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ORIGINAL ARTICLE

DNA methylation in spermatozoa as a prospective marker in andrology

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SUMMARY

Recent studies have shown associations of aberrant DNA methylation in spermatozoa with idiopathic infertility. The analysis of DNA methylation of specific genes could therefore serve as a valuable diagnostic marker in clinical andrology. For this purpose, rapid and reliable detection methods, reference values and the temporal stability of spermatozoal DNA methylation need to be established and demonstrated. In this prospective study, swim-up purified semen samples from 212 consecutive patients (single samples), 31 normozoospermic volunteers (single samples) and 10 normozoospermic volunteers (four samples at days 1, 3, 42 and 45 plus a fifth sample after 180–951 days) were collected. Spermatozoal DNA was isolated, bisulphite converted and DNA methylation was analysed by pyrosequencing. DNA methylation of the maternally imprinted gene *MEST* was measured in samples of 212 patients and 31 normozoospermic volunteers and the temporal stability of eight different genes and two repetitive elements was examined in consecutive samples of 10 normozoospermic volunteers. *MEST* DNA methylation was significantly associated with oligozoospermia, decreased bi-testicular volume and increased FSH levels. A reference range for spermatozoal *MEST* DNA methylation (0–15%) was established using the 95th percentile of DNA methylation in normozoospermic volunteers. Using this reference range, around 23% of our patient cohort displayed an aberrant *MEST* DNA methylation. This epigenetic aberration was found to be significantly associated with bi-testicular volume, sperm concentration and total sperm count. DNA methylation in normozoospermic volunteers was stable over a time period of up to 951 days in contrast to classical semen parameters. Our data show that *MEST* DNA methylation fulfils the prerequisites to be used as routine parameter and support its use during andrological workup if a prognostic value can be shown in future.

INTRODUCTION

In Europe 10–15% of couples are affected by infertility. Approximately 50% of reported cases are accounted for by male factor infertility, resulting in a prevalence of about 7 % of all men (Nieschlag *et al.*, 2010). Among these, 4% of all cases and up to 20% of azoospermic patients display genetic reasons for their infertility (Tüttelmann *et al.*, 2011). The currently known and routinely analysed genetic causes for infertility are chromosomal aberrations, microdeletions of the azoospermia factor (AZF) – loci and mutations of the *CFTR* (cystic fibrosis transmembrane conductance regulator) gene (Nieschlag *et al.*, 2010; Tüttelmann *et al.*, 2011). Besides genetic causes, aberrant patterns of DNA methylation or histone modification called epimutations seem to play a role in infertility.

Spermatozoa possess germ cell–specific epigenetic patterns that are obtained by modifications like DNA methylation and histone to protamine transitions during spermatogenesis (Hajkova *et al.*, 2002; Santos & Dean, 2004; Trasler, 2009; Jenkins & Carrell, 2011). One important aspect of the germ cell–specific epigenetic pattern is genomic imprinting. It describes heritable molecular differences between the alleles of maternal and paternal origin which are mediated by DNA methylation (Reik *et al.*, 1987; Sapienza *et al.*, 1987). In germ cells, imprinted genes are highly methylated either in spermatozoa (paternally imprinted genes) or in oocytes (maternally imprinted genes) (Tycko, 1997).

Early primordial germ cells (PGCs) display a somatic-like methylation pattern with a high degree of genome-wide DNA methylation. When the PGCs enter the genital ridge a wave of

genome-wide DNA demethylation occurs and ensures an equivalent epigenetic state of the germ cells of both sexes prior to the establishment of sex-specific epigenetic germ line modifications (Hajkova *et al.*, 2002; Biermann & Steger, 2007). In the male germ line, the re-methylation of the genome by *de novo* DNA methyltransferases DNMT3A, DNMT3B and DNMT3L starts after prenatal mitotic arrest and is probably completed either in spermatogonia A at the earliest or in pachytene spermatocytes at the latest. This wave of re-methylation establishes male-specific imprints and specific DNA methylation patterns (Kerjean *et al.*, 2000; Hajkova *et al.*, 2002; Yamazaki *et al.*, 2003; Biermann & Steger, 2007; Oakes *et al.*, 2007; Marques *et al.*, 2011).

After fertilization, the genome of the zygote undergoes a second genome-wide wave of demethylation followed by *de novo* methylation and establishment of somatic DNA methylation patterns around the time of implantation (Mayer *et al.*, 2000; Reik *et al.*, 2001; Haaf, 2006). Only imprinted genes are unaffected by the demethylation wave after fertilization. This ensures the maintenance of their germ line-specific DNA methylation patterns and a parent-of-origin specific gene expression which plays an important role in embryonic development (Trasler, 2009; Bartolomei & Ferguson-Smith, 2011; Jenkins & Carrell, 2011).

We and others have shown strong associations of aberrant DNA methylation of several imprinted genes in spermatozoa with idiopathic male infertility (Kobayashi *et al.*, 2007; Marques *et al.*, 2008; Hammoud *et al.*, 2010; Poplinski *et al.*, 2010; Rajender *et al.*, 2011; Sato *et al.*, 2011). In detail, hypermethylation of the maternally imprinted gene *MEST* and hypomethylation of the paternally imprinted gene *H19* were significantly associated with decreased sperm count and in the case of *MEST* with decreased sperm motility and abnormal morphology (Poplinski *et al.*, 2010). As a result of these findings, proper DNA methylation patterns of spermatozoa seem to be essential for male fertility.

