

Epigenetic heterogeneity of developmentally important genes in human sperm: Implications for assisted reproduction outcome

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Abbreviations: ART, assisted reproductive technology; DBS, deep bisulfite sequencing; ICSI, intracytoplasmic sperm injection; IMSI, intracytoplasmic morphologically selected sperm injection; LD, limiting dilution; OAT, oligoasthenoteratozoospermia; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism.

The molecular basis of male infertility is poorly understood, the majority of cases remaining unsolved. The association of aberrant sperm DNA methylation patterns and compromised semen parameters suggests that disturbances in male germline epigenetic reprogramming contribute to this problem. So far there are only few data on the epigenetic heterogeneity of sperm within a given sample and how to select the best sperm for successful infertility treatment. Limiting dilution bisulfite sequencing of small pools of sperm from fertile donors did not reveal significant differences in the occurrence of abnormal methylation imprints between sperm with and without morphological abnormalities. Intracytoplasmic morphologically selected sperm injection was not associated with an improved epigenetic quality, compared to standard intracytoplasmic sperm injection. Deep bisulfite sequencing (DBS) of 2 imprinted and 2 pluripotency genes in sperm from men attending a fertility center showed that in both samples with normozoospermia and oligoasthenoteratozoospermia (OAT) the vast majority of sperm alleles was normally (de) methylated and the percentage of epimutations (allele methylation errors) was generally low (<1%). However, DBS allowed one to identify and quantify these rare epimutations with high accuracy. Sperm samples not leading to a pregnancy, in particular in the OAT group, had significantly more epimutations in the paternally methylated *GTL2* gene than samples leading to a live birth. All 13 normozoospermic and 13 OAT samples leading to a child had <1% *GTL2* epimutations, whereas one (7%) of 14 normozoospermic and 7 (50%) of 14 OAT samples without pregnancy displayed 1–14% *GTL2* epimutations.

Introduction

The ever-increasing shift in parental age is associated with a growing number of couples with fertility problems seeking assisted reproductive technologies (ARTs). Although overall 5–10% of all men are infertile, currently known genetic causes account only for a small proportion of them.^{1,2} In male germ cells, the number of *de novo* mutations increases with paternal age and the number of spermatogonial cell divisions.³ Since copying of the epigenetic information (in particular of DNA methylation patterns) during cell division is much more error-prone than replication of the DNA sequence itself,^{4,5} it is plausible that epimutations are also accumulating in aging male germ cells.

A genome-wide demethylation and remethylation wave occurs in the mammalian germline. Around the time when the primordial germ cells reach the genital ridge, the methylation patterns of previous generations are erased. New parent-specific methylation patterns are set during male and female germ-cell differentiation.^{6,7} In the male germline, remethylation is initiated after prenatal mitotic arrest and completed postnatally during pachytene.^{8,9} This implies that in IVF/ICSI sperm all paternal methylation imprints must be established correctly. A second demethylation and remethylation wave occurs after fertilization to restore totipotency in embryonal cells.^{10,11} Only 100–200 imprinted genes (out of >22,000 genes) escape this postzygotic reprogramming and maintain their germline methylation patterns and parent-specific activities.¹²

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Accumulating experimental evidence links epigenetic modifications affecting DNA methylation and histone-protamine configuration in sperm to male infertility.^{13,14} Aberrant sperm DNA methylation patterns, mainly in imprinted genes have been associated with poor semen parameters and male infertility¹⁵⁻¹⁸ as well as with abortions¹⁹ and ART outcome.²⁰ Genome-wide analyses with methylation arrays suggest broad methylation changes in both imprinted and non-imprinted genes in sperm from infertile and older men, but none with large effect size.²¹⁻²⁴ Most candidate gene and genome-wide approaches have measured the average methylation level of genes/loci in samples consisting of millions of sperm. The observed changes in mean methylation, e.g., of an imprinted gene, could be due to an increased rate of single CpG methylation errors at random positions of each or most sperm or to allele methylation errors. Since it is usually the density of methylated CpGs rather than individual CpGs that turns a gene "on" or "off," only alleles with the majority of CpGs being aberrantly (de)methylated (allele methylation errors) are considered as epimutations, whereas single CpG errors are most likely without functional consequences.^{25,26} Plasmid bisulfite sequencing of a limited number of sperm DNA alleles suggests the existence of true epimutations of both paternally (e.g., *H19*) and maternally methylated alleles (e.g., *MEST*) in sperm of infertile men.^{15,27} Here we employed different techniques for in depth methylation analysis of individual DNA molecules in different classes of sperm.

Results

Methylation analysis of single or a few sperm

Sperm for intracytoplasmic sperm injection (ICSI) are usually selected under a standard microscope (magnification ~400×) on the basis of their motility and morphology. Intracytoplasmic morphologically selected sperm injection (IMSI) requires a much higher magnification (~6000×) for better morphological assessment.²⁸ We employed limiting dilution (LD) bisulfite pyrosequencing²⁶ to analyze single-allele methylation of one paternally

methylated (*GTL2*) and 2 maternally methylated (*LIT1* and *PEG3*) imprinted genes in 4 different classes of sperm from fertile donors: IMSI+ are the "best" sperm selected under a high-powered microscope. They display normal morphology and do not contain any vacuoles. IMSI- sperm have large vacuoles. IMSI-/ are sperm with vacuoles and morphological abnormalities. ICSI sperm appear to be normal at lower magnification but still may contain vacuoles. **Table 1** and **Figure 1** present the number of alleles without CpG methylation errors (all analyzed CpGs show the correct sperm methylation imprint), with single CpG methylation errors (one, rarely 2 CpGs are aberrant), and >50% CpG methylation errors (epimutations). All together, we recovered 382 individual alleles in the IMSI+, 120 in the IMSI-, 73 in the IMSI/-, and 168 in the ICSI group. Neither the number of single CpG nor the number of allele methylation errors (>50% aberrant CpGs) differed significantly between groups. It is interesting to note that only 1 of 104 (1%) analyzed *LIT1* alleles (in all 4 groups) and 1 of 303 (0.3%) *PEG3* alleles showed single CpG errors, compared to 62 of 336 (18.5%) *GTL2* alleles. Similarly, none (0%) of the 104 *LIT1* alleles and only 3 of 303 (1%) *PEG3* alleles were classified as epimutations, whereas 13 of 336 (3.9%) *GTL2* alleles were aberrantly demethylated. Single CpG errors (Fisher's exact test; $P < 0.0001$) and epimutations ($P < 0.05$) were significantly more frequent in *GTL2* than in *LIT1* and *PEG3*.

