Asian Journal of Andrology (2014) 16, 669–674

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PERSPECTIVE

Male Health

Bringing epigenetics into the diagnostics of the andrology laboratory: challenges and perspectives

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Recent studies have shown significant associations of aberrant DNA methylation in spermatozoa with idiopathic male infertility, increased frequency of spontaneous abortions and imprinting disorders. Thus, the analysis of DNA methylation of specific genes in spermatozoa has the potential to become a new valuable diagnostic marker in clinical andrology. This perspective article discusses the current state and value of DNA methylation analysis in the diagnostic setup of infertile men and outlines challenges and perspectives. It highlights the potential of DNA methylation in andrological diagnostics and its putative benefit in the examination of hitherto idiopathic infertile patients is described.

Asian Journal of Andrology (2014) **16**, 669–674; doi: 10.4103/1008-682X.125412; published online: 30 May 2014

Keywords: DNA methylation; male infertility; spermatogenesis

INTRODUCTION

Worldwide 10%–15% of couples are affected by infertility which can be attributed to various factors. Approximately 50% of reported cases are accounted for by male factor infertility, resulting in a prevalence of about 7% of all men.[1–3] These numbers highlight the need for reliable diagnostic tools and further investigations of potential treatments. The classical andrological diagnostic procedure starts with the clinical and biochemical/endocrinological examination of the patient and is followed by semen analyses. These procedures have remained relatively unchanged for decades and consist of the inspection of macroscopic and microscopic appearance of the semen and the determination of sperm number, motility, vitality and morphology. Furthermore leukocyte peroxidase activity is measured and a mixed antiglobulin reaction test is recommended.[4] As in 4% of infertile patients and 20%–30% of azoospermic patients established genetic causes might underlie their infertility, patients are also routinely screened for chromosomal aberrations, microdeletions of the azoospermia factor-loci and mutations of the *CFTR* gene.[3,5] In addition, further genetic causes for infertility are suspected like copy number variants (CNVs).[6–8] In this context, Tüttelmann and colleagues investigated the role of CNVs in male infertility by analyzing normozoospermic controls and patients with idiopathic severe oligozoospermia or with Sertoli-cell-only syndrome. Although the mean number of CNVs were comparable between all groups, they found recurring CNVs which were present only in patients with severe oligozoospermia (10 CNVs), only in Sertoli-cell-only syndrome (3 CNVs) or in both groups with spermatogenic failure (1 CNV), but not in controls.[6] In addition, the analysis of X-linked CNVs in men with different sperm count demonstrated 73 CNVs being related to spermatogenesis. Interestingly, the patients had a higher excessive rate of deletions/person and a higher mean sequence loss/person resulting in a higher global burden of deletions in patients compared to controls.[7] Furthermore, Lopes *et al.*[8] analyzed recently CNVs in the genome of idiopathic infertile men and normozoospermic controls and found that a man's risk of idiopathic spermatogenic failure is changed by rare autosomal deletions (by 10%), rare X-linked CNVs (by 29%) and rare Y-linked duplications (by 88%). In summary, all described CNVs mark candidate genes which possibly cause spermatogenic failure or enhance the risk for it and thus represent novel targets for future investigations.

Apart from CNVs several single nucleotide polymorphisms and gene polymorphisms have also been described to be associated with poor male reproductive parameters. Such variants were detected in numerous genes including *PRM1* (protamine 1),[9] *DAZL* (deleted in azoospermia-like),[10] *MTHFR* (methylenetetrahydrofolate reductase)[11,12] and the *GST* (Glutathione S-transferases) genes.[13]

However, 30%–40% of infertile patients are still characterized as idiopathic as the underlying (molecular) reason for their infertility is not known.[2,3] Due to the high percentage of idiopathic male infertility new insights and approaches concerning the molecular and genetic nature of impaired spermatogenesis are urgently needed to improve diagnosis and treatment. Currently, the development of novel technologies such as whole-genome sequencing and single-cell sequencing[14–16] lead to exciting new findings in this research field and inspires the search for novel biomarkers (e.g. genes, proteins and metabolites) which could be used in the diagnosis of male infertility.[17,18]

