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Complete List of Authors:	Urdinguio, Rocio; Institute of Oncology of Asturias (IUOPA-HUCA), Epigenetics Bayon, Gustavo; Institute of Oncology of Asturias (IUOPA-HUCA), Epigenetics Dmitrijeva, Marija; Institute of Oncology of Asturias (IUOPA-HUCA), Epigenetics Toraño, Estela; Institute of Oncology of Asturias (IUOPA-HUCA), Epigenetics Bravo, Cristina; Institute of Oncology of Asturias (IUOPA-HUCA), Epigenetics Fraga, Mario; Institute of Oncology of Asturias (IUOPA-HUCA), Epigenetics; National Center for Biotechnology, CNB-CSIC, Immunology and Oncology Bassas, Lluís; Service-Fundació Puigvert, Laboratory of Seminology and Embryology, Andrology Larriba, Sara; IDIBELL, CGMM Fernandez, Agustin; Institute of Oncology of Asturias (IUOPA-HUCA), Epigenetics
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Aberrant DNA methylation patterns of spermatozoa in men with unexplained infertility

Short title: Aberrant epigenetic patterns in male infertility

Rocío G. Urdinguio[1†], Gustavo F. Bayón[1†], Marija Dmitrijeva[1], Estela G. Toraño[1], Cristina Bravo[1], Mario F. Fraga[1,2], Lluís Bassas[3], Sara Larriba[4*] and Agustín F. Fernández[1*].

[1]Cancer Epigenetics Laboratory, Institute of Oncology of Asturias (IUOPA), HUCA, Universidad de Oviedo, 33006, Oviedo, Spain.
[2]Department of Immunology and Oncology, National Center for Biotechnology, CNB-CSIC, Cantoblanco, 28049 Madrid, Spain.
[3]Laboratory of Seminology and Embryology, Andrology Service-Fundació Puigvert, 08025 Barcelona, Spain
[4]Human Molecular Genetics Group-IDIBELL, 08908 L'Hospitalet de Llobregat, Barcelona, Spain

[†]These authors contributed equally to this work

*Correspondence to:
Agustin F. Fernandez: affernandez@hca.es; Sara Larriba: slarriba@idibell.cat

Abstract

Study question:

Are there DNA methylation alterations in sperm that could explain the reduced biological fertility of male partners from couples with unexplained infertility?

Summary answer: DNA methylation patterns, not only at specific loci but also at Alu Yb8 repetitive sequences, are altered in infertile individuals compared to fertile controls.

What is known already: Aberrant DNA methylation of sperm has been associated with human male infertility in patients demonstrating either deficiencies in the process of spermatogenesis or low semen quality.

Study design, size, duration: Case and control prospective study. This study compares 46 sperm samples obtained from 17 normospermic fertile men and 29 normospermic infertile patients.

Participants/materials, setting, methods: Illumina Infinium HD Human Methylation 450K arrays were used to identify genomic regions showing differences in sperm DNA methylation patterns between 5 fertile and 7 infertile individuals. Additionally, global DNA methylation of sperm was measured using the Methylamp Global DNA Methylation Quantification Ultra kit (