In addition to LD analysis of small pools of morphologically selected sperm, we developed a multiplex assay for methylation analysis of 2 spermatogenesis (*BOLL* and *CATSPER1*) genes, which are unmethylated in sperm, 2 pluripotency (*NANOG* and *OCT4*) genes, which are methylated in sperm, and 4 (paternally methylated *GTL2* and maternally methylated *LIT1*, *PEG3*, and *SNRPN*) imprinted genes. Single sperm were selected under low magnification (ICSI criteria) from samples of 3 fertile men with normal semen parameters and 8 infertile men with OAT syndrome, respectively. **Figure 2** shows the methylation patterns of 5 individual sperm, 2 without and 3 with epimutations. All together, we recovered 94 alleles from 61 sperm of infertile men, 8 (8.5%) of which showed single CpG errors and 11 (12%) of which showed >50% CpG

Table 1. Methylation status of imprinted genes in human sperm

Gene	Allele methylation	Number (percentage) of alleles			
		IMSI+	IMSI-	IMSI/-	ICSI
<i>GTL2</i>	No CpG errors	133 (78.7%)	49 (86.0%)	26 (72.2%)	53 (71.6%)
	Single CpG errors	30 (17.8%)	6 (10.5%)	8 (22.2%)	18 (24.3%)
	>50% CpG errors	6 (3.5%)	2 (3.5%)	2 (5.6%)	3 (4.1%)
<i>LIT1</i>	No CpG errors	54 (100%)	19 (95.0%)	5 (100%)	25 (100%)
	Single CpG errors	0 (0%)	1 (5.0%)	0 (0%)	0 (0%)
	>50% CpG errors	0 (0%)	0 (0%)	0 (0%)	0 (0%)
<i>PEG3</i>	No CpG errors	157 (98.7%)	43 (100%)	31 (96.9%)	68 (98.6%)
	Single CpG errors	0 (0%)	0 (0%)	1 (3.1%)	0 (0%)
	>50% CpG errors	2 (1.3%)	0 (0%)	0 (0%)	1 (1.4%)
All genes	No CpG errors	344 (90%)	111 (92.5%)	62 (84.9%)	146 (86.9%)
	Single CpG errors	30 (7.9%)	7 (5.8%)	9 (12.3%)	18 (10.7%)
	>50% CpG errors	8 (2.1%)	2 (1.7%)	2 (2.8%)	4 (2.4%)

IMSI+ sperm have normal morphology and no vacuoles; IMSI- sperm contain vacuoles; IMSI/- sperm are morphologically abnormal and contain vacuoles; ICSI sperm were selected at lower magnification.

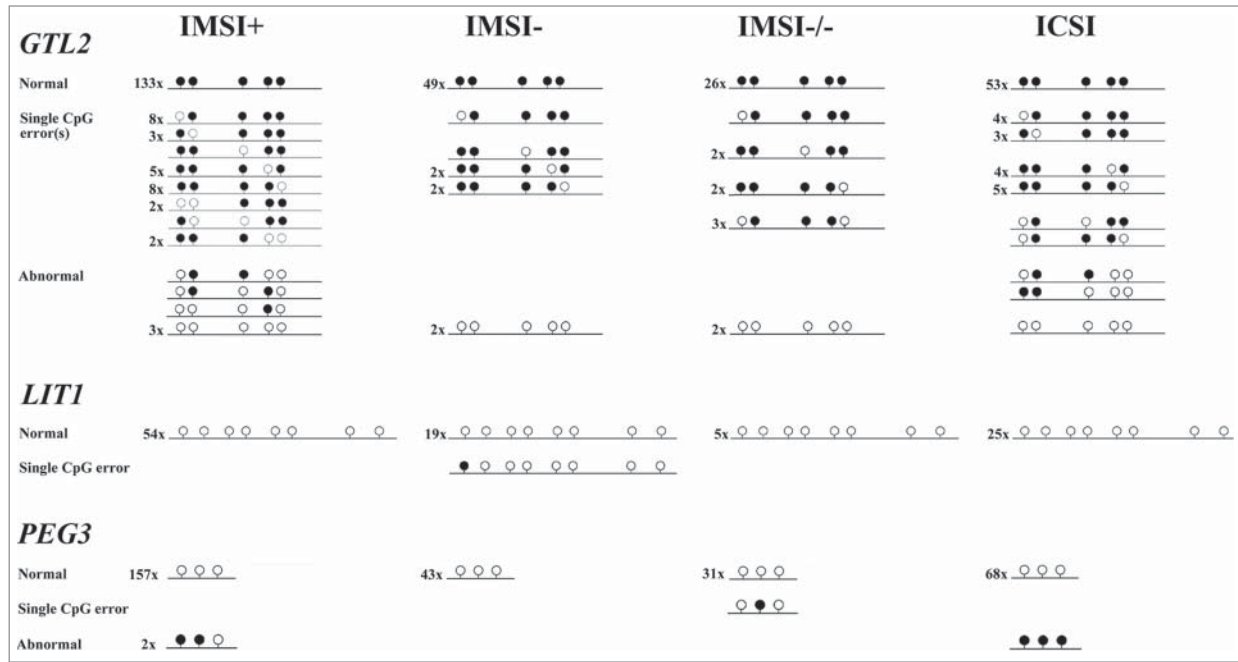


Figure 1. Methylation patterns of 743 individual DNA molecules (336 for the paternally methylated *GTL2*, 104 for the maternally methylated *LIT1*, and 303 for the maternally methylated *PEG3* gene) in different groups of sperm from fertile donors. IMSI+ are the "best" sperm selected at high magnification, IMSI- sperm contain vacuoles, IMSI-/- sperm show abnormal morphology and vacuoles, and ICSI sperm appear to be normal at standard magnification. Each line represents the methylation pattern of an individual DNA molecule. The number at the left-hand side indicates how many independent alleles of this type were recovered. Open circles represent unmethylated and filled circles methylated CpG sites. In normal alleles, all CpGs show the correct sperm methylation imprint. Alleles with single CpG errors contain one or at most 2 aberrant CpG sites. Abnormal alleles have more than 50% aberrantly (de)methylated CpG sites, indicative of an epimutation.

errors (Table 2). Most epimutations (8 of 11) were found in imprinted genes. Eighty alleles were recovered from 40 sperm of fertile men, 2 (2.5%) of which showed single CpG errors and 5 (6%) epimutations, all in imprinted genes (Table 2). Overall, 11 of 61 (18%) OAT sperm and 5 of 35 (14%) of sperm from fertile men displayed epimutations. Although men with OAT syndrome showed a higher rate of epimutations and alleles with single CpG errors, these differences were not statistically significant.

