, in general, arises earlier and upregulates abnormal transcription, which is linked to chromosomal instability and loss of imprinting, whereas abnormal hypermethylation in CpG-promoters, such as in most cancer tissues, can result in transcriptional silencing following cell division (Egger *et al.* 2004; Dodge *et al.* 2005; Robertson 2005; Weber *et al.* 2007; Deaton & Bird 2011). However, currently no publications have reported whether the methylation status at *TET1–3* CpG-promoters will affect their mRNA synthesis. In this study, we demonstrated that low *TET1–3* mRNA levels in mature spermatozoa were closely associated with poor spermiogram profiles. Moreover, *TET2* and *TET3* mRNA levels were linked with ICSI outcome. Therefore, it is valuable to elucidate the influences of *TET1–3* CpG-promoter methylation status on their corresponding mRNA transcription.

Our results indicated that sperm *TET1–2* CpG-promoters, in general, were unmethylated, regardless of men with low (healthy donors) and high (subfertile patients) mRNA expression. Nevertheless, compared to the donors with high *TET3*-mRNA level exhibiting hpyomethylated *TET3* CpG-promoters in sperm cells, two subfertile patients with low *TET3*-mRNA expression showed partially methylated *TET3* CpG-promoters in ejaculated sperm. The regulation of the CpG-promoter methylation status, therefore, might affect *TET3*-mRNA expression, but not *TET1* and *TET2*. Interestingly, consistent

with our previous results that sperm *TET3*-mRNA level was associated with fertilization but not pregnancy, the fertilization rates of these two patients, who also suffered from severe oligozoospermia with a sperm count below 10 Mil/ml, were both below 70%, but one female partner was pregnant after ICSI treatment. Furthermore, they also had aberrant methylation at the *H19* imprinted gene based on COBRA results. This finding was in line with several studies reporting that aberrant methylation at the imprinted gene *H19* was frequently observed in subfertile patients with severe oligospermia (Kerjean *et al.* 2000; Marques *et al.* 2004; Kobayashi *et al.* 2007; Marques *et al.* 2008). Nevertheless, because of the limited material in this study, there is a need for a larger cohort study. Although TETs are known to adjust other mRNAs synthesis (Wu *et al.* 2011; Pastor *et al.* 2013), further studies are required to investigate the mechanisms of *TET*-mRNA transcriptional regulation.

In conclusion, these findings led to the hypothesis that severely oligozoospermic patients not only carried abnormal methylated imprinted gene, but also suffered from aberrantly partially methylated *TET3* CpG-promoter in sperm cells, which might down-regulate sperm *TET3*-mRNA transcription and further impede fertilization during ICSI procedure.

## 4.5 DNA methylation governing enzymes might be associated with the methylation pattern of the imprinted gene *H19* in sperm cells

Since the first report of successful pregnancy achieved by ICSI (Palermo *et al.* 1992), this technique has become the most common method of choice for infertile patients at reproductive age, especially for male factor infertility. However, because of neglecting the natural elimination of defective spermatozoa during ICSI procedure, it may account for a higher risk of transmitting incorrect genomic information to the offspring resulting in

aberrant embryo cell divisions, congenital malformations and hereditary disorders (Johnson 1998; Wennerholm *et al.* 2000; Ericson & Kallen 2001; Devroey & Van Steirteghem 2004). Indeed, various clinical studies reported an increased incidence of rare imprinting deregulation in children conceived with ART, such as Beckwith–Wiedemann syndrome (BWS; 11p15) (DeBaun *et al.* 2003; Maher *et al.* 2003) and Angelman syndrome (AS; 15q11-q13) (Clayton-Smith & Laan 2003; Ludwig *et al.* 2005).

Genomic imprinting is an epigenetic phenomenon involving DNA methylation without altering the genetic sequence, by which certain genes are expressed in a parental-of-origin dependent manner leading to allele-specific expression due to the different methylation status of CpG dinucleotides at differentially methylated regions (DMRs) (Reik & Walter 2001; Barlow & Bartolomei 2014). DNA methylation is a heritable epigenetic modification, which might represent the most valuable epigenetic information transmitted from sperm cells to oocytes (Steger 1999; Jones & Takai 2001). Recently, the high influence of sperm methylome was emphasized by the finding in zebrafish indicating maternal genome was reprogrammed to match the paternal methylation after fertilization (Jiang *et al.* 2013; Potok *et al.* 2013). Appropriate genomic imprinting plays an important role in embryonic development, placental function and neurological processes and behavior (Surani 1998; Tilghman 1999). Up to now, the methylation patterns of several imprinted genes have been well determined, *H19* is one of the best characterized examples, which encodes for an untranslated RNA presenting completely methylated (repressed) in the paternal allele and fully unmethylated (expressed) in the maternal allele (Bartolomei *et al.* 1993; Tremblay *et al.* 1995). Several studies reported a close association of hypospermatogenesis with abnormal genomic imprinting of the *H19* gene. Specifically, aberrant methylation patterns at the *H19* gene were observed in oligozoospermic patients, especially for severe oligozoospermia (< 10 Mil/ml),