In addition, associations of aberrant DNA methylation patterns with an increased frequency of spontaneous abortions as well as imprinting disorders in assisted reproductive techniques (ART) have been described (DeBaun *et al.*, 2003; Gicquel *et al.*, 2003; Maher *et al.*, 2003; Halliday *et al.*, 2004; Benchaib *et al.*, 2005; Amor & Halliday, 2008; Pliushch *et al.*, 2010; El Hajj *et al.*, 2011; Ankolkar *et al.*, 2012; Rotondo *et al.*, 2012) which delineate the potential impact of DNA methylation on pregnancy outcome and embryo development.

Thus, the analysis of the DNA methylation of specific genes in spermatozoa could constitute an important tool in the andrological examination as a novel diagnostic parameter and prognostic factor for the outcome of any ART treatment.

All studies performed so far have been retrospectively designed and none have evaluated whether epigenetic analysis is suitable and feasible in the routine andrological laboratory. Essential information regarding the extent to which DNA methylation analysis can be used is completely lacking. For example, it is still unknown whether DNA methylation represents a constant parameter over time or fluctuates similar to sperm counts. DNA methylation may not be necessarily stable over time as epigenetic changes might be involved in the late onset of diseases and reflect age-related effects or environmental exposures (Jiang *et al.*, 2004; Bjornsson *et al.*, 2008). Some studies have already described that DNA methylation patterns in blood and buccal cells may change over time and after exposure to environmental factors (Fraga *et al.*, 2005; Bjornsson *et al.*, 2008; Baccarelli *et al.*, 2009; Torrone *et al.*, 2012), whereas others suggested that DNA methylation is stable (Cortessis *et al.*, 2011) and loci dependent (Heijmans *et al.*, 2007; Byun *et al.*, 2012). In addition, little is known about the inter-individual variation in gene-specific DNA methylation patterns and reference values of DNA methylation in spermatozoa are missing (Schneider *et al.*, 2010; Talens *et al.*, 2010).

Given the lack of essential data that are needed to assess the suitability and feasibility of DNA methylation analysis in andrological units, we conducted a prospective study involving infertile men and performed an epigenetic analysis of spermatozoa. Protocols were developed and modified allowing for rapid, reliable and cost effective DNA methylation analysis. A reference range was established and the clinical significance of DNA methylation determined. Finally, the temporal stability of DNA methylation in spermatozoa – a crucial parameter for the predictive power of clinical epigenetic analysis – was ascertained in semen samples of 10 volunteers in 10 genes over a period of up to 951 days.

SUBJECTS AND METHODS

We collected semen samples from 238 consecutive (mainly Caucasian) patients seeking advice for couple infertility between April and October 2011 at the Department of Clinical Andrology of the Centre of Reproductive Medicine and Andrology (Münster, Germany), a tertiary-referral centre. All patients underwent a complete physical examination. Patients were excluded from our study if they had an established genetic cause of male infertility (chromosomal aberrations, AZF-deletions), history of or current malignant disease or some surgical intervention of the genitourinary system. Consequently, 26 patients were excluded, resulting in the final study group comprising 212 patients (Table 1).

In addition, single semen samples from 31 healthy, normozoospermic (as per World Health Organization criteria, (World Health Organization, 2010) volunteers (mean age ± SD: 34.5 ± 7.2 years) were collected as control group (Table S1). In order to measure the temporal stability of spermatozoal DNA methylation, four consecutive semen samples from 10 normozoospermic healthy volunteers (P1–P10) (mean age ± SD: 30.4 ± 6.0 years) were obtained at four time points over a period of 45 days (because of practical reasons on days 1, 3, 42 and 45) and analysed. From eight of these 10 volunteers, a fifth sample was collected after a period of 180–951 days (P1: 255 days; P2: 951 days; P3: 180 days; P4: 933 days; P5: 948 days; P7 and P8: 683 days; P10: 688 days). To avoid any influence of the individual abstinence time on the inter-individual variability the abstinence time was similar (2–3 days) for all volunteers.

Ethical approval

All subjects provided written informed consent and agreed to the analysis of genetic material as approved by the Ethics Committee of the University and the state medical board (reference number of Institutional Review Board approval: 4 I Nie).

Measurement of hormones

Serum testosterone levels were measured using a commercial ELISA kit (DRG Instruments GmbH, Marburg, Germany) and

serum levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were determined by highly specific time-resolved fluoroimmunoassays (Autodelfia; Perkin-Elmer, Inc., Wallac Inc., Turku, Finland).

Testicular volume

Sonographic measurements of the testes were performed by applying a high-frequency 12-MHz convex scanner (ultrasound scanner type 2002 ADI; BK Medical, Gentofte, Denmark). Volume was calculated by the ellipsoid method (Nieschlag *et al.*, 2010).

Semen analysis

Measurements of sperm count, motility and morphology were carried out according to the WHO guidelines 2010 (World Health Organization, 2010). In brief, semen samples were collected in modified graduated glass measuring cylinders and semen volume was measured. Semen samples were liquidated at 37 °C and analysed on prewarmed (37 °C) slides. Sperm motility was calculated by classifying the spermatozoa as grade a (fast progressive motility), b (slow progressive motility), c (motility with an absence of progression) or d (no movement). Progressive sperm motility was defined as percentage of spermatozoa classified as grade a and b from all spermatozoa counted. Sperm concentration was measured by a haemocytometer chamber. Afterwards, total sperm count was calculated as sperm volume × sperm concentration. Sperm morphology was analysed by Papanicolaou staining of dried semen smears and subsequent classification of the spermatozoa as normal, head defects, midpiece defects and tail defects.