Deep bisulfite sequencing (DBS)

Methylation analysis of small pools of cells or even individual cells is relatively time-consuming and only a limited number of individual DNA molecules (several dozen to several hundred) can be sequenced. Next generation sequencing of bisulfite-treated DNA samples can generate methylation data of several hundred to

several thousand individual alleles each of different target genes and multiple samples in a single experiment. We used DBS²⁹ for methylation analysis of 2 pluripotency (*NANOG* and *OCT4*) and 2 (paternally methylated *GTL2* and maternally methylated *PEG3*) imprinted genes in 54 sperm samples from men

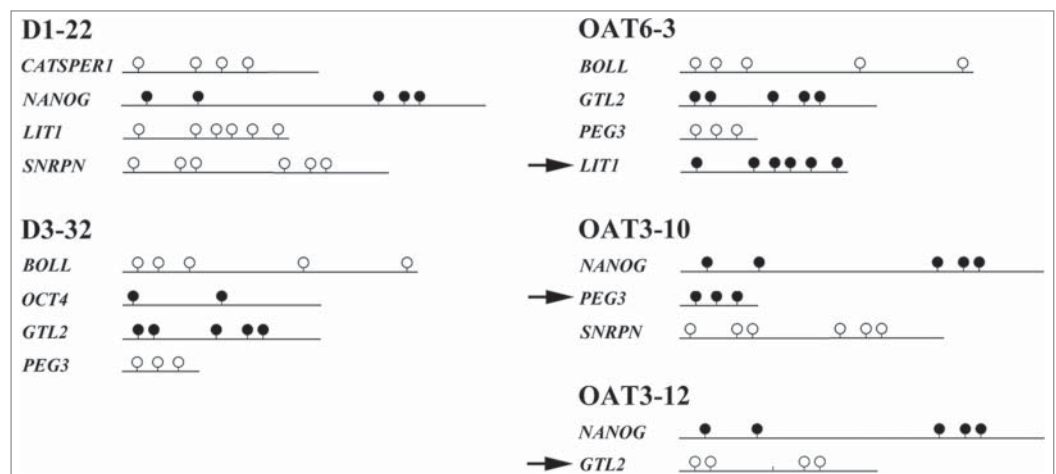


Figure 2. Methylation analysis of single sperm. Alleles recovered from the same sperm are grouped together. Sperm 22 of fertile donor 1 (D1–22) and sperm 32 from donor 3 (D3–32) display normal methylation patterns in all 4 recovered alleles. Sperm 3 of OAT patient 6 (OAT6–3) displays an abnormally methylated *LIT1* allele, sperm 10 and 12 of OAT patient 3 (OAT3–10 and –12) display an abnormally methylated *PEG3* and an abnormally demethylated *GTL2* allele, respectively. Sperm epimutations are indicated by arrows.

Table 2. Methylation analysis of 8 genes in single sperm

	Sperms from infertile men with oligoasthenoteratozoospermia			Sperms from fertile donors with normal semen parameters		
	Allele methylation			Allele methylation		
	No CpG error	Single CpG errors	>50% CpG errors	No CpG error	Single CpG errors	>50% CpG errors
Spermatogenesis genes (2)	13	0	3 (19%)	13	0	0
<i>BOLL</i>	1	0	3	3	0	0
<i>CATSPER1</i>	12	0	0	10	0	0
Pluripotency genes (2)	11	1 (8%)	0	10	0	0
<i>NANOG</i>	7	1	0	5	0	0
<i>OCT4</i>	4	0	0	5	0	0
Imprinted genes (4)	51	7 (11%)	8 (12%)	50	2 (3.5%)	5 (9%)
<i>GTL2</i>	18	6	2	16	2	1
<i>LIT1</i>	10	0	2	10	0	0
<i>PEG3</i>	14	1	3	15	0	4
<i>SNRPN</i>	9	0	1	9	0	0
All genes (8)	75	8 (8.5%)	11 (12%)	73	2 (2.5%)	5 (6%)

(attending a fertility center) with normal semen parameters (N) and OAT syndrome (O). In both groups, approximately half of the samples had led to a live birth after ICSI (N+ and O+) and the other half had not (N- and O-). On average, each sample yielded 1164 (range 101–4455) reads for *GTL2*, 1473 (297–6759) for *PEG3*, 581 (50–1537) for *OCT4*, and 976 (77–2697) for *NANOG* (Table 3). The mean methylation (number of methylated CpGs divided by the total number of CpGs in all analyzed alleles) was $94.6 \pm 2.7\%$ for *GTL2*, $1.5 \pm 0.3\%$ for *PEG3*, $92.3 \pm 0.4\%$ for *OCT4*, and $96.4 \pm 0.2\%$ for *NANOG*. Since DBS analyzes a larger number of CpGs (8 for *GTL2*, 19 for *PEG3*, 5 for *OCT4*, and 8 for *NANOG*) than LD, sperm alleles with no or a single CpG methylation error were considered as normal and alleles with >50% aberrant CpGs as abnormal (epimutations). Mixed alleles contained at least 2 but <50% CpG sites with the wrong (oocyte) methylation imprint.

Across all genes and samples, the majority (>85%) of alleles displayed normal methylation patterns (Table 3). The percentage of mixed alleles differed significantly (ANOVA with Bonferroni post hoc test; $P < 0.001$) between the 4 studied genes, being the highest (6.5%) for *GTL2* and the lowest (1.2%) for *PEG3* (Fig. 3). The percentage of abnormal alleles did not differ significantly between genes. For most DBS measurements (4 genes x 54 samples), the percentage of abnormal alleles was lower than 1%, in one fifth of the cases even 0% (Table 3). In 22 (10%) of the 224 measurements the percentage of abnormal alleles ranged from 1–3%, in 15 (7%) cases from 3–10%, and in 2 (1%) cases was >10%.

After correction for multiple testing, pluripotency genes did not show significant differences (Table 4) between the 4 studied groups (N+, N-, O+, and O-). In contrast, the percentage of abnormally demethylated *GTL2* alleles was significantly ($P < 0.0001$) higher in sperm samples (N- and O-) with negative ART outcome than in those (N+ and O+) leading to a child after ICSI. Box plots show a higher variation in the percentage of *GTL2* epimutations in sperm samples with negative ART outcome, especially in the O- group than in sperm samples leading to live-birth (Fig. 4). Sperm samples from men with OAT

syndrome and negative ART outcome (O-) had significantly more *GTL2* epimutations than all other groups ($P < 0.0001$, compared to both N+ and O+; $P = 0.02$ for N-). To a lesser extent, the percentage of abnormally methylated *PEG3* alleles also appeared to differ between groups (Table 4). Interestingly, we could not find any difference in methylation of the 4 studied genes between normozoospermic and OAT samples leading to a child (N+ versus O+).

In addition, we used binary logistic regression models to test the predictive values of percentages of normal, mixed and/or abnormal alleles of the 4 studied genes for ART outcome (live-birth of a child vs. no pregnancy). For *NANOG*, *OCT4* and *PEG3*, the predictive values were low, ranging from 52% to 58%. A model based on the percentages of abnormal and normal *GTL2* alleles (excluding intermediate alleles as covariate) had the highest predictive value (72%).