and in a small number of normozoospermic subfertile patients (Marques *et al.* 2004; Kobayashi *et al.* 2007; Marques *et al.* 2008; Boissonnas *et al.* 2010). These findings were consistent with our results, as we found only one normozoospermic healthy donor revealing aberrantly methylated *H19* gene. However, this man was not married and did not prepare to become a father via natural conception. Interestingly, one study revealed that male idiopathic infertility was strongly associated with aberrant methylation of the *H19* gene (Poplinski *et al.* 2010). Moreover, the outcome of ART treatment using sperm exhibiting abnormal at DNA methylation pattern of the *H19* gene was generally poor (Kobayashi *et al.* 2007). Our data were in line with this finding demonstrating that 71.43% (5/7) of patients carrying an aberrant methylation pattern at the *H19* gene had low FR (< 70%), whereas 57.14% (4/7) of female partners were pregnant. Nevertheless, a reduced DNA methylation level might be also observed at the aberrant *H19*-DMRs in placentas from pregnancies (Nelissen *et al.* 2013). These evidences highlight the need for more research into the mechanisms of aberrant methylation of imprinted genes. Therefore, we aimed to investigate the association of DNA methylation governing enzymes (TET1–3, DNMT1 and DNMT3A) with the methylation pattern of the imprinted gene *H19* in mature spermatozoa.

In the human germ line, DNA methylation of the imprinted gene *H19* should be first erased at an early fetal stage by TET enzymes, while the paternal specific imprint is completely re-established through the activity of DNMTs during spermatogonial differentiation in the adult testis (Davis *et al.* 2000; Kerjean *et al.* 2000; Saitou *et al.* 2012; Kohli & Zhang 2013). Two waves of genome-wide DNA demethylation regarding imprint erasure were reported in male germ line involved in the function of Tet1–3 enzymes (Kohli & Zhang 2013). In the first wave of dynamic global demethylation, Tet3 protein mainly accounts for the unique process of imprint erasure in the paternal pronucleus shortly after fertilization, whereas the second wave in

primordial germ cells was regulated by Tet1 and Tet2 (Gu *et al.* 2011; Inoue & Zhang 2011; Li *et al.* 2011; Yamaguchi *et al.* 2012; Hackett *et al.* 2013). Currently, several studies reported the dynamics of DNA demethylation in human preimplantation embryos and prenatal germline shares tremendous similarities with those of mouse at the equivalent stages (Guo *et al.* 2014; Smith *et al.* 2014; Gkountela *et al.* 2015). Our previous results showed that TET1–3 proteins were also closely related to human spermatogenesis, which were detected in human purified sperm cells as well. Therefore, we studied the association of relative *TET1–3* mRNA levels with the methylation status of the imprinted gene *H19* in human mature spermatozoa. No differences were found between patients with Ab-Me-*H19* and N-Me-*H19*. This finding indicates that TETs might not affect the methylome of postnatally imprinted genes in human germ line after the re-establishment of imprints, which could be mainly responsible for DNA demethylation of imprinted gene in prenatal embryonic development.

On the other hand, human mRNA and protein expression of DNMT1 and DNMT3A were both detected in all stages of spermatogenic cells from spermatogonia to mature spermatozoa acting as a maintenance and *de novo* DNA methyltransferase respectively during replication (Omisanjo *et al.* 2007; Marques *et al.* 2011). Conditional knockout of *Dnmt1* in the mouse model reveals Dnmt1 protein is responsible for genome-wide DNA methylation maintenance (Howell *et al.* 2001; Hirasawa *et al.* 2008). Meanwhile, a germline-specific gene-knockout study indicates that Dnmt3a enzyme has a central role for all known paternally methylated loci in the *de novo* methylation process, except for the Rasgrf1 locus, whose imprint is re-established by Dnmt3b (Kaneda *et al.* 2004; Kato *et al.* 2007). Our data indicated that severely reduced *DNMT3A* level in mature spermatozoa might be associated with aberrantly methylated *H19* gene based on the evidences that *DNMT3A* mRNA level in the patients with Ab-Me-*H19* was the lowest compared to the

subfertile patients with N-Me-*H19* and the donors with N-Me-*H19*. However, relative *DNMT1* mRNA level showed no differences in purified sperm cells among donors with N-Me-*H19* and patients with N-Me-*H19 and* Ab-Me-*H19*. Previous observations in mouse and human showed that *H19* methylation is completed by the mature spermatozoa stage (Kerjean *et al.* 2000; Li *et al.* 2004). Therefore, this finding supports the hypothesis that abundant *de novo* methylation is required for the maintenance of methylation patterns after their establishment. Moreover, overview of individual values of mRNA levels in donors and patients showed multiple low *TET1–3*, *DNMT1* and *DNMT3A* mRNA expressions were observed simultaneously in the Pat-Ab-Me-*H19* group, which led to the assumption that the comprehensive defects of DNA methylation governing enzymes rather than unique enzyme result in aberrant methylation of the imprinted genes in mature spermatozoa. Nevertheless, several potential limitations of this part should be considered as well. Due to the limited materials in this study, further studies on a large cohort are necessary to strengthen these findings. Furthermore, our findings were restricted to one specific imprinted gene *H19*, therefore, a multi-gene investigation, covering both maternally and paternally expressed imprinted gene should be performed.