For all sperm parameter duplicates of 200 spermatozoa for each sample were measured and the mean provided. As per to the WHO guidelines 2010, sperm counts ≥39 mill., ejaculate volume ≥1.5 mL, progressive motility ≥32% and normal morphology ≥4% was defined as normozoospermic. An external quality control was performed in the framework of QuaDeGA (Mallidis *et al.*, 2012).

Swim-up purification

Swim-up purification of all semen samples was performed to avoid contamination by somatic cells. Semen was diluted 1 : 1 in Sperm Preparation Medium (Origio, Måløv, Denmark), centrifuged (390 ***g*** for 10 min), then the supernatant removed and the pellet washed in 2 mL Sperm Preparation Medium (390 ***g***, 10 min). After washing, 1 mL Sperm Preparation Medium was slowly added to the pellet and incubated for 1 h at 37 °C and 5% CO_2. After incubation, 500 μL of the supernatant containing motile spermatozoa was collected for subsequent analysis (World Health Organization, 2010). In order to ensure that the swim-up purified sperm samples were not contaminated by somatic cells, some random samples were examined by light microscopy.

Isolation of DNA and bisulphite conversion

DNA from 1×10^6 swim-up purified spermatozoa of 10 volunteers (consecutive samples) was isolated using the Master-Pure DNA Purification Kit (EPICENTRE Biotechnologies, Madison, WI, USA) and bisulphite converted by the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany). Faster processing of the DNA from at least 1×10^5 swim-up purified spermatozoa per each of the patients and volunteers (control group) samples was performed by using the EpiTect Plus LyseAll Bisulfite Kit (Qiagen). All protocols were performed following the manual's instructions.

In order to ensure that bisulphite conversion was complete, the DNA methylation levels of bisulphite-converted control samples from different sources (blood, spermatozoa) were compared to the expected values. Furthermore, control dispensations at non-cytosine–guanine dinucleotide (CpG) sites were used in all pyrosequencing assays (Figure S1) to rule out insufficient bisulphite conversion as recommended by the manufacturer (Qiagen).

Amplification of differentially methylated regions of genes

Differentially methylated regions (DMRs) of the paternally imprinted genes *MEG3* (*maternally expressed gene* 3, chromosome 14; 100,345,300-100,345,900 Ensembl version (E.v.) 54) and *H19* (*imprinted maternally expressed transcript (non-protein coding)*, chromosome 11; 1977647–1977878, E.v. 54), the maternally imprinted genes *LIT1* (*long QT intronic transcript 1*, chromosome 11; 2,677,751–2,677,873, E.v. 54), *SNRPN* (*small nuclear ribonucleoprotein N*, chromosome 15; 22,751,105–22,751,342, E.v. 54) and *MEST* (*mesoderm specific transcript*, chromosome 7; 129919302-129919521, E.v. 54), the spermatogenesis-specific genes *VASA* (*DEAD (Asp-Glu-Ala-Asp) box polypeptide 4*, chromosome 5; 55033903 – 55034028, Retrieved from NCBI build 37.1), *DAZL* (*deleted in azoospermia-like*, chromosome 3; 16646000 – 16647000, E.v. 58) and *BOLL* (*boule-like (Drosophila)*, chromosome 2; 198,358,403–198,358,764, E.v. 54) and the repetitive elements *ALU* [*short interspersed elements* (*SINEs*)] and *LINE1* (*long interspersed elements*) (according to Yang *et al.*, 2004) were analysed. PCR primers and the specific PCR thermocycling conditions are listed in the supplemental material (Data S1).

DNA methylation analysis

Quantitative DNA methylation analysis was performed by pyrosequencing (PyroMark Q24 System; Qiagen). The analysed sequences comprised two CpGs for *H19*, three CpGs for *MEST, SNRPN, LIT1, BOLL, ALU* and *LINE1* and four CpGs for *VASA, MEG3* and *DAZL* and the DNA methylation levels of each gene were described by the mean of these CpGs. Prior to this study, all of the analysed CpGs were validated as DMRs by bisulphite sequencing of several human blood and sperm samples.

By establishing the pyrosequencing assays, the sensitivity and reproducibility of each assay was calculated. The analysis of control DNA with different percentages (0, 25, 50, 75 and 100%) of methylation (Qiagen) and of several human blood and sperm samples was used to estimate the sensitivity. The reproducibility was measured by analysing numerous of these samples in duplicates. If the values of the replicated analyses were significantly different from the previous analyses or from the expected values (control DNA), this pyrosequencing assay was not used for DNA methylation analyses in this study.

Moreover, all pyrosequencing assays included control dispensations at non-CpG sites to technically monitor and rule out insufficient bisulphite conversion. Pyromark Q24 software (PyroMark Q24 2.0.6.20; Qiagen) was used for analysis. The sequencing primers are listed in the supplemental material (Data S2).

Statistical analysis

Clinical data of patients with normal and hypermethylated *MEST* were compared using Mann–Whitney *U*-test as data were not normally distributed. Associations of clinical data and *MEST* DNA methylation were assessed by linear regression. Sperm concentration and count were log-transformed to approximate normal distribution. Non-parametric Spearman-Rho correlations of *MEST* DNA methylation with other genes were approved as values were not normally distributed. Temporal stability data were normally distributed and analysed by one-way ANOVA followed by Bonferroni's Multiple Comparison post-test.

All statistical calculations were performed with GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, USA) or Stata/SE software (version 9.1; StataCorp LP, College Station, TX, USA). Differences were considered significant if $p < 0.05$.