A single nucleotide polymorphism (SNP rs71594112) in the *GTL2* amplicon allowed us to distinguish the 2 parental alleles. Of the 54 analyzed sperm samples 34 were homozygous TT, 4 homozygous CC, and 16 heterozygous TC. When using a more stringent filter setting (than for standard methylation analysis of low complexity bisulfite-converted DNA) to avoid SNP-mistyping, 9 of the 16 heterozygous samples still displayed multiple *GTL2* alleles with epimutations. In five of these informative samples (17N-, 18N-, 20N-, 39O+, and 49O-) the *GTL2* epimutations were confined to a single parental allele, whereas in 4 cases (26N-, 40O+, 45O-, and 47O-) both the T and the C allele were affected. Since we did not have parental DNAs, the parental origin of the T and C allele, respectively, in a given sample could not be determined.

Discussion

IMSI has been developed to pick out the "best" sperm of samples from men with compromised semen parameters, using a 10- to 15-times higher magnification than ICSI to assess sperm

Table 3. Number and methylation patterns of sperm alleles analyzed by deep bisulfite sequencing

ID	GTL2 alleles				PEG3 alleles				NANOG alleles				OCT4 alleles			
	No.	% normal	% mixed	% abnormal	No.	% normal	% mixed	% abnormal	No.	% normal	% mixed	% abnormal	No.	% normal	% mixed	% abnormal
Sperm samples from men with normal semen parameters (N) leading to a live-birth (+)																
1N+	1550	90.2	9.4	0.4	1353	98.9	0.8	0.3	683	96.3	2.8	0.9	522	93.7	5.8	0.6
2N+	752	94.2	5.5	0.4	835	98.8	1.2	0.0	584	97.4	2.0	0.3	272	95.6	4.0	0.4
3N+	1458	94.5	5.5	0.0	1049	99.2	0.8	0.0	1027	97.3	2.0	0.7	578	97.2	2.6	0.2
4N+	1560	94.0	6.0	0.0	1628	98.5	1.5	0.1	1217	98.2	1.8	0.0	1027	95.3	4.5	0.2
5N+	776	97.9	1.9	0.1	760	99.0	1.1	0.0	493	97.8	1.4	0.8	196	94.4	3.6	2.0
6N+	541	87.6	12.2	0.2	753	98.9	0.7	0.4	1711	97.3	2.1	0.7	339	95.9	3.8	0.3
7N+	440	92.7	7.1	0.2	646	97.2	1.1	1.7	298	98.7	1.3	0.0	115	94.8	4.4	0.9
8N+*	515	92.8	7.0	0.2	830	99.6	0.1	0.2	1219	96.9	2.6	0.5	444	93.9	5.4	0.7
9N+	941	95.2	4.7	0.1	1559	99.4	0.6	0.0	970	94.5	3.6	1.9	775	96.4	3.5	0.1
10N+*	1314	94.1	5.7	0.2	1269	99.6	0.4	0.0	1343	96.5	3.1	0.4	1139	95.6	4.3	0.1
11N+	508	94.7	4.7	0.6	633	98.6	4.7	0.6	1168	92.1	3.6	4.3	272	86.0	5.9	8.1
12N+	2157	95.3	4.4	0.3	638	98.3	1.1	0.6	1472	98.1	1.4	0.5	464	97.0	3.0	0.0
13N+	1465	94.8	5.2	0.0	3850	99.3	0.7	0.1	649	98.3	1.5	0.2	50	92.0	2.0	6.0
Sperm samples from men with normal semen parameters (N) not leading to a pregnancy (-)																
14N-	2256	93.9	5.8	0.3	4673	99.5	0.5	0.0	2187	95.7	2.8	1.6	686	91.8	7.4	0.7
15N-	2076	92.2	7.6	0.3	3123	99.1	0.7	0.2	2607	94.5	4.7	0.8	189	93.7	5.3	1.1
16N-	731	95.2	4.4	0.6	907	99.9	0.1	0.0	694	97.6	2.2	0.3	310	96.5	2.9	0.7
17N-*	613	92.0	7.3	0.7	569	99.1	0.9	0.0	396	97.5	2.3	0.3	236	94.5	4.7	0.9
18N-*	2524	93.7	5.6	0.7	2049	98.6	0.5	0.9	2109	97.3	2.4	0.7	1573	97.1	2.4	0.5
19N-	1304	92.2	7.0	0.8	1116	98.5	1.1	0.5	911	94.7	3.8	1.4	766	95.8	3.8	0.4
20N-*	2327	94.4	5.5	0.2	1972	99.9	0.1	0.0	1616	96.9	2.8	0.3	1086	95.2	4.4	0.4
21N-	1338	93.7	6.1	0.2	1272	99.6	0.6	0.0	875	94.5	2.7	2.7	633	95.9	4.0	0.2
22N1	762	93.8	6.0	0.1	985	99.6	0.4	0.0	601	95.0	3.7	1.3	534	94.9	4.9	0.2
23N-	538	93.7	4.8	1.5	633	99.4	0.6	0.0	251	97.2	2.8	0.0	325	93.5	5.9	0.6
24N-	1577	93.7	5.8	0.5	2476	99.7	0.2	0.0	1389	94.8	3.67	1.5	774	92.4	6.5	1.2
25N-	972	94.8	5.3	0.0	1509	98.9	1.1	0.0	684	93.3	4.8	1.9	509	96.9	3.0	0.2
26N-*	1464	93.4	5.9	0.7	2029	98.6	1.3	0.2	1165	97.8	2.2	0.1	777	98.5	1.4	0.1
27N-*	789	94.0	5.5	0.4	1033	98.8	1.2	0.0	819	97.0	1.8	1.2	541	95.8	3.9	0.4
Sperm samples from men with oligoasthenoteratozoospermia (O) leading to a live-birth (+)																
28O+	2171	96.0	3.9	0.0	4095	97.8	1.2	1.0	1681	97.2	2.5	0.3	883	96.8	3.2	0.0
29O+	519	90.8	8.9	0.4	612	99.5	0.5	0.0	599	92.5	6.7	0.9	186	93.6	6.5	0.0
30O+	750	90.3	9.6	0.1	893	98-8	1.2	0.0	705	98.4	1.6	0.0	133	94.7	5.3	0.0
31O+	1503	92.8	6.9	0.4	1285	99.1	0.9	0.0	761	97.6	1.2	1.2	742	97.2	2.6	0.3
32O+	604	93.9	6.0	0.2	987	99.2	0.5	0.3	734	98.1	1.9	0.0	309	95.8	4.9	0.3
33O+	1263	96.0	4.0	0.1	1212	98.5	1.4	0.1	1008	98.1	1.4	0.5	638	95.8	4.1	0.2
34O+	4455	94.0	5.7	0.3	806	98.3	1.7	0.0	620	96.0	0.8	3.2	696	99.0	0.9	0.1
35O+	963	93.5	6.4	0.1	511	99.0	0.8	0.2	1023	92.8	3.4	3.8	215	91.2	7.9	0.9
36O+	2032	96.3	3.5	0.2	793	99.8	0.3	0.0	1119	91.7	3.8	4.5	231	94.8	3.9	1.3
37O+*	1246	94.0	5.7	0.3	1775	99.4	0.5	0.1	1027	97.8	2.0	0.3	1045	95.9	3.9	0.2
38O+*	513	90.7	9.4	0.0	1103	99.3	0.7	0.0	1487	95.2	2.6	2.2	431	86.5	9.5	3.9
39O+*	920	95.8	3.8	0.4	1354	99.5	0.4	0.2	705	91.2	4.8	4.0	678	96.9	2.7	0.4
40O+*	1125	87.0	12.3	0.7	1856	99.5	0.4	0.1	1542	97.3	2.3	0.3	1161	95.7	4.1	0.3