# 5. SUMMARY

Epigenetic reprogramming during male germ cell differentiation plays a crucial role in regulating genome functions at critical stages of development. DNA methylation profiles are mainly established and maintained by DNMT family members, whereas TET dioxygenases are essential for active DNA demethylation in the paternal pronuclei and in ESCs.

The present study focused on the expression of TET1–3 enzymes during human spermatogenesis and in mature spermatozoa. Furthermore, it should be clarified whether aberrant expression of *TET1–3, DNMT1* and *DNMT3A* mRNAs are associated with aberrant imprinting of the *H19* gene, male subfertility and poor outcome of ICSI treatment.

During normal human spermatogenesis *TET1–3* mRNAs are present in the cytoplasm of pachytene spermatocytes from stage I to stage V, while TET1–3 proteins are successively expressed: TET2 is expressed in the cytoplasm of late pachytene spermatocytes of stage V, then TET1 can be observed in the nuclei of step 1 round spermatids at stage I, and TET3 in the nuclei of step 3 round spermatids at stage III. 5hmC appears only in step 5 elongated spermatids. All three TETs are still detectable at the mRNA and protein level in mature sperm cells in considerable amounts.

*TET1–3*, *DNMT1* and D*NMT3A* mRNA levels in spermatozoa are significantly reduced in subfertile patients. In addition, they are closely associated with male age and routine semen parameters. *TET2* and *TET3* mRNA levels in mature sperm cells are significantly associated with pregnancy and fertilization rate after ICSI treatment, respectively. Aberrant methylation of *TET3,* but not *TET1–2*, CpG-promoter might down-regulate *TET3*-mRNA transcription. Finally, DNA methylation reprogramming enzymes, especially *DNMT3A*, might be associated with the methylation pattern of the

imprinted gene *H19* in human mature spermatozoa.

Our data provide first hints that TETs enzymes and DNA-demethylation are essential for male germ cell differentiation. In addition, *TET1–3*, *DNMT1* and *DNMT3A* mRNA levels in human mature spermatozoa seems to be important for male fertility and success of ART treatment.

# 6. ZUSAMMENFASSUNG

Die epigenetische Reprogrammierung während der männlichen Keimzelldifferenzierung spielt für die Regulation der Genexpression eine entscheidende Rolle. Während die Methylierung der DNA vornehmlich durch Enzyme der DNMT-Familie etabliert und aufrecht erhalten wird, erfolgt die active Demethylierung der DNA im paternalen Pronukleus und in embryonalen Stammzellen durch TET Dioxygenasen.

In der vorliegenden Studie wurde die Expression der TET1–3 Enzyme während der Spermatogenese und in ejakulierten menschlichen Spermien untersucht. Darüber hinaus sollte geklärt werden, ob eine aberrante Expression von *TET1–3*, *DNMT1* und *DNMT3A* mRNA mit einem abnormalen Imprinting des H19 Gens bzw. männlicher Subfertilität und/oder dem Ergebnis einer ICSI-Behandlung assoziiert ist.

Während der normalen Spermatogenese konnten *TET1–3* mRNAs im Zytoplasma von pachytänen Spermatozyten (Stadium I-V) nachgewiesen werden, wohingegen die TET1–3 Proteine ein sukkzessives Expressionsmuster zeigten. So wurde TET2 im Zytoplasma von späten pachytänen Spermatozyten (Stadium V) exprimiert. TET1 hingegen war in den Zellkernen von step 1 runden Spermatiden (Stadium I) und TET3 in den Zellkernen von step 3 runden Spermatiden (Stadium III) nachweisbar. 5hmC war ausschließlich in step 5 elongierten Spermatiden (Stadium V) zu sehen. Alle drei TET Enzyme sind zudem sowohl auf mRNA-, als auch auf Protein-Ebene in ejakulierten Spermien nachzuweisen.

In subfertilen Patienten ist die Menge an *TET1–3*, *DNMT1* und *DNMT3A* mRNAs in Spermien significant reduziert. Darüber hinaus zeigt die mRNA-Menge eine Korrelation mit dem Alter des Mannes und mit Routine-Samenparametern. Die Menge an *TET2* bzw. *TET3* in Spermien ist

zudem significant mit der Schwangerschaftsrate bzw. Der Fertilisierungsrate nach ICSI korreliert. Eine abnormale Methylierung von CpG-Inseln im *TET3* Genpromoter, aber nicht in den Promotoren von *TET1* und *TET2*, könnte somit die Transkription der *TET3* mRNA herunter regulieren. Schließlich lassen unsere Ergebnisse eine Assoziation von *DNMT3A* mit dem Methylierungsmuster des paternalen Imprintinggens *H19* in Spermien vermuten.

Zusammenfassend lässt sich feststellen, dass die Ergebnisse dieser Studie erste Hinweise dafür liefern, dass TET Enzyme und DNA-Demethylierung eine wichtige Rolle in der männlichen Keimzelldifferenzierung spielen. Darüber hinaus scheint die Menge an *TET1–3*, *DNMT1* und *DNMT3A* mRNA in Spermien einen Einfluss auf die männliche Fertilität bzw. den Erfolg einer ICSI-Behandlung zu haben.