Among them recent descriptions of a strong association between aberrant DNA methylation in spermatozoa and idiopathic male infertility[19–23] indicate that epigenetics might have a strong impact on the quantitative and qualitative aspects of spermatogenesis. This assumption is further strengthened by studies outlining a possible predictive power of spermatozoal DNA methylation in pregnancy outcome.[24–27] Thus, the analysis of DNA methylation of specific genes in spermatozoa could serve as a new valuable and noninvasive diagnostic marker in clinical andrology. We therefore believe that it is timely to

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Received: 24 September 2013; Revised: 11 November 2013; Accepted: 04 December 2013

consider the value of epigenetics in the diagnostic setup of infertile men and to critically evaluate the current challenges and perspectives of the approach.

DNA METHYLATION AND SPERMATOGENESIS

Epigenetics describes mitotically and/or meiotically heritable changes in gene function beyond the DNA sequence itself such as DNA methylation, histone modification, miRNA and non-coding RNAs.[28,29] Spermatozoa possess specific DNA methylation patterns which are obtained during early stages of spermatogenesis.[30,31] In this context, some genes become differentially 'imprinted' by DNA methylation being important for fertilization and subsequent embryogenesis.[32,33] Several recent studies have shown a strong association of aberrant DNA methylation (epimutations) of some imprinted genes in spermatozoa with idiopathic infertility.[19–23,34–36] Interestingly, men with moderate or severe oligospermia often displayed abnormalities in both maternal and paternal imprints.[19] Furthermore, Marques *et al.*[20] described that nearly 50% of patients of an oligozoospermic cohort had defective DNA methylation of the paternally imprinted gene *H19* and/or the maternally imprinted gene *MEST*. Our group demonstrated that low sperm counts are associated with aberrant DNA methylation of *H19* and more so of *MEST*. Additionally, aberrant DNA methylation of *MEST* was found to be associated with decreased progressive sperm motility and poor sperm morphology.[22] Two other studies showed that men with severe or moderate oligozoospermia display more aberrant imprints than normozoospermic men.[23,34] Furthermore, Camprubí and colleagues demonstrated that infertile patients had more aberrant imprints than fertile men.[36] Studies by Hammoud *et al.*[21] and Minor *et al.*[35] supported these findings by demonstrating that DNA methylation patterns in sperm of infertile patients were considerably altered at *H19* and various additional imprinted loci (*LIT1, MEST, SNRPN, PLAGL1* and *PEG3*). As several studies described associations of male infertility with spermatozoal DNA methylation of *H19* and/or *MEST*, we performed meta-analyses of these studies (if suitable data for a meta-analysis were available) demonstrating that it is more likely for infertile patients to have aberrant *MEST* (odds ratio: 3.4, 95% confidence interval: 1.98–5.84, $P < 0.0001$) or *H19* (odds ratio: 14.62, 95% confidence interval: 7.34–29.12, $P < 0.0001$) DNA methylation in spermatozoa compared to fertile men (**Figure 1**).

Apart from imprinted genes, other studies described associations of aberrant DNA methylation of non-imprinted genes and CpG loci with oligozoospermia, abnormal sperm morphology and reduced sperm motility.[37–41]

Epigenetic aberrations of spermatozoa have been described to be possibly inheritable to the offspring[42] and aberrant DNA methylation patterns are known to be responsible for imprinting disorders (e.g. Beckwith-Wiedeman syndrome and Angelman syndrome). Interestingly, these aberrant imprints and imprinting disorders seem to occur more frequently after assisted reproductive techniques (ARTs) than after natural conception-although the prevalence of these disorders is still extremely low.[43–49] In general, the epigenome of the spermatozoa seems to have an impact on embryo development[50,51] and epimutations of the spermatozoa could be the cause for miscarriages as associations of spontaneous abortions and aberrant spermatozoal DNA methylation have been described.[26,27]