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Table 3. Number and methylation patterns of sperm alleles analyzed by deep bisulfite sequencing (Continued)

ID	GTL2 alleles			PEG3 alleles			NANOG alleles			OCT4 alleles						
	No.	% normal	% mixed	% abnormal	No.	% normal	% mixed	% abnormal	No.	% normal	% mixed	% abnormal	No.	% normal	% mixed	% abnormal
Sperm samples from men with oligoasthenoteratozoospermia (O) not leading to a pregnancy (-)																
410-	2351	86.4	9.2	4.5	4634	88.6	7.6	3.9	2095	95.2	3.9	0.9	1102	89.6	9.2	1.3
420-	1761	92.0	7.0	1.0	6959	97.4	1.1	1.5	1404	91.0	3.9	5.1	668	85.2	8.4	6.4
430-	949	96.8	3.2	0.0	937	99.8	0.2	0.0	775	98.1	1.9	0.0	262	96.6	3.4	0.0
440-	145	97.9	1.4	0.7	406	99.3	0.7	0.0	135	96.3	3.7	0.0	n.d.	n.d.	n.d.	n.d.
450-*	961	91.6	7.8	0.6	1273	97.8	1.4	0.8	886	96.3	2.4	1.4	680	95.2	3.6	1.2
460-	1031	93.4	5.3	1.3	1066	97.2	2.0	0.8	1010	95.8	2.4	1.8	523	95.1	4.3	0.6
470-*	1068	94.9	4.6	0.6	1078	98.8	1.2	0.0	904	97.6	0.9	1.6	865	87.1	10.7	2.2
480-	101	88.1	8.9	3.0	297	86.5	1.4	12.1	160	92.5	6.3	1.3	n.d.	n.d.	n.d.	n.d.
490-*	559	91.1	8.4	0.5	942	97.8	1.6	0.6	1278	98.1	1.9	0.0	270	94.8	4.4	0.7
500-	194	78.9	7.2	13.9	786	85.4	7.8	6.9	77	96.1	2.6	1.3	n.d.	n.d.	n.d.	n.d.
510-*	884	88.0	11.4	0.6	1300	99.9	0.2	0.0	724	90.9	6.1	3.0	890	82.4	9.2	8.4
520-	704	87.5	11.5	1.0	1220	94.0	5.7	0.3	3421	98.4	1.6	0.0	856	86.8	11.7	1.5
530-	932	95.4	4.1	0.5	1110	98.8	1.1	0.1	606	94.7	4.8	0.5	461	95.0	4.3	0.7
540-*	245	86.9	11.4	1.6	305	92.5	1.3	6.2	144	88.9	5.6	5.6	n.d.	n.d.	n.d.	n.d.

Normal alleles do not contain CpG errors or, in rare cases, a single CpG error. Mixed alleles contain at least 2 CpG errors, but the majority (> 50%) of CpGs shows the correct methylation imprint. In abnormal alleles, the majority of CpGs shows an aberrant methylation imprint. Samples indicated by a * are heterozygous for GTL2 SNP rs7159412.

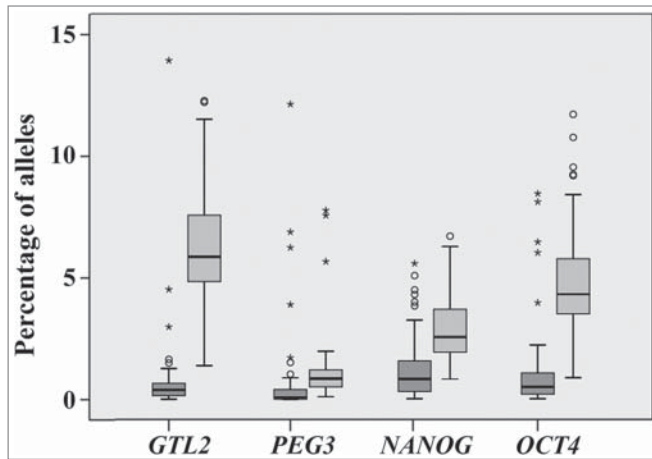


Figure 3. Box plots showing the percentages of alleles with abnormal (dark gray) and mixed (light gray) methylation patterns for *GTL2*, *PEG3*, *NANOG*, and *OCT4* in 54 sperm samples. The median is represented by a horizontal line. The bottom of the box indicates the 25th percentile, the top the 75th percentile. Outliers are shown as circles and extreme outliers as stars.

morphology. Vacuoles can be present in sperm of fertile and infertile men. So far, there is no conclusive evidence whether or not these vacuoles are associated with functional abnormalities such as DNA damage, chromatin decondensation, immaturity, and/or fertilization problems.³⁰⁻³² Some studies reported higher pregnancy rates and lower abortion rates using IMSI.^{28,33-35} However, a recent meta-analysis did not find evidence for an improved ART outcome using IMSI, compared to standard ICSI.³⁶ This is consistent with our observation that the epimutation rate (of 3 imprinted genes) in the "best" sperm (IMSI+), sperm with vacuoles (IMSI-) and morphological abnormalities (IMSI-/-) is comparable to that of ICSI sperm. At the single sperm level the presence of vacuoles or morphological abnormalities did not correlate with epigenetic abnormalities. Low levels of

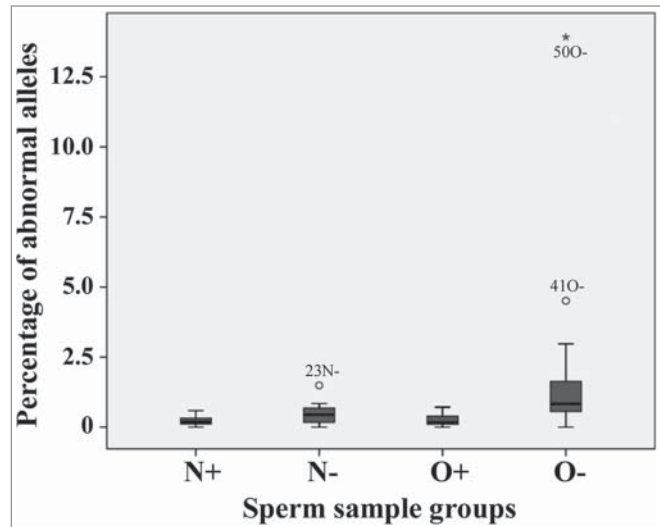


Figure 4. Box plots showing the percentages of abnormal *GTL2* alleles (>50% unmethylated CpGs) in sperm samples of men with normal semen parameters with (N+) and without child (N-), respectively, and men with OAT syndrome with (O+) and without child (O-), respectively. The median is represented by a horizontal line. The bottom of the box indicates the 25th percentile, the top the 75th percentile. Outliers are shown as circles and extreme outliers as stars.

epimutations (2–4%) were found in all 4 classes of sperm tested. In addition to LD bisulfite sequencing which studies methylation of individual DNA molecules from small pools of sperm, we have developed a multiplex PCR assay for methylation analysis of up to 8 genes in single sperm. When we compared individual sperm from men with OAT and fertile controls, we found a higher rate of epimutations in the OAT sperm, but the majority of sperm in both groups showed normal methylation patterns for the studied genes.