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# 8. SUPPLEMENTARY INFORMATION

## 8.1 Chemicals

| | |
|---|---|
| Acetic acid | Roth, Karlsruhe |
| Acrylamide/Bisacrylamide (37.5:1) | Bio-Rad, Munich |
| Agarose | Roth, Karlsruhe |
| Ammonium persulfate (APS) | Sigma-Aldrich, Steinheim |
| Ampicillin | Roth, Karlsruhe |
| Bovine serum albumin (BSA) | Roth, Karlsruhe |
| Cyclohexylamino propanesulphonic acid (CAPS) | Roth, Karlsruhe |
| Chloroform | Roth, Karlsruhe |
| Citric acid | Merck, Darmstadt |
| Dextran sulphate sodium salt | Sigma-Aldrich, Steinheim |
| Diethypyrocarbonate (DEPC) | Sigma-Aldrich, Steinheim |
| Dithiothreitol (DTT) | Roth, Karlsruhe |
| Ethanol | Sigma-Aldrich, Steinheim |
| Ethylenediamine tetraacetic acid (EDTA) | Roth, Karlsruhe |
| Formaldehyde | Sigma-Aldrich, Steinheim |
| Formamide | Sigma-Aldrich, Steinheim |
| Glycerol | Roth, Karlsruhe |
| Glycin | Roth, Karlsruhe |
| Glycogen | Invitrogen, Karlsruhe |
| Hydrogen chloride (HCl), 2 N | Roth, Karlsruhe |
| Isopropanol | Sigma-Aldrich, Steinheim |
| LB medium | Roth, Karlsruhe |
| Levamisole | Sigma-Aldrich, Steinheim |
| Lithium 3,5-diiodosalicylate | Sigma-Aldrich, Steinheim |
| Lithium chloride (LiCl) | Roth, Karlsruhe |
| Magnesium chloride ($MgCl_2$) | Merck, Darmstadt |

| | |
|---|---|
| Methanol | Sigma-Aldrich, Steinheim |
| β-Mercaptoethanol | AppliChem, Darmstadt |
| N,N,N',N'-Tetramethylethylenediamine (TEMED) | Roth, Karlsruhe |
| Non-fat dry milk | Roth, Karlsruhe |
| Orange G | Merck, Darmstadt |
| Paraformaldehyde (PFA) | Merck, Darmstadt |
| Phenol/chloroform | Roth, Karlsruhe |
| Phenylmethylsulfonylfluorid (PMSF) | Roth, Karlsruhe |
| Phosphate buffered saline (PBS) | Sigma-Aldrich, Steinheim |
| Ponceau S solution | Sigma-Aldrich, Steinheim |
| Protease inhibitor cocktail tablets | Sigma-Aldrich, Steinheim |
| Roti®-Phenol | Roth, Karlsruhe |
| Sodium dodecyl sulfate (SDS) solution, 10% (W/V) | Bio-Rad, Munich |
| sodium acetate (NaOAc) | Roth, Karlsruhe |
| Sodium chloride (NaCl) | Roth, Karlsruhe |
| Sodium dodecyl sulfate (SDS) | Roth, Karlsruhe |
| Tris-base | Roth, Karlsruhe |
| Tris-HCl | Roth, Karlsruhe |
| Tri-sodium citrate | Merck, Darmstadt |
| Triton X-100 | Sigma-Aldrich, Steinheim |
| Tween-20 | Roth, Karlsruhe |
| Xylol | VWR, Darmstadt |

## 8.2 Reagents and buffers

### 8.2.1 Cell culture

| | |
|---|---|
| Dulbecco's Modified Eagle Medium (DMEM) | Life Technologies, Darmstadt |
| Dulbecco's PBS (1×) w/o Ca2+ & Mg2+ | Gibco, Darmstadt |
| Fetal calf serum | Biochrom AG, Berlin |

| | |
|---|---|
| Penicillin/Streptomycin | Gibco, Darmstadt |
| RPMI 1640 medium | Life Technologies, Darmstadt |
| Synth-a-Freeze cryopreservation | Gibco, Darmstadt |
| TrypLE™ Express reagent | Gibco, Darmstadt |

### 8.2.2 IHC, ICC and IF

| | |
|---|---|
| DAB enhanced liquid substrate system | Sigma-Aldrich, St. Louis |
| SIGMAFAST Fast Red | Sigma-Aldrich, St. Louis |
| Vectashield® Mounting Medium with DAPI | Vector, Burlingame |
| VECTASTAIN Elite ABC-AP system | Vector, Peterborough |
| VECTASTAIN Elite ABC-Peroxidase system | Vector, Peterboroug |

**PBS buffer (0.01 M), pH 7.4:** 1 PBS tablet in 200 ml deionized water.

**2% Triton/PBS buffer, pH 7.4:** 2 ml Triton X-100 in 98 ml PBS buffer.

**3% BSA buffer:** 3 g BSA in 100 ml 2% Triton/PBS buffer.

**10 × Tris-HCl buffer, pH 7.4:** 121.1 g Tris-HCl and 58.4 g NaCl in 1 l deionized water.

**Citrate buffer, pH 6.0:** 18 ml stock solution A (21.01 g citric acid, 1 l deionized water), 82 ml stock solution B (29.41 g tri-sodium citrate, 1 l deionized water) and 900 ml deionized water.