These findings have led to the concept that spermatozoal DNA methylation has some potential predictive power for pregnancy outcome.[24–27] In this context, a study by El Hajj *et al.*[25] demonstrated significantly higher ($P < 0.001$) *ALU* DNA methylation in sperm samples which led to pregnancy and live birth; whereas, significantly lower ($P = 0.027$) *ALU* DNA methylation levels were found in spontaneous abortions. However, Benchaib *et al.*[24] demonstrated by immunostaining of 5-methylcytosine that global DNA methylation of human spermatozoa was associated to pregnancy rate but not to fertilization rate or rate of good quality embryos. Furthermore, DNA methylation aberrations of *H19* and the *MTHFR* promoter in paternal spermatozoa were described in cases of spontaneous abortions, suggesting an impact of spermatozoal DNA methylation on pregnancy outcome.[26,27] In **Supplementary Table 1** the studies which analyzed potential associations of spermatozoal DNA methylation with poor semen parameters or pregnancy outcome are summarized.

In addition, retained histones could play an essential role in the association of aberrant DNA methylation with male infertility. During spermatogenesis histones are replaced by protamines; however, this process is not completed and up to 15% of the histones remain in spermatozoal DNA.[52,53] These retained histones mark genes and loci that play a role in development (e.g. imprinted genes) and can be epigenetically modified.[54,55] Failures in histone retention and modification or aberrations in protamine distribution might not only have an impact on male fertility as already described in several studies,[56–59] but also could affect spermatozoal DNA methylation

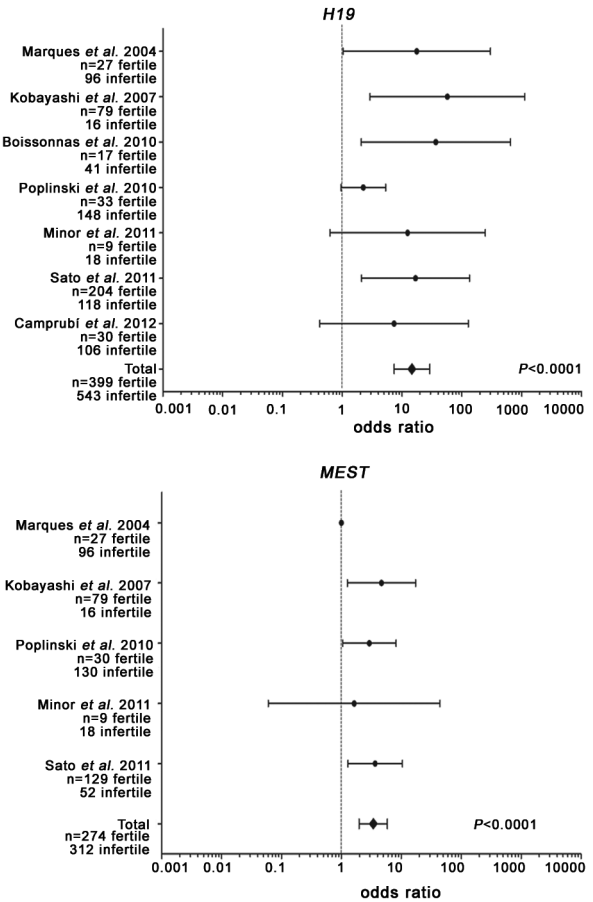

Figure 1: Meta-analyses of studies which analyzed normal and abnormal DNA methylation of *H19* and *MEST* in spermatozoa of infertile men in comparison to these values of fertile men. For each study the odds ratio with 95% confidence interval is shown. Odds ratio >1 means that it is more likely for infertile men to have aberrant imprints than for fertile men, odds ratio <1 signifies that it is less likely for infertile men to have aberrant imprints compared to fertile men.

patterns. Indeed, aberrant spermatozoal DNA methylation has been already shown in men with abnormal protamine replacement.[21,40,60]

Recently, DNA hydroxymethylation was described as an additional type of DNA methylation, but it is still obscure if 5-hydroxymethylcytosine is only an intermediate in the removal of 5-methylcytosine or a separate epigenetic mark.[61] Until now, most of the conventional methods for DNA methylation analysis can distinguish only between methylated and unmethylated cytosine, but not between different kinds of methylation. Thus, methods have been and will be developed to investigate 5-hydroxymethylation.[62,63] With these new techniques, future studies will be able to elucidate the role of hydroxymethylation in andrology as this DNA modification could also play an essential role in spermatogenesis and embryo development.