Because both LD and single sperm methylation analysis are time-consuming and expensive, only a limited number of

Table 4. Between-group differences in the percentage of normal, mixed and abnormal alleles

Allele methylation	Genes	Four-group comparison	Pairwise comparisons						(N+ and N-) vs (O+ and O-)	(N+ and O+) vs (N- and O-)
			N+ vs N-	N+ vs O+	N+ vs O-	N- vs O+	N- vs O-	O+ vs O-		
Normal	<i>GTL2</i>	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	<i>PEG3</i>	0.01	n.s.	n.s.	0.03	n.s.	0.004	0.02	n.s.	n.s.
	<i>NANOG</i>	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	<i>OCT4</i>	n.s.	n.s.	n.s.	n.s.	n.s.	0.04	n.s.	n.s.	n.s.
Mixed	<i>GTL2</i>	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	<i>PEG3</i>	0.02	n.s.	n.s.	0.02	n.s.	0.002	0.04	0.05	n.s.
	<i>NANOG</i>	n.s.	0.02	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.02
	<i>OCT4</i>	n.s.	n.s.	n.s.	0.05	n.s.	n.s.	n.s.	n.s.	n.s.
Abnormal	<i>GTL2</i>	<0.0001*	0.03	n.s.	<0.0001*	n.s.	0.02	<0.0001*	n.s.	<0.0001*
	<i>PEG3</i>	0.05	n.s.	n.s.	n.s.	n.s.	0.02	0.05	n.s.	n.s.
	<i>NANOG</i>	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	<i>OCT4</i>	0.05	n.s.	n.s.	n.s.	n.s.	0.01	0.02	n.s.	n.s.

N+ and N- represent sperm samples from men with normal semen parameters, with and without child, respectively;

O+ and O- samples from men with OAT syndrome, with and without child, respectively.

n.s. = not significant; * indicates *P* values (Mann-Whitney test) which remain significant after correction for multiple testing.

samples and/or alleles can be analyzed, making it difficult to reach statistical significance. In contrast, DBS can generate methylation information on a large number of individual DNA molecules (from different target genes and different samples) in a single experiment. Unlike bisulfite pyrosequencing, which determines the average methylation level of a large number of DNA molecules at single CpG resolution,²⁶ DBS can distinguish between normally and abnormally methylated sperm alleles and, thus, detect rare epimutations in a much larger pool of normal alleles ("needles in a haystack"). In this context, it is important to emphasize that for imprinted genes the maternal alleles from somatic cells would mimic epimutations in sperm. Thus, even a very minor contamination of a given sperm sample with somatic cells interferes with correct data interpretation. For the quantification of sperm epimutations, meticulous care has to be taken to avoid contamination with somatic DNA, i.e., from lymphocytes and epithelial cells in the ejaculate. To this end, the swim-up sperm fraction was purified further by density gradient centrifugation and the resulting sperm purity was then checked by inverted light microscopy.

In all (normozoospermic and OAT) samples the vast majority of alleles of all 4 studied genes showed normal methylation patterns and in most (>80%) cases the percentage of epimutations was very low (<1%). Interestingly, the percentage of mixed alleles with multiple single CpG methylation errors differed significantly between genes (1.2% for *PEG3*, 2.9% for *NANOG*, 4.8% for *OCT4*, and 6.5% for *GTL2*). Since the same bisulfite-converted sperm DNAs were used for all measurements, this is unlikely due to a technical artifact, i.e., different rates of bisulfite conversion errors. Moreover, LD bisulfite sequencing also showed a significantly increased number of CpG methylation errors in *GTL2*, compared to *LIT1* and *PEG3*. Thus, some genes may be more prone than others to stochastic methylation errors in sperm, e.g., by less stringent maintenance of the methylation imprints established in the male germline.

When looking for possible epigenetic signatures for sperm quality, the 2 pluripotency genes did not correlate with semen parameters (normozoospermia versus OAT) or ART outcome (live-birth vs. no pregnancy). In contrast, the percentage of *GTL2* epimutations was significantly lower in sperm samples producing a child than in those that did not. The association of *GTL2* epimutations with negative ART outcome was highly significant ($P < 0.0001$) for OAT sperm samples (O+ versus O-); a similar trend ($P = 0.03$) was observed for normozoospermic samples (N+ vs. N-). *GTL2* is the only studied gene with a paternal methylation imprint that must be maintained after fertilization. *PEG3* is demethylated on the paternal allele in both sperm and embryo, whereas the methylated *NANOG* and *OCT4* sperm alleles become actively demethylated after fertilization.^{10,11} It is tempting to speculate that transmission of correct paternal methylation imprints into the embryo are more crucial for further development and, therefore, a better predictor of sperm quality and ART success than genes that are either demethylated in sperm or become demethylated after fertilization. However, we need information on more sperm genes to test this hypothesis.

In most analyzed samples, sperm epimutations were found at low frequency in all 4 studied genes. The epimutations in different genes most likely arose in different precursor cells, resulting in extensive epigenetic mosaicism in the germline of a given male. There is no evidence that epimutations in different genes accumulate in the same germ cell and its clonal descendants. First, multiplex methylation analysis of single sperm (Fig. 2) usually revealed only a single epimutated allele and normal methylation patterns in the additional recovered alleles. Second, the percentage of epimutations determined by DBS usually varied between the 4 studied genes in a given sperm sample. For example, sample 13 (N+) showed 0% *GTL2*, 0.1% *PEG3*, 0.2% *NANOG*, and 6% *OCT4* allele methylation errors; sample 48 (O-) 3% *GTL2*, 12% *PEG3*, and 1.3% *NANOG* epimutations. Our results are consistent with the view that in most cases of male infertility with an epigenetic component there may be only a relatively modest increase in the number of epimutations at the individual gene level, but if multiple genes are affected, this may result in a large proportion of sperm with compromised epigenome. A conceptually related DBS study³⁷ demonstrated much higher epimutation rates in 7 OAT sperm samples, affecting 4 imprinted genes more or less equally. This may be explained by technical differences or, more likely, by preselection of sperm samples with known gross epigenetic abnormalities. The 27 OAT samples in our study were only selected on the basis of semen parameters and ART outcome and, therefore may be more representative of the "average" infertile man attending a fertility center.