### 8.2.3 WB

| | |
|---|---|
| Coomassie stain | ROTH, Karlsruhe |
| Laemmli sample buffer (4×) | Bio-Rad, Munich |
| Pierce BCA Protein Assay Kit | Thermo Scientific, Waltham |
| Pierce ECL Western blotting substrate | Thermo Scientific, Waltham |
| Protein size markers | Life Technologies, Darmstadt |

**Lysis buffer for protein extraction, pH 7.4:** 10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 × Protease inhibitor cocktail and 1% Triton X-100.

**30% Acrylamide solution:** Acrylamide/Bisacrylamide = 37.5:1.

**10% APS:** 50 mg APS in 500 µl deionized water.

**7.5% separating gel:** 4.85 ml water, 2.5 ml 1.5 M Tris-HCl (pH 8.8), 100 µl 10% SDS, 2.5 ml 30% Acrylamide, 50 µl 10% APS and 25 µl TEMED.

**4% stacking gel:** 3 ml water, 1.25 ml 0.5 M Tris-HCl (pH 6.8), 50 µl 10% SDS, 0.65 ml 30% Acrylamide, 25 µl 10% APS and 12.5 µl TEMED.

**Laemmli loading buffer:** 90 µl Laemmli sample buffer (4×) and 10 µl β-mercaptoethanol.

**10 × Electrophoresis buffer, pH 8.3:** 30.3 g Tris-base, 144 g Glycin and 10 g SDS in 1 l deionized water.

**10 × Tris/CAPS buffer, pH 9.6:** 25 g CAPS and 20.5 g Tris-base in 282.5 ml deionized water.

**Cathode buffer:** 0.75 ml 10% SDS and 74.25 ml 1 × Tris/CAPS buffer.

**Anode buffer:** 7.5 ml methanol and 42.5 ml 1 × Tris/CAPS buffer.

**10 × Tris buffered saline (TBS), pH 7.4:** 24.2 g Tris-base and 80 g NaCl in 1 l deionized water.

**1 × TBS/T washing buffer:** 50 µl Tween-20 in 100 ml 1 × TBS buffer.

**Blocking buffer, pH 7.4:** 5 g non-fat dry milk in 100 ml 1 × TBS/T buffer.

### 8.2.4 ISH

| | |
|---|---|
| Denhardt’s solution (50×) | Sigma-Aldrich, Steinheim |

| | |
|---|---|
| Digoxigenin (DIG) RNA Labeling Kit (SP6/T7) | Roche, Mannheim |
| NBT/BCIP reagent | KPL, Gaithersburg |
| NucleoSpin Gel and PCR clean-up kit | Macherey Nagel, Düren |
| pGEM-T Vector | Promega, Mannheim |
| QIAfilter Plasmid Midi Kit | Qiagen, Hilden |
| QIAprep Spin Miniprep Kit | Qiagen, Hilden |
| Salmon sperm DNA | Thermo Scientific, Waltham |
| E. coli XL1-Blue strain competent cells | Stratagene, Heidelberg |
| Yeast t-RNA | Thermo Scientific, Waltham |

**LB/Ampicillin medium:** 20 g LB medium and 1660 µl Ampicillin (60 mg/ml) in 1 l double distilled water.

**PBSM buffer, pH 7.4:** 5 mM $MgCl_2$ in 0.01 M PBS in DEPC water.

**Proteinase K (20 mg/ml):** 40 mg Proteinase K in 2 ml PBSM buffer.

**20 × SSC buffer, pH 7.0:** 88.23 g sodium citrate and 175.29 g NaCl in 1 l DEPC water.

**Hybridization buffer:** 50% deionized formamide, 10% dextran sulphate, 2 × SSC, 1 × Denhardt's solution, 10 µg/ml salmon sperm DNA and 10 µg/ml yeast t-RNA.

**1 × TNMT buffer, pH 7.5:** 100 mM Tris-HCl, 150 mM NaCl, 4.38 mM $MgCl_2$ and 0.5% Triton X-100 in DEPC water.

**1 × NTB buffer, pH 9.6:** 150 mM NaCl, 100 mM Tris-HCl and 50 mM $MgCl_2$ in DEPC water.

### 8.2.5 PCR

| | |
|---|---|
| DNase I, RNase-free | Thermo Scientific, Waltham |
| dNTP Mix | Promega, Mannheim |

| | |
|---|---|
| EDTA (0.5 M), pH 8.0 | Thermo Scientific, Waltham |
| iScript™ cDNA Synthesis kit | Bio-Rad, Munich |
| GelRed Nucleic Acid Gel Stain | Biotium, Fremont |
| GeneRuler DNA Ladder Mix | Thermo Scientific, Waltham |
| M-MLV Reverse Transcriptase | Promega, Mannheim |
| MyTaq™ mix | Bioline, Taunton |
| Oligo dT | Promega, Mannheim |
| PeqGOLD TriFast | VWR, Erlangen |
| RNase inhibitor | Promega, Mannheim |
| Rotor-Gene SYBR Green PCR kit | Qiagen, Hilden |
| RNeasy Plus Micro and Mini Kits | Qiagen, Hilden |