CHALLENGES FOR THE IMPLEMENTATION OF DNA METHYLATION ANALYSIS IN ANDROLOGICAL DIAGNOSTICS

Although as of today numerous studies support the potential utility of DNA methylation in andrological workup, none of these studies have evaluated whether epigenetic analysis is suitable or for that matter feasible in a routine andrological lab.

Essential requirements for the application of DNA methylation analysis in the daily clinical routine are still missing. For this purpose and as one of the first steps, time- and cost-effective technologies which provide dependable and reliable results have to be established. Several different technologies (which are to greater or lesser extent time- and cost-effective) for DNA methylation analysis such whole-genome bisulfite sequencing, genome-wide DNA methylation arrays and locus-specific DNA methylation analysis such as pyrosequencing or methylation-specific PCR-based analyses are currently available and summarized in **Table 1**. Alternatively, already available commercial tests for DNA methylation analysis in routine diagnostics could be used. These tests are often based on bisulfite conversion of DNA and subsequent methylation specific real-time PCRs of the relevant gene(s).[64]

Some technologies are limited in their sensitivity of DNA methylation detection (immunostaining) or in the amount of investigated CpGs (e.g. pyrosequencing or methylation-specific PCR-based analysis); however, these methods can be performed at affordable costs. In contrast, technologies such as whole-genome bisulfite sequencing or high resolution methylation arrays are able to measure multitudinous sequences across the genome, but are requiring sophisticated bioinformatic settings and are run at relatively high costs. If considering a clinical/andrological setting, a cost-effective, easy to perform and still informative technology seems preferable. Thus, a locus-specific DNA methylation analysis might provide sufficient information and is more rapid and less expensive than whole-genome analyses.

Another important aspect which has to be considered when analyzing DNA methylation in spermatozoa is the purification of semen samples. Independent of which methods are being applied, it is of crucial importance that the sample consists of spermatozoa only and is not contaminated by somatic cells or bacteria. One commonly used method, also recommended by the World Health Organization, is to perform swim-up purification before semen analysis which provides a relatively pure fraction of motile sperm.[4]

Further specific issues that need to be addressed are whether spermatozoal DNA methylation patterns represent a constant parameter over time and to which extent intra- and/or interindividual variations of gene-specific DNA methylation patterns exist. For the use as a diagnostic parameter, spermatozoal DNA methylation should ideally be temporally stable within individuals and should only display a small variability between healthy men.

Table 1: List of frequently used methods for DNA methylation analysis

Type of DNA methylation analysis	*Method*	*Characteristics*
Global	High-performance liquid chromatography	⊕ Highly quantitative and reproducible ⊖ Requires large amounts of high-quality genomic DNA
	Bisulfite sequencing of repetitive elements	⊕ Requires very little DNA ⊕ Quantitative, if a sufficient number of clones is sequenced ⊖ Labor-intensive
	5-mC antibody staining	⊕ Easy to perform ⊖ Less sensitive ⊖ Not very quantitative
Genome-wide	Whole-genome bisulfite sequencing	⊕ Comprehensive genomic coverage ⊕ High quantitative accuracy ⊖ High costs
	Genome-wide DNA methylation arrays	⊕ Enable to measure DNA methylation levels of preselected cytosines throughout the genome ⊕ Lower per-sample cost compared with whole-genome bisulfite sequencing ⊖ Limited genomic coverage ⊖ High setup cost for designing customized microarrays
	Restriction landmark genome scanning	⊕ Simultaneous DNA methylation analysis of thousands of loci ⊖ Dependent of specific restriction enzyme cutting sites ⊖ Limited genome coverage and sensitivity
	Methylated DNA immunoprecipitation	○ Can be followed by DNA microarrays or next-generation sequencing ⊖ Requires large amounts of genomic DNA and antibody
Gene-specific	Bisulfite sequencing of specific genes ('gold standard')	⊕ Requires very little DNA ⊕ Quantitative, if a sufficient number of clones is sequenced ⊕ Information on allele-specific methylation possible ⊖ Labor-intensive
	Pyrosequencing	⊕ Internal control (control dispensations) and accurate quantification of multiple CpG methylation sites in the same reaction ⊕ Time-effective ⊖ Only analysis of short to medium-length DNA sequences
	Methylation sensitive PCR	⊕ Rapid and very sensitive technique ⊕ Requires low quantity and quality of DNA ⊖ Not quantitative (in case of MethyLight: quantitative by real-time PCR)
	Bisulfite PCR followed by restriction analysis (COBRA)	⊕ Requires low quantity of DNA ⊕ Quantitative ⊖ Provides data only for specific restriction enzyme cutting sites