In approximately half of the sperm samples that were heterozygous for a *GTL2* SNP, epimutations were confined to one of the 2 parental alleles, consistent with a single hit in a precursor cell. In the other half, both alleles were affected, suggesting (at least) 2 hits, one in the paternal and one in the maternal allele in different precursor cells. Sperm epimutations can result from incomplete erasure of the parental methylation patterns in primordial germ cells, failures in the establishment of paternal methylation imprints after erasure (from prenatal mitotic arrest to postnatal pachytene stage), or failures in the maintenance of established methylation patterns (from pachytene to mature sperm). At least for the paternally methylated *GTL2* gene, epimutations (abnormal demethylation) can affect both parental alleles.

Overall, DBS is a high-throughput technique, which allows one to identify and precisely quantify the number of epimutations which amount to just a few percent (in most cases <1%) of all DNA target molecules in a given sperm sample. Our results show that in both normozoospermic and OAT samples the vast majority of alleles display normal methylation patterns (at least for the 4 studied genes). For our best marker gene of ART outcome, *GTL2*, the average percentage of abnormally demethylated alleles was 0.2% in normozoospermic (N+) and 0.3% in OAT (O+) samples leading to a child, compared to 0.5% in normozoospermic (N-) and 1.8% in OAT (O-) samples not leading to a pregnancy. Although these between-group differences are highly significant ($P < 0.0001$), most conventional techniques for methylation analysis, including bisulfite plasmid sequencing,

LD analysis of small sperm pools, and bisulfite pyrosequencing of sperm DNA, are unable to detect such small increments in epimutations in individual genes. Massive parallel sequencing of thousands of individual DNA target molecules in a biological sample can overcome this problem. From a clinical point of view, it is helpful to define thresholds for the prediction of ART outcome. When looking at our data set (Table 3), an empirical threshold of 1% *GTL2* epimutations seems to be practicable. None (0%) of the 26 samples (N+ and O+) leading to a child, but 8 (29%) of 28 samples (N- and O-) with negative ART outcome had $\geq 1\%$ *GTL2* epimutations. Combined with classical semen parameters, the predictive power is even better: 7 (50%) of 14 OAT samples not leading to a child (O-) displayed an increased *GTL2* epimutation rate.

Materials and Methods

Sperm samples

The study was approved by the Ethics Committee at the Medical Faculty of Wuerzburg University and informed consent was obtained from all participating subjects. Sperm samples for DBS were collected from 54 couples undergoing infertility treatment at the Fertility Center Wiesbaden and pseudonymized before methylation analysis. Twenty-seven samples were from men with repeatedly normal semen parameters and 27 from men with OAT syndrome. Following ICSI, 13 samples of each group led to a live birth and 14 did not lead to a pregnancy. Semen parameters were measured according to the WHO guidelines.³⁸ OAT was assumed in males with sperm concentration fewer than 15×10^6 /ml (oligozoospermia), fewer than 32% spermatozoa with progressive motility (asthenozoospermia), and fewer than 4% spermatozoa with normal morphology (teratozoospermia).

The left-over swim-up sperm fraction (excess material) was frozen at -80°C until further use. After thawing, sperm cells were purified by PureSperm 40/80 (Nidacon, Mölndal, Sweden) gradient centrifugation. Purified sperm were incubated for 2 h at 56°C (on a thermomixer) with 100 mM TrisCl, 10 mM EDTA, 500 mM NaCl, 1% SDS, 2% β -mercaptoethanol, and 100 μl proteinase K (>600 mAU/ml). Additional 20 μl proteinase K were added and incubated for another 2 h at 56°C . DNA was isolated with the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) following the recommendations of the manufacturer. DNA concentration was measured using a NanoDrop 2000c spectrophotometer (PepLab, Erlangen, Germany). Bisulfite conversion of sperm DNA was performed using the EZ DNA Methylation kit (Zymo Research Corporation, Irvine, CA, USA).

For LD bisulfite pyrosequencing,²⁶ pools of 10 sperm each (in 10 μl PBS) were selected from sperm samples of 3 healthy donors with normal semen parameters and proven fertility. A high-powered microscope (magnification $6000\times$) for IMSI was used to pick out sperm based on morphological criteria. IMSI+ sperm were motile, had normal morphology and did not contain vacuoles,³⁹ IMSI- sperm had large vacuoles, and IMSI-/- showed morphological (head and/or neck) abnormalities as well as vacuoles. ICSI sperm were picked out at lower magnification

($400\times$) according to standard criteria (normal motility and morphology but small inclusions in sperm head cannot be excluded). For single sperm analysis, 80 swim-up sperm from 3 fertile donors with normal semen parameters and 144 sperm from 8 infertile men with OAT syndrome were collected in 224 individual tubes.

Deep bisulfite sequencing

The PyroMark Assay Design 2.0 software (Biotage, Uppsala, Sweden) was used for primer design (Supplemental Table 1). The *GTL2* target sequence contained a T/C-SNP (rs71594112; chr.14:100,809,346 bp) with a minor allele frequency of 0.13. Cis-regulatory regions of 2 pluripotency (*NANOG* and *OCT4*) genes, one paternally methylated (*GTL2*) and one maternally methylated (*PEG3*) imprinted gene were amplified in a first round polymerase chain reaction (PCR) from bisulfite-treated sperm DNA. The 25 μl reaction mixture consisted of 2.5 μl $10\times$ PCR buffer with MgCl_2 (Roche Diagnostics, Mannheim, Germany), 0.5 μl (10 mM dNTPs) PCR Grade Nucleotide Mix (Roche Diagnostics), 0.2 μl (5 U/ μl) FastStart Taq DNA Polymerase (Roche Diagnostics), 1 μl (10 pmol/ μl) of forward and reverse primer (Metabion, München-Planegg, Germany) as well as 1 μl bisulfite-converted sperm DNA. For *GTL2*, 2.5 μl $10\times$ PCR buffer without MgCl_2 and 4 μl MgCl_2 (25 mM) were used. PCR was carried out with an initial denaturation step at 95°C for 5 min, 40 (for *NANOG* and *PEG3*) and 50 cycles (*OCT4* and *GTL2*), respectively, of 95°C for 30 s (denaturation), primer-specific annealing temperature (56°C for *NANOG*, 60°C for *GTL2* and *PEG3*, and 62°C for *OCT4*) for 30 s, and 72°C for 45 s (elongation), and a final extension step at 72°C for 10 min. Sample-specific multiplex identifiers (MIDs), 454 Titanium A and B sequences, and key (TCAG) sequences were added in a second round PCR. After initial denaturation at 95°C for 10 min, 45 cycles of 95°C for 20 s, and 72°C for 45 s (annealing and elongation) with a final elongation step at 72°C for 7 min were performed.