**6 × DNA loading buffer with Orange G:** 1% (w/v) Orange G, 40% sucrose

**10 × TAE buffer, pH 8.0:** 48.4 g Tris base, 11.42 ml Glacial acetic acid, 20 ml 0.5 M EDTA in deionized water up to 1l

**2% agarose gel:** 1.8 g Agarose, 100 ml 1× TAE-Puffer

### 8.2.6 COBRA and Bisulfite pyrosequencing

| | |
|---|---|
| EpiMark® Bisulfite Conversion Kit | New England Biolabs, Ipswich |
| PyroMark Annealing Buffer | Qiagen, Hilden |
| PyroMark Binding Buffer | Qiagen, Hilden |
| PyroMark Denaturation Solution | Qiagen, Hilden |
| PyroMark Gold Q24 Reagents | Qiagen, Hilden |
| PyroMark Wash Buffer | Qiagen, Hilden |
| Streptavidin-coated Sepharose beads | GE Healthcare, Freiburg |

**Lysis buffer for DNA extraction, pH 8.0:** 10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.5% SDS, 1 mM DTT and 1.5 mg/ml proteinase K.

## 8.3 Enzymes

| | | |
|---|---|---|
| Bsh1236I (BstUI) (10 U/µl) | Thermo Scientific, Waltham | ER0922 |
| CpG methyltransferase (M.SssI) | Thermo Scientific, Waltham | EM0821 |
| DNase I, RNase-free (1U/ul) | Thermo Scientific, Waltham | EN0525 |
| NcoI (10 U/µl) | Thermo Scientific, Waltham | ER0575 |
| NotI (10 U/µl) | Thermo Scientific, Waltham | ER0595 |
| Proteinase K | Roth, Karlsruhe | 7528.2 |

## 8.4 Antibodies

### Primary antibodies

| | | | |
|---|---|---|---|
| Rabbit anti TET1 | GeneTex, San Antonio | GTX124207 | 1: 400 (IHC)<br>1: 2000 (WB) |
| Rabbit anti TET2 | Abcam, Cambridge | ab94580 | 1: 400 (IHC) |
| Rabbit anti TET2 | GeneTex, San Antonio | GTX124205 | 1: 1000 (WB) |
| Rabbit anti TET3 | Novus, Littleton | NBP2-13427 | 1: 400 (IHC) |
| Rabbit anti TET3 | GeneTex, San Antonio | GTX121453 | 1: 1000 (WB) |
| Rat anti 5hmC | Abcam, Cambridge | ab106918 | 1: 300 (IF) |

### Secondary antibodies

| | | | |
|---|---|---|---|
| Donkey anti rat IgG | Abcam, Cambridge | ab150153 | 1: 500 (IF) |
| Goat anti rabbit IgG | Dako, Glostrup | E0432 | 1: 200 (IHC) |
| Goat anti rabbit IgG-HRP | GeneTex, San Antonio | GTX213110-01 | 1: 5000 (WB) |
| Sheep anti Dig-AP | Roche, Mannheim | 11093274910 | 1: 500 (ISH) |

## 8.5 Equipments

| | |
|---|---|
| Benchtop Centrifuge (Universal 320) | Hettich, Germany |
| Biological Safety Cabinet (MSC-Advantage) | Thermo, Germany |
| Cell culture CO2 incubator (Heracell150i ) | Thermo, Germany |
| Eppendorf BioPhotometer plus | Eppendorf, Germany |

| | |
|---|---|
| Eppendorf Thermo Mixer | Eppendorf, Germany |
| FUSION-FX7 advanced | Vilber Lourmat, Germany |
| Gel documentation system (BioDocAnalyze) | Biometra, Germany |
| Heating block (LS 2) | VLM, Germany |
| Heidolph Reax 2000 shaker | Heidolph, Germany |
| HERAfreeze™ HFU T Series -86°C Freezer | Thermo, Germany |
| Incubating Mini Shaker | VWR, Germany |
| Invitrogen Power Ease® 500 Power Supply | Thermo, Germany |
| Leica ICC50 HD microscope | Leica, Germany |
| Magnetic Stirrer (BLSH0007) | IKAMAG, Germany |
| Mastercycler | Eppendorf, Germany |
| METTLER AE 240 balance | Mettler, Germany |
| Microbiological incubator (SERIES 6000) | Heraeus, Germany |
| Microlitre Centrifuge (MIKRO 220R) | Hettich, Germany |
| Microwave oven | LG, South Korea |
| Milli-Q® Direct Water Purification System | Merck KGaA, Germany |
| Mini-PROTEAN Tetra Cell | Bio-Rad, Germany |
| NanoDrop ND-1000 | PEQLAB, Germany |
| pH meter (S20K) | Mettler-Toledo, Switzerland |
| Phase-contrast inverted microscope (CK2) | Olympus, Germany |
| Power PAC 200 | Bio-Rad, Germany |
| PyroMark Q24 Cartridge | Qiagen, Germany |
| Pyromark Q24 instrument | Qiagen, Germany |
| Rotor-Gene Q PCR cycler | Qiagen, Germany |
| Standard Mini-Centrifuge | Fisherbrand, Germany |
| T100$^{TM}$ Thermal Cycler | Bio-Rad, Germany |
| Trans-Blot SD Semi-Dry Transfer Cell | Bio-Rad, Germany |
| Ultra Turrax | IKA, Germany |
| Water bath (JB Aqua 5plus) | Grant, Germany |