RESULTS

Introduction of *MEST* DNA methylation analysis in clinical diagnostics

The maternally imprinted gene *MEST* and its aberrations were already described in detail in several studies and among all genes it displayed the strongest association of aberrant DNA methylation with oligozoospermia, abnormal morphology and decreased motility (Kobayashi *et al.*, 2007; Marques *et al.*, 2008; Hammoud *et al.*, 2010; Poplinski *et al.*, 2010; Sato *et al.*, 2011). Owing to the association of *MEST* DNA methylation and idiopathic male infertility, this imprinted gene seems to be a sensitive parameter for spermatogenesis and male fertility – besides its role for correct embryo development. Thus, we decided to study *MEST* DNA methylation with respect to its potential as andrological marker in 31 volunteers and 212 consecutive patients.

Comparison of *MEST* DNA methylation in normozoospermic and oligozoospermic patients

According to their andrological evaluation, the 212 analysed patients were separated in 78 normozoospermic and 134 oligozoospermic men. Following this stratification, bi-testicular volume and sperm concentration/count, progressive motility and normal morphology were significantly lower in the oligozoospermic group, as well as serum levels of LH and FSH were significantly higher. The obtained median value of the *MEST* DNA methylation was borderline significantly higher ($p = 0.0499$) in the oligozoospermic group (11.3%) than in the normozoospermic group (10.2%) (Table 1). All other parameters analysed were comparable between both groups.

Factors possibly influencing semen parameters were present in subgroups of patients: 10.3% (22 of 212) had a history of cryptorchidism, 16.0% (34) had varicocoeles and 28.8% (61) had current or former genitourinary infections. However, no differences in *MEST* DNA methylation were found between patients either with or without these factors (all $p > 0.4$).

Reference range for *MEST* DNA methylation

In order to utilize *MEST* DNA methylation of spermatozoa as an andrological parameter, the definition of normal range values is necessary. The given standard definition of a reference range confers to an interval which comprises 95% of the values of a control group (Marshall & Bangert, 2008). Thus, the 95th percentile of the normozoospermic control group was chosen as upper reference value for normal *MEST* DNA methylation which based on our data was found to be 15%. With this upper reference value the normal range of *MEST* DNA methylation was defined as 0–15%.

Due to this reference range, 23.1% (49/212) of the patients showed aberrant *MEST* DNA methylation. By separating these 212 patients in normozoospermic (78/212) and oligozoospermic (134/212) men, 18% (14/78) of the normozoospermic men and 26.1% (35/134) of the oligozoospermic men displayed *MEST* DNA methylation above the reference range (Fig. 1). The maximum *MEST* DNA methylation was 23.3% in the normozoospermic and 50% in the oligozoospermic group indicating that only oligozoospermic patients had extreme aberrations of *MEST* DNA methylation.

Applying the 15% *MEST* DNA methylation cut-off sensitivity and negative predictive values to predict oligozoospermia were rather low with 0.26 and 0.39 respectively. In contrast to this, specificity and positive predictive values were high with 0.82 and 0.71.

Association of *MEST* DNA methylation and clinical parameters

The patient cohort was stratified according to a cut-off value of 15% *MEST* DNA methylation (95th percentile of the normozoospermic control group) yielding group sizes of 163 men with <15% *MEST* DNA methylation and 49 men displaying ≥15% *MEST* DNA methylation.

By comparing several clinical parameters of both groups the median of the FSH levels (4.7 U/L vs. 5.5 U/L, $p = 0.248$) did not vary significantly between these groups, but was slightly higher in patients with aberrant *MEST* DNA methylation. In contrast, bi-testicular volume (42 mL vs. 36 mL, $p = 0.014$), sperm concentration (20 mill/mL vs. 3.6 mill/mL, $p < 0.0001$) and total sperm count (85.5 mill. vs. 17.8 mill., $p < 0.0001$) were highly significantly different (Fig. 2).

In the entire patient cohort, *MEST* DNA methylation was negatively associated with bi-testicular volume ($r = -0.22$, $p = 0.0021$), sperm concentration ($r = -0.47$, $p < 0.0001$) and total sperm count ($r = -0.48$, $p < 0.0001$) and positively related to FSH level ($r = 0.22$, $p = 0.0016$) (Fig. 3). Furthermore, a negative association was found with progressive sperm motility ($r = -0.22$, $p = 0.0017$), normal morphology ($r = -0.17$, $p = 0.0151$) and spermatogenic efficiency (total sperm count per bi-testicular volume) ($r = -0.22$, $p = 0.0021$). In contrast, levels of LH and Testosterone were not related to *MEST* DNA methylation.

Analysis of additional methylated genes

Besides *MEST,* the paternally imprinted genes *MEG3* and *H19* could also serve as potential andrological markers as recent studies showed associations of aberrant DNA methylation of those genes with poor sperm quality (Kobayashi *et al.*, 2007; Poplinski *et al.*, 2010). In addition, the DNA methylation of the spermatogenesis-specific gene *VASA* could be of interest for andrological purposes owing to previously described associations of decreased *VASA* expression in spermatozoa with oligozoospermia (Guo *et al.*, 2007). In order to determine potential

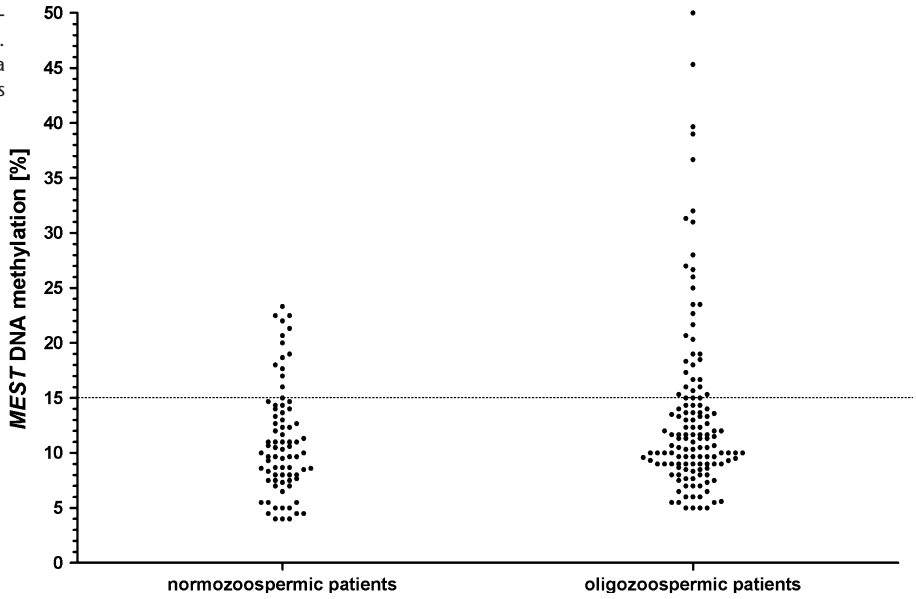


Figure 1 *MEST* DNA methylation values of 78 normozoospermic and 134 oligozoospermic patients. The 95th percentile of *MEST* DNA methylation in a normozoospermic control group (15%) is set as upper reference value for further analyses.

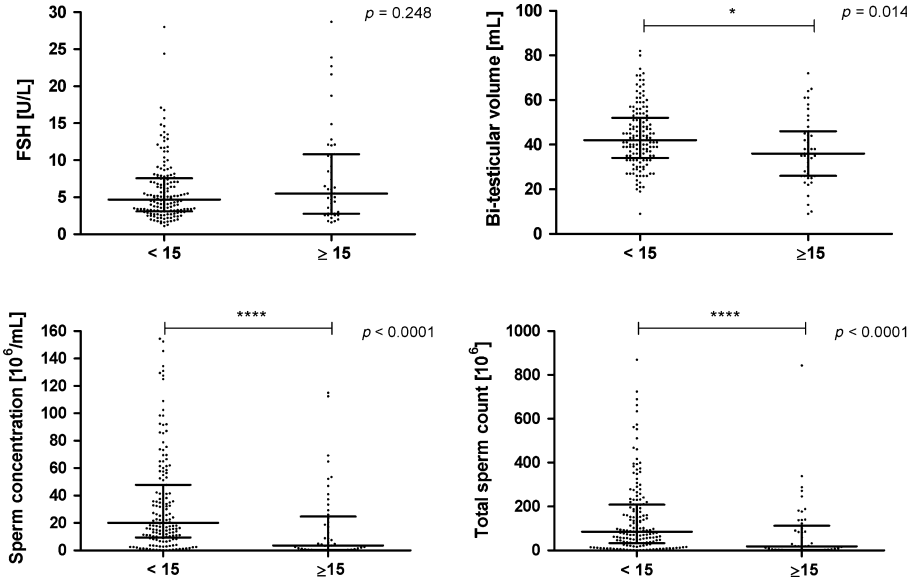


Figure 2 Serum FSH, bi-testicular volume, sperm concentration and total sperm count of 163 men with normal *MEST* DNA methylation compared with 49 men with abnormal *MEST* DNA methylation. Statistical analysis was performed by Mann–Whitney *U*-test, medians with interquartile ranges are shown, *$p < 0.05$, ****$p < 0.0001$.

associations with other genes, we performed DNA methylation analysis of *VASA, MEG3* and *H19 (*in addition to *MEST)* in the first 37 patients of our study.

The analysis of spermatozoal DNA methylation of these genes in 37 patients (Table S2) displayed *MEST* and *VASA* DNA methylation levels that were highly correlated ($r = 0.941$, $p < 0.001$), whereas the DNA methylation levels of *H19* and *MEG3* were not associated with *MEST* DNA methylation (*H19*: $r = -0.083$, $p = 0.212$, *MEG3*: $r = 0.213$, $p = 0.207$). Thus, *VASA* DNA methylation could potentially serve as another marker in the andrological evaluation.

Temporal stability of DNA methylation in spermatozoa

To assess temporal stability of DNA methylation patterns in spermatozoa, DNA methylation patterns of eight genes and two repetitive elements were examined in four consecutive mid-term semen samples (days 1, 3, 42 and 45, $n = 10$) and one long-term semen sample (after a period of 180–951 days; $n = 8$) of normozoospermic volunteers. Among these genes, two were paternally (*H19, MEG3*) and three maternally imprinted (*MEST, SNRPN, LIT1*) genes as they indicate parent-of-origin expression. In addition, three genes were spermatogenesis-specific (*VASA, DAZL, BOLL*) and two repetitive elements (*ALU, LINE1*) were analysed to define the genome-wide DNA methylation.

The five analysed imprinted genes as well as the three selected spermatogenesis-specific genes showed no changes in their DNA methylation status over time (Fig. 4A). Similar to this the DNA methylation of the repetitive elements *ALU* and *LINE1* were found to be stable over this period. In contrast to DNA methylation patterns, semen volume, total sperm count, sperm concentration and progressive sperm motility were highly fluctuating over time (Fig. 4A).