Prior to DBS second-round amplification products were purified using Agencourt AMPure XP Beads (Beckman Coulter, Krefeld, Germany) and quantified using the NanoDrop 2000c spectrophotometer. After pooling, dilution and emulsion PCR (emPCR), amplification products were sequenced on a Roche/454 GS Junior system, following the protocol described in the Roche emPCR Amplification Method and Sequencing Method Manual. Sequencing runs were processed using a special filter setting (bisRecommendation filter) for low complexity bisulfite-converted DNA that is depleted of C (sense) or G (antisense strand) nucleotides. Briefly, the default "filterOnlyAmplicons" that is applied when using the "full processing for amplicons" option in the Roche Genome Sequencer application was modified to increase the yield of reads from GC-poor bisulfite-treated DNA.²⁹ Sequence reads were then analyzed further using the Amplifyzer program.⁴⁰ For SNP-typing the more stringent filter setting of the Roche Genome Sequencer application was used (instead of the bisRecommendation filter).

LD bisulfite pyrosequencing

DNA was extracted from pools of 10 sperm each and bisulfite-treated with the EZ DNA methylation direct kit (Zymo Research Corporation), which is particularly suited for small amounts of DNA. Bisulfite-treated DNA was eluted in 10 μ l of elution buffer, diluted to a final volume of 200 μ l and evenly distributed into 20 wells of a 96-well plate. Additionally, 4 negative controls were added for each PCR reaction. Multiplex (*GTL2*, *LIT1* and *PEG3*) PCR was performed in 25 μ l reactions, each well containing 2.5 μ l 10 \times PCR buffer with MgCl₂, 0.5 μ l (10 mM dNTPs) PCR Grade Nucleotide Mix, 0.2 μ l (5 U/ μ l) FastStart Taq DNA Polymerase, and 0.33 μ l (33 μ M) forward and reverse outer primers (Metabion) (Supplemental Table 1). PCR was carried out with an initial denaturation step at 95°C for 5 min, 35 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 45 s, and a final extension step at 72°C for 5 min. Nested singleplex PCRs for each of the studied genes were performed using 1 μ l of the first-round multiplex PCR product as a template. The 25 μ l reaction volume consisted of 2.5 μ l 10 \times PCR buffer with MgCl₂, 0.5 μ l PCR Grade Nucleotide Mix, 0.2 μ l FastStart Taq DNA Polymerase, and 1 μ l (10 pmol/ μ l) forward and reverse inner primers. Cycling conditions were as follows: 95°C for 5 min, 32 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 45 s, and a final extension of 7 min at 72°C. For *LIT1*, 40 cycles with an annealing temperature of 54°C were performed. The nested PCRs (4 μ l each) were run on 2% agarose gels to visualize reactions yielding a product representing a single DNA molecule (allele) in the starting sample. Because bisulfite-treated DNA is heavily degraded, the number of wells containing an amplifiable DNA template was always markedly lower than the number of DNA molecules in the starting sample.

Pyrosequencing was performed on a Pyromark Q96MD system with the PyroGold SQA reagent kit (Qiagen). The Pyromark Q-CpG software was used to quantify methylation of individual CpGs. Sequestration of individual DNA molecules from the starting sample in separate PCR reactions reduces the methylation status of the targeted sequence to a binary state: an analyzed CpG site is either methylated (100%) or not (0%). However, because the actual measurements also depend on the sequence context and other factors, methylation values of <20% are typical for unmethylated and >80% for methylated sites. Lollipop diagrams were generated using BiQ Analyzer.⁴¹

Single sperm analysis

Following bisulfite treatment with the EZ DNA methylation direct kit, the DNA of a single sperm was eluted in 10 μ l. Multiplex (*BOLL*, *CATSPER1*, *OCT4*, *NANOG*, *GTL2*, *LIT1*, *PEG3*, and *SNRPN*) PCR of single sperm DNA was performed in 25 μ l reactions containing 2.5 μ l 10 \times PCR buffer without MgCl₂, 4 μ l (25 mM) MgCl₂, 0.5 μ l (10 mM dNTPs) PCR Grade Nucleotide Mix, 0.2 μ l (5 U/ μ l) FastStart Taq DNA Polymerase, and 0.33 μ l (33 μ M) of each forward and reverse outer

primer (Supplemental Table 1). PCR was carried out with an initial denaturation at 95°C for 5 min, 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 45 s, and a final extension step at 72°C for 5 min. Nested PCRs were generally performed using 2.5 μ l 10 \times PCR buffer with MgCl₂, 0.5 μ l PCR Grade Nucleotide Mix, 0.2 μ l FastStart Taq DNA Polymerase, 1 μ l (10 pmol/ μ l) forward and reverse inner primer, and 1 μ l multiplex PCR product. For *BOLL*, 2.5 μ l 10 \times PCR Rxn Buffer without MgCl₂ (Invitrogen, Darmstadt, Germany), 0.75 μ l (50 mM) MgCl₂, 0.5 μ l PCR Grade Nucleotide Mix, and 0.2 μ l (5 U/ μ l) Platinum Taq DNA Polymerase (Invitrogen) were used. Cycling conditions were initial denaturation at 95°C for 5 min, 32 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 5 min. For *BOLL*, 45 cycles and an annealing temperature of 58°C; for *CATSPER1*, 40 cycles and 60°C; for *LIT1*, 35 cycles and 57°C were used.

Statistical analyses

All descriptive and inferential statistical analyses were performed with IBM SPSS version 22.0.0.0 (<http://www.spss.com>). Depending on data distribution, the Mann-Whitney test or ANOVA with Bonferroni post hoc test was applied for group comparisons. *P* values were adjusted for multiple testing by the Bonferroni method. *P* < 0.05 was considered as significant. To estimate the predictive value of DBS data (Table 3), a binary logistic regression analysis was performed using "live-birth" versus "no pregnancy" as outcome variable and the percentages of normal, mixed and abnormal alleles of the 4 studied genes as covariates.

Outlook

A predictive test based on *GTL2* alone (using a 1% threshold) has a high specificity (no false positives in this pilot study) but relatively low sensitivity (50% for the OAT and 30% for all samples). This may still be inadequate for a stand-alone test in a clinical setting. However, it is feasible to identify additional epigenetic signatures for ART outcome, e.g., by genome-wide methylation analyses or to combine classical and epigenetic semen parameters, improving sensitivity. Considering that multiple sperm samples and marker genes can be analyzed in a single next generation sequencing run, epigenetic signatures can provide an additional layer of information for assessing sperm quality before infertility treatment. With an improved epigenetic marker panel, DBS may become a powerful tool for differential diagnosis of male infertility and the prediction of ART outcome.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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