## 8.6 *TET1–3, DNMT1* and *DNMT3A* mRNA levels in donor swim-up and non-swim-up sperm samples

In order to confirm that the non-swim-up sperm samples from the healthy donors (*TET1*, n = 58; *TET2–3*, n = 63; *DNMT1 and DNMT3A*, n = 36) were also suitable for this study, we performed the following experiment: freshly isolated RNA from swim-up sperm cells of 12 healthy donors were analyzed for *TET1–3*, *DNMT1* and *DNMT3A* mRNAs by RT-qPCR. The results showed that no differences of *TET1* (1.28 ± 0.16 (± 0.57) versus 1.49 ± 0.28 (± 2.15); mean ± SEM (± SD), $P > 0.05$), *TET2* (0.95 ± 0.15 (± 0.54) versus 0.90 ± 0.11 (± 0.84), $P > 0.05$), *TET3* (1.63 ± 0.32 (± 1.11) versus 1.67 ± 0.21 (± 1.63), $P > 0.05$), *DNMT1* (1.19 ± 0.17 (± 0.59) versus 1.59 ± 0.17 ( ± 1.04), $P > 0.05$) and *DNMT3A* (2.01 ± 0.48 (± 1.66) versus 2.36 ± 0.17 (± 1.03), $P > 0.05$) mRNA levels were found between the swim-up samples and the non-swim-up samples (Table 7). Therefore, we conclude that the non-swim-up sperm samples were also suitable for this study.

**Table 7 *TET1–3*, *DNMTA* and *DNMT3A* mRNA levels in swim-up and non-swim-up sperm samples from healthy donors determined by RT-qPCR**

| Gene | Swim-up | Non-swim-up | *P*-value |
|---|---|---|---|
| *TET1* | 1.28 ± 0.16 (± 0.57), n = 12 | 1.49 ± 0.28 (± 2.15), n = 58 | $P = 0.24 > 0.05$ |
| *TET2* | 0.95 ± 0.15 (± 0.54), n = 12 | 0.90 ± 0.11 (± 0.84), n = 63 | $P = 0.36 > 0.05$ |
| *TET3* | 1.63 ± 0.32 (± 1.11), n = 12 | 1.67 ± 0.21 (± 1.63), n = 63 | $P = 0.57 > 0.05$ |
| *DNMT1* | 1.19 ± 0.17 (± 0.59), n = 12 | 1.59 ± 0.17 (± 1.04), n = 36 | $P = 0.29 > 0.05$ |
| *DNMT3A* | 2.01 ± 0.48 (± 1.66), n = 12 | 2.36 ± 0.17 (± 1.03), n = 36 | $P = 0.20 > 0.05$ |

Relative mean values ± SEM (± SD), *P*-values of Mann-Whitney *U*-test and number of analyzed samples are given.

# 9. ABBREVIATIONS

| | |
|---|---|
| 5caC | 5-carboxycytosine |
| 5fC | 5-formylcytosine |
| 5hmC | 5-hydroxymethylcytosine |
| 5hmU | 5-hydroxymethyluracil |
| 5mC | 5-methylcytosine; |
| A | Asthenozoospermia |
| AID | Activation-induced deaminase |
| AM | Active modification |
| APS | Ammonium persulfate |
| ART | Assisted reproductive technology |
| BCA | Bicinchoninic acid |
| BER | Base-excision repair |
| bp | base pair |
| BSA | Bovine serum albumin |
| $^{o}$C | Degree Celsius |
| C | Cytosine |
| cDNA | Complementary DNA |
| CIS | Carcinoma-in situ |
| COBRA | Combined bisulfite restriction analysis |
| cRNA | Complementary RNA |
| Cys-rich | Cysteine-rich region |
| DAB | 3,3’-Diaminobenzidine |
| DAPI | 4′,6-diamidino-2-phenylindole |
| DEPC | Diethypyrocarbonate |
| DIG | Digoxygenin |
| DMRs | Differentially methylated regions |
| DNA | Desoxyribonucleic acid |
| DNase | Desoxyribonuclease |
| DNMT | DNA methyltransferase |
| dNTP | 2’-deoxynucleoside-5’-triphosphate |
| DSBH | Double-stranded β-helix |
| DTT | Dithiothreitol |
| EDTA | Ethylene diamine tetraacetic acid |
| ESC | Embryonic stem cells |
| F | Forward primer |
| FR | Fertilization rate |
| g | gram |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GCCs | Germ cell cancer tissues |
| h | hour |
| HE | Hematoxylin-eosin |

| | |
|---|---|
| ICC | Immunocytochemistry |
| ICSI | Intracytoplasmic sperm injection |
| IF | Immunofluorescence |
| IHC | Immunohistochemistry |
| ISH | In-situ hybridization |
| kDa | kilo Dalton |
| LB | Lysogeny Broth |
| M.SssI | CpG methyltransferase |
| MEF | Mouse embryonic fibroblasts |
| mg | milligram |
| min | minute |
| Mil | Million |
| ml | milliliter |
| mM | millimolar |
| NTTs | Normal testicular tissues |
| NZS | Normozoospermia |
| O | Oligozoospermia |
| PBS | Phosphate buffered saline |
| PD | Passive dilution |
| PFA | Paraformaldehyde |
| PGCs | Primordial germ cells |
| PM | Primary magnification |
| PVDF | Polyvinylidene difluoride |
| qPCR | quantitative PCR |
| R | Reverse primer; |
| RNA | Ribonucleic acid |
| RNase | Ribonuclease |
| ROC | Receiver operating characteristic |
| rpm | revolutions per minute |
| RT-PCR | Reverse transcription-polymerase chain reaction |
| RT-qPCR | Quantitative reverse transcription PCR |
| RT | Room temperature |
| SB | Scale bars |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulfate |
| sec | second |
| SEM | Standard error of mean |
| TDG | Thymine DNA glycosylase |
| TEMED | Tetramethylethylenediamine |
| TET | Ten eleven translocation |
| Tm | Primer melting temperature; |
| WB | Western-blot analysis |
| µg | microgram |
| µl | microliter |

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# 11. LIST OF OWN PUBLICATIONS

## 1. Publications (articles in English)

- Ni K., Dansranjavin T., Rogenhofer N., Oeztuerk N., Deuker J., Bergmann M., Schuppe H.C., Wagenlehner F., Weidner W., Steger K. & Schagdarsurengin U. TET enzymes are successively expressed during human spermatogenesis and their expression level is pivotal for male fertility. *Hum Reprod* 31, 1411-24. (**2016**, **originally from this thesis**)
- Ni K., Spiess A.N., Schuppe H.C. & Steger K. The impact of sperm protamine deficiency and sperm DNA damage on human male fertility: a systematic review and meta-analysis. *Andrology*. (**2016**)
- Ni K., Steger K., Yang H., Wang H., Hu K., Zhang T. & Chen B. A comprehensive investigation of sperm DNA damage and oxidative stress injury in infertile patients with subclinical, normozoospermic, and astheno/oligozoospermic clinical varicocoele. *Andrology*. (**2016**)
- Ni K., Steger K., Yang H., Wang H., Hu K. & Chen B. Expression and role of leptin under hypoxic conditions in human testis: organotypic in vitro culture experiment and clinical study on patients with varicocele. *J Urol* 193, 360-7. (**2015**)
- Ni K., Steger K., Yang H., Wang H., Hu K. & Chen B. Sperm protamine mRNA ratio and DNA fragmentation index represent reliable clinical biomarkers for men with varicocele after microsurgical varicocele ligation. *J Urol* 192, 170-6. (**2014**)

## 2. Publications (articles in Chinese)

- Ni K, Chen B, Li H, Wang H, Yang H, Hu K, Han Y, Wang Y & Huang Y. Treatment of varicocele infertility men patients of different Chinese medical syndrome types by integrative medicine treatment selection: a primary research. *Chinese Journal of Integrated Traditional and Western Medicine* 33, 324-9. (**2013**)

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- Ni K & Chen B. Role of oxidative stress in the pathogenesis of infertile male with varicocele. *Chinese Journal of Andrology* 26, 10-3. (**2012**)
- Ni K & Chen B. The effects and impairments of TNF-α on semen quality. *Chinese Journal of Andrology* 26, 20-2. (**2012**)

## 3. Conference Abstracts

- Annual American Urological Association (AUA) Congress (Apr. **2016**), San Diego, USA. Expression of Ten-eleven-translocation (TET) enzymes is associated with male fertility. [Poster presentation]
- Annual European Association of Urology (EAU) Congress (Mar. **2015**), Madrid, Spain. Sperm protamine mRNA ratio and DNA fragmentation index represent reliable clinical biomarkers for men with varicocele after microsurgical varicocele ligation. [Poster presentation]
- Annual American Urological Association (AUA) Congress (May **2015**), New Orleans, USA. Expression and role of leptin under hypoxic conditions in human testis: organotypic in vitro culture experiment and clinical study on patients with varicocele. [Poster presentation]
- International Giessen Graduate Centre for the Life Sciences (GGL) annual congress (Sep. **2015**), Giessen, Germany. Expression of Ten eleven translocation enzyme 3 during human spermatogenesis and its role in male fertiltiy. [Poster presentation]
- International Giessen Graduate Centre for the Life Sciences (GGL) annual congress (Sep. **2014**), Giessen, Germany. Detection of TET2 and TET3 in human testis tissue and ejeculates. [Poster presentation]

# 12. EHRENWÖRTLICHE ERKLÄRUNG

Ich erkläre: Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus Liebig Universität Giessen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

Giessen, July 2016

**Kai Ni**