COBRA: combined bisufite-PCR restriction analysis

Until now only a few studies have analyzed the temporal stability as well as the inter- and intraindividual variability of DNA methylation in spermatozoa. While one study described that DNA methylation in spermatozoa is stable over a short period of time (up to 1 year),[65] others found locus-, cell- and age-dependent differences.[66,67] Concerning inter- and intraindividual variability, Krausz *et al.*[68] recently analyzed multitudinous cytosine positions across the human genome by using high resolution Infinium 450K methylation arrays and demonstrated

that DNA methylation in spermatozoa is highly conserved between normozoospermic men and stable in different subpopulations of the same individual.

In addition, quantitative relevant reference values of DNA methylation in spermatozoa need to be established[69,70] to classify patients' spermatozoal DNA methylation into 'normal' and 'abnormal'. Such a reference should be calculated by studying a large, healthy control group from which the normal range is defined.[71] Recently, our group established a first reference range for normal spermatozoal *MEST* DNA methylation (0%–15%) based on the 95th percentile of *MEST* DNA methylation of 31 highly selected men with normal andrological parameters.[72] In the future, multicenter studies in several countries on different continents need to be performed in order to establish reference values based on different ethnicities and laboratory techniques.

Nevertheless, prior to introduction into clinical diagnostics the clinical benefit of each novel biomarker has to be evaluated. Although the number of potential biomarkers is increasing due to new technologies, only few of them are appropriate for the use in clinics since associations with clinical parameters do not always result in clinical importance. In addition, some markers are not valid for all patients, but only for a specific subgroup which could present a limitation of a clinical marker.[18]

As diagnostic protocols and performance can be quite different in several laboratories and diagnoses can vary widely in both accuracy and precision, standardizations of protocols, diagnostic procedures, the interpretation of results and patients reports are necessary. Furthermore, internal and external quality control procedures should be set up and performed. One example for this standardization and quality control assessment is the diagnostic performance for Y-chromosomal microdeletions (azoospermia factor). For this the European Academy of Andrology and the European Molecular Genetics Quality Network have teamed up together and supported the publication of Laboratory guidelines[73] (http://www.emqn.org/bpguidelines.php). In this context, societies such as Asian Society of Andrology or European Society of Human Reproduction and Embryology could also have a leading role in the establishment of such a quality assessment scheme for DNA methylation analysis in spermatozoa.

PERSPECTIVES

Several studies have already shown that aberrant DNA methylation in spermatozoa is associated with oligozoospermia, abnormal sperm morphology and decreased progressive motility (**Figure 2**); however, the underlying mechanisms and exact nature of associations are still unknown. For example it is unknown whether DNA methylation in spermatozoa is influenced by the origin of infertility which could be due to either central or testicular causes. By the same token it is currently unclear to which extent epimutations affect qualitative and quantitative aspects of spermatogenesis and *vice versa*.