Inter-individual variability in DNA methylation

The range of inter-individual variability was determined by analysing the DNA methylation patterns of 10 selected loci in 10 individuals. The DNA methylation of the repetitive element *ALU*

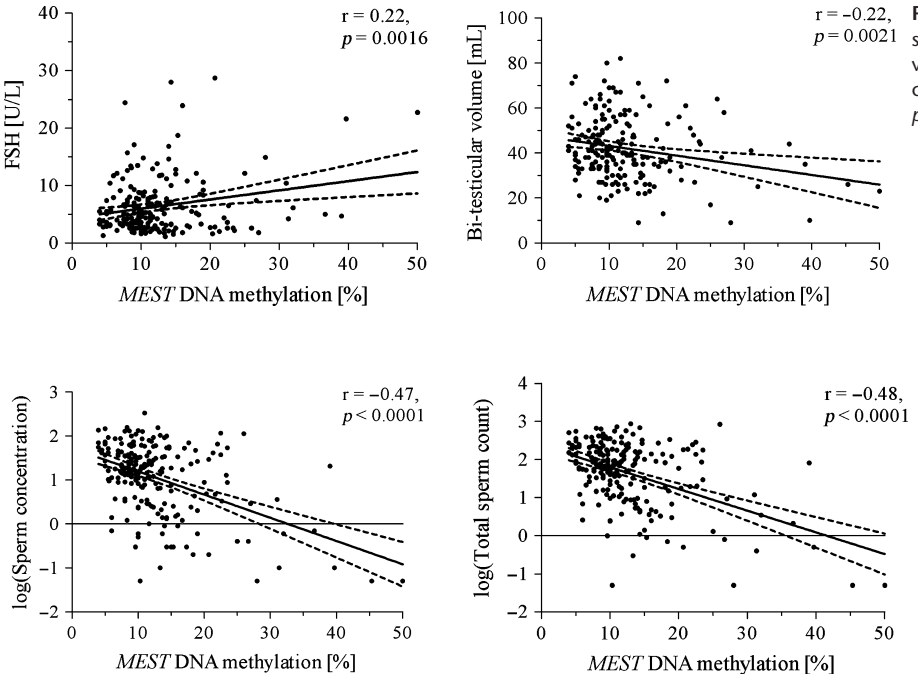


Figure 3 Correlation of *MEST* DNA methylation in spermatozoa of 212 men and FSH level, bi-testicular volume, sperm concentration and total sperm count (*r*: Spearman's rho correlation coefficient; *p* = significance).

showed only minor differences between individuals whereas the DNA methylation of the repetitive element *LINE1* varied between the individuals (range of DNA methylation: 41.33–79%), but not over time and within individuals. The DNA methylation patterns of three analysed spermatogenesis-specific genes and five analysed imprinted genes including *MEST* showed only low intra-individual variations (Fig. 4A).

DISCUSSION

Thus far, several studies have reported that aberrations of the germ cell specific DNA methylation patterns are associated with oligozoospermia, reduced progressive sperm motility and abnormal sperm morphology (Kobayashi *et al.*, 2007; Marques *et al.*, 2008; Hammoud *et al.*, 2010; Poplinski *et al.*, 2010; Sato *et al.*, 2011). Besides the strong association of aberrant DNA methylation of the maternally imprinted gene *MEST* with idiopathic male infertility (Kobayashi *et al.*, 2007; Poplinski *et al.*, 2010; Sato *et al.*, 2011), this current study displayed associations of *MEST* DNA methylation with clinical parameters such as serum FSH and bi-testicular volume, making it to a potential marker in andrology. Moreover, spermatozoal DNA methylation seems not only to be an informative parameter for spermatogenesis but also – and this makes it unique – for subsequent fertilization processes such as pregnancy success and outcome as described by previous studies (Benchaib *et al.*, 2005; El Hajj *et al.*, 2011; Ankolkar *et al.*, 2012; Rotondo *et al.*, 2012). In two of these studies, the predictive power of spermatozoal DNA methylation for pregnancy outcome was described (Benchaib *et al.*, 2005; El Hajj *et al.*, 2011). Another study reported the association of hypermethylated *MEST* in chorionic villi with spontaneous abortions (Zheng *et al.*, 2011). As these described DNA methylation aberrations in embryonic tissue could be because of epimutations in the paternal genome (Kobayashi *et al.*, 2009), the analysis of spermatozoal DNA methylation could help to reduce the number of spontaneous abortions.

Hitherto used parameters only enable the examination of fertility but cannot predict pregnancy rates or outcome, making DNA methylation a potentially better parameter in andrological examinations. As there are only few genetic causes for male infertility (e.g. Y-chromosomal deletions, chromosomal aberrations) which are currently screened for (Tüttelmann *et al.*, 2011), the introduction of an epigenetic analysis into the workup of an andrological clinic would be a promising supplement or even prognostic marker in the examination of hitherto idiopathic infertile men. For this purpose and to strengthen our idea, further studies for longitudinal data on fertility capacity and pregnancy outcome in relation to spermatozoal *MEST* DNA methylation are needed.

Introduction of *MEST* DNA methylation analysis in clinical diagnostics

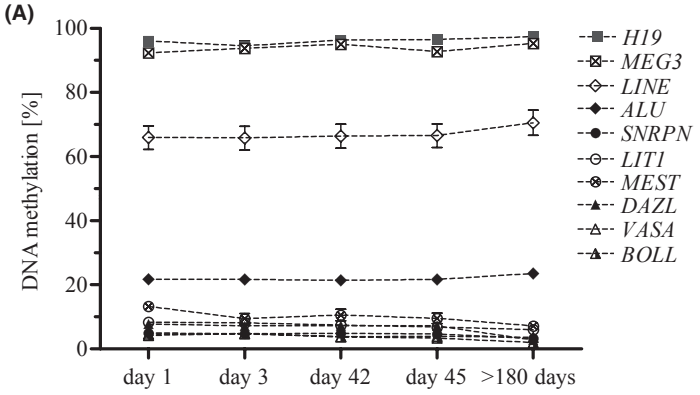
For this study, modified protocols which facilitate a more rapid analysis of DNA methylation in spermatozoa were established. Optimized protocols for DNA methylation enabled to screen swim-up purified sperm samples within short time (11 h). Furthermore, pyrosequencing assays were designed to maximize sensitivity and reproducibility, thereby allowing to perform single assays for each sample at a high level of reliability and low running costs.

Although our pyrosequencing approach only analyses few CpG sites within the studied *MEST* sequence, comparative sequencing studies revealed that all CpG sites of the DMR have undergone the same changes either normal or aberrant. This finding very likely excludes the possibility that observed DNA methylation changes are as a result of aberrant methylation of single CpG sites.

Comparison of *MEST* DNA methylation in normozoospermic and oligozoospermic patients

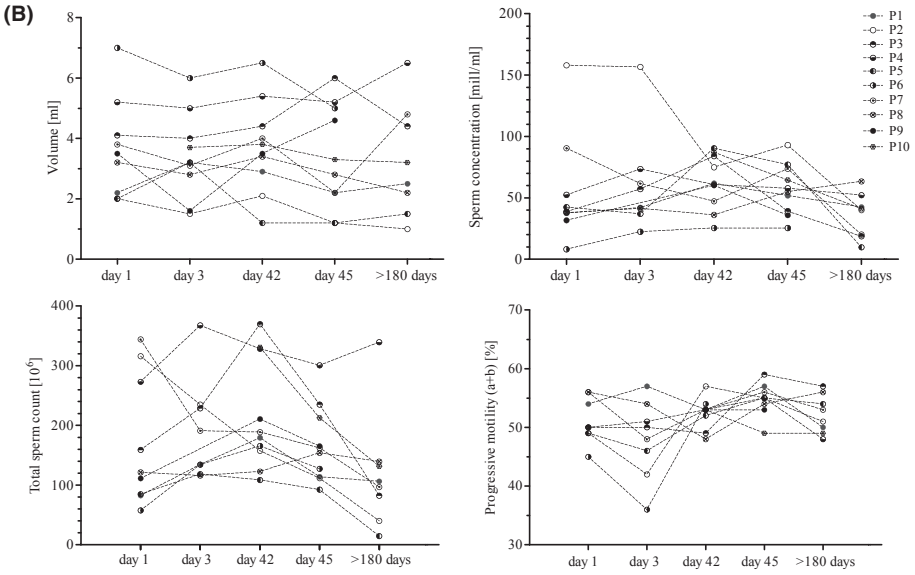
Our comparison of normozoospermic and oligozoospermic patients showed only borderline significant differences in the

Figure 4 (A) Temporal stability of DNA methylation of two paternally imprinted genes (*MEG3*, *H19*), three maternally imprinted genes (*SNRPN*, *LIT1*, *MEST*), three spermatogenesis-specific genes (*VASA*, *DAZL*, *BOLL*) and two repetitive elements (*ALU*, *LINE1*) over a time period of at least 45 days. Mean [%] ± SEM of 10 (for days 1, 3, 42 and 45) and eight (for the period ≥180 days) normozoospermic men are shown. (B) Time course of semen parameters of 10 (for days 1, 3, 42 and 45) and eight (for the period ≥180 days) normozoospermic men.



DNA methylation: Mean [in %] ± SEM of ten normozoospermic volunteers.

	H19		*MEG3*		*SNRPN*		*LIT1*		*MEST*	
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
day 1	96.05	1.21	92.35	1.05	4.95	0.84	8.33	0.79	13.23	1.43
day 3	94.56	1.62	93.78	0.36	4.68	0.70	8.08	0.53	9.42	1.67
day 42	96.33	0.76	95.00	0.54	4.87	0.75	7.45	0.52	10.58	1.80
day 45	96.44	0.73	92.73	1.10	4.63	0.59	6.86	0.26	9.56	1.65
> 180 days	97.38	0.25	95.28	0.56	3.29	0.47	6.00	0.63	7.17	1.46
	DAZL		*VASA*		*BOLL*		*ALU*		*LINE1*	
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
day 1	7.75	1.18	4.60	0.63	4.19	0.62	21.70	0.22	65.93	3.71
day 3	7.15	1.05	4.67	0.56	4.38	0.64	21.64	0.18	65.80	3.69
day 42	7.28	1.19	3.80	0.40	3.67	0.47	21.37	0.15	66.37	3.73
day 45	7.15	1.32	3.93	0.46	3.37	0.48	21.67	0.17	66.53	3.71
> 180 days	2.75	0.28	3.56	0.48	2.31	0.28	23.54	0.71	70.58	3.95



MEST DNA methylation. This is different to previous findings showing clear-cut significant differences between oligozoospermic patients and a highly selected normozoospermic control group (Poplinski *et al.*, 2010). As even the normozoospermic patients of our study were seeking advice for fertility problems, additional parameters such as DNA methylation seem to influence the fertility. The lack of detailed information about female factor or couple fertility of our patient cohort, limits the interpretation of our results with respect to the underlying cause of their infertility.

Additionally, the sharp differences between patients and controls in previous studies could be because of contamination by leucocytes and immature germ cells as not all studies performed the necessary procedure to purify spermatozoa from somatic

cells before DNA methylation analysis. For our current study, only swim-up purified spermatozoa were analysed which reflects the routine clinical procedure for subsequent ART in which preferential motile spermatozoa are used. This selection should not cause any bias as previous experiments of our group demonstrated that DNA methylation does not differ between the upper motile and the lower immotile fraction (data not shown). Moreover, a recent study of Krausz *et al.* (2012) showed highly stable sperm DNA methylation patterns in different quality-fractioned sperm populations of the same individual.

Normal range of *MEST* DNA methylation as diagnostic marker

The requirement of reference values for DNA methylation was already pointed out in several previous studies (Schneider *et al.*, 2010; Talens *et al.*, 2010). In this study, we for the first time defined a reference range for normal *MEST* DNA methylation in spermatozoa based on a control group of 31 healthy normozoospermic men with 0–15%.

By determining a reference range, we could demonstrate that hypermethylation of *MEST* is associated with significantly lower bi-testicular volume, sperm concentration and total sperm count. Stratifying our patient cohort according to a cut-off value of ≥15% yielded 49 men of 212 men, indicating that 23.1% of our patient cohort had an aberrant *MEST* DNA methylation (18% of the normozoospermic group and 26.1% of the oligozoospermic cohort).

The fact that aberrant *MEST* DNA methylation was predominantly found in oligozoospermic men was also reflected by the high specificity and positive predictive power of the 15% *MEST* threshold methylation value.

By these calculations, it has to be considered that some of the normozoospermic patients may be overlapping with the control group (concerning DNA methylation). However, the inclusion of the normozoospermic patients into the control group was rejected as it would not only lead to a wider reference range but also would introduce a strong bias towards DNA methylation abnormalities associated with idiopathic normozoospermic infertility.

However, the establishment and use of our reference value by other laboratories could only be the first step as it might be dependent on a variety of contributing factors such as different techniques and equipment and genetic backgrounds. As our study was conducted in a mainly Caucasian population and differences in the DNA methylation by ethnicity have been described (Zhang *et al.*, 2011), reference values might differ depending on the ethnic origin. In order to establish a worldwide reference value multicentre studies involving several countries to include ethnic differences are needed.

The fact that our study revealed that *MEST* DNA methylation seems to be a valuable indicator in andrology, does not preclude other genes from future studies. For example, in principle, *VASA* could represent a valid marker for DNA methylation with comparable potency corroborated by our finding that *MEST* and *VASA* DNA methylation are significantly associated.

MEST as a stable prognostic spermatogenic marker

The ideal parameter for the andrological workup of infertile men would be stable over time and not display inter-individual variations. Several studies have already determined the stability of global and loci-specific DNA methylation in different tissues and blood and described changes of DNA methylation over time or after exposures to environmental factors (Fraga *et al.*, 2005; Bjornsson *et al.*, 2008; Baccarelli *et al.*, 2009; Bollati *et al.*, 2009). However, some of the changes were either minor or loci dependent (Heijmans *et al.*, 2007; Byun *et al.*, 2012; Murphy *et al.*, 2012). In some studies even no differences over a short period of time were found (Fraga *et al.*, 2005). The latter one is in agreement with our previous findings that even after mid-term cryopreservation of spermatozoa the DNA methylation patterns of nine genes were stable (Kläver *et al.*, 2012).

Only few studies analysing the temporal stability of DNA methylation in spermatozoa are currently available. Cortessis *et al.* (2011) described that DNA methylation in spermatozoa is stable while Flanagan *et al.* (2006) found locus-, cell- and age-dependent differences in DNA methylation in spermatozoa. Krausz *et al.* (2012) recently demonstrated that DNA methylation in spermatozoa is highly conserved between normozoospermic men and stable in different subpopulations of the same individual. In our study, we found that DNA methylation of eight selected genes and two repetitive elements was highly stable over a time period of up to 951 days – unlike classical semen parameters which exhibited well-known high intra-individual variations (Nieschlag *et al.*, 2010).

CONCLUSION

Taken together, our data strongly support that *MEST* DNA methylation may serve as an additional parameter in the andrological workup in the future. As a result of our findings, we recommend a reference range for *MEST* DNA methylation of 0–15%, which possibly has to be adjusted to ethnicity and laboratory equipment and methods. However, further studies of the role of spermatozoal *MEST* DNA methylation in subsequent fertilization, embryo development and pregnancy outcome are needed.

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DISCLOSURE STATEMENT

The authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

Ruth Kläver designed and performed the experiments, collected and analysed data and wrote the manuscript. Frank Tüttelmann performed statistical analyses of the collected data and helped in writing the manuscript. Andreas Bleiziffer assisted in the design of the study as well as pyrosequencing assays and in performing experiments. Thomas Haaf helped in the design

and interpretation of pyrosequencing assays. Sabine Kliesch was responsible for patient and donor care. Jörg Gromoll designed and supervised the study and provided financial as well as administrative support.

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SUPPORTING INFORMATION

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

Table S1. Clinical parameters of the normozoospermic control groups. Data are presented as mean ± SD and median (5th to 95th percentile).
Table S2. DNA methylation levels of *MEST, VASA, H19* and *MEG3* in 37 patients.
Data S1. Information on PCR conditions.
Data S2. Information on pyrosequencing conditions.
Figure S1. Representative pyrosequencing analysis for *MEST* in (A) blood and (B) spermatozoa.