In addition, the knowledge of spermatozoal DNA methylation status could provide important information to subsequent fertilization, pregnancy success and possible outcome. Thus far 'classical' semen parameters only provide little information on the fertility capacity and cannot predict pregnancy rates or outcome, making DNA methylation a potentially invaluable tool in andrological examinations which in turn might yield a novel diagnostic parameter and prognostic factor for the outcome of any ART treatment.

The epigenetic status of spermatozoa could also affect the offspring's health by potential transmission of epimutations. Besides imprinting disorders (as already described above) epigenetic aberrations can have severe consequences on genomic stability and gene expression

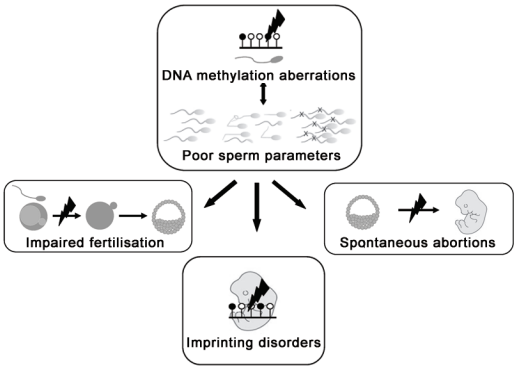

Figure 2: Aberrant imprints and abnormal semen parameters are associated with impaired fertilization, imprinting disorders and spontaneous abortions.

and thereby could cause serious diseases such as lupus, asthma, malignancies and several neurological disorders. For some of these diseases, commercial DNA methylation tests are already available now.[64,74] However, it is still unknown if epimutations of spermatozoa can be transmitted to the offspring and affect its health as there are two main events in which epigenetic patterns are erased and renewed: during gametogenesis and in the early zygote after fertilization. These two epigenetic reprogramming events avoid in principle the transmission of parental DNA methylation aberrations to the offspring; but the reprogramming in the early zygote does not include imprinted genes making the transmission of spermatozoal epimutations to the first offspring (F1-generation) possible.[75]

It is also very clear that more studies are needed to determine to what extent DNA methylation analysis will be useful as diagnostic factor for male (in)fertility and as a prognostic factor for pregnancy. If information on the DNA methylation status could provide further hitherto unknown insights into the fertility capacity of the patient it would significantly improve the diagnostics for couples with longstanding history of male idiopathic infertility.

In the decade of whole genome sequencing, technologies in the field of epigenetics are rapidly developing. It is to be expected that methods for DNA methylation will probably be improved within the upcoming years and a cost-effective analysis for diagnostic procedures will be available soon. On the horizon one might even think about DNA methylation analysis of one single living sperm using techniques such as Raman microscopy,[76,77] which could be used for ART. This would revolutionize the examination and treatment of infertile couples and increase the success of ART as sperm could be systematically selected for its potential to induce pregnancy.

Taken together, the current findings point to the potential of the approach; however, we encourage further investigations of the role of spermatozoal DNA methylation and subsequently fertilization, embryo development and pregnancy outcome to establish a novel prognostic parameter in the andrological workup of infertile men. Novel methods which enable the time- and cost-effective sequencing of the spermatozoal epigenome could make the epigenetic analysis of spermatozoa a standard measurement in andrology. If andrologists, andrological societies and the genetic quality assessment groups would combine their forces and activities we are convinced that this would lead to a new level in the diagnostic setup of male infertility.

AUTHOR CONTRIBUTIONS

RK helped in designing the concept of this article, performed literature research and wrote the manuscript. JG planned this article, designed

the concept of this article, helped in literature research and writing the manuscript and supervised this project. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

ACKNOWLEDGMENTS

Our work was supported by Graduate Program Cell Dynamics and Disease (CEDAD) and the International Max Planck Research School-Molecular Biomedicine (IMPRS-MBM) and by German Research Foundation (Research Unit 'Germ cell potential' (FOR 1041)).

Supplementary information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

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How to cite this article: Kläver R, Gromoll J. Bringing epigenetics into the diagnostics of the andrology laboratory: challenges and perspectives. *Asian J Androl* 30 May 2014. doi: 10.4103/1008-682X.125412. [Epub ahead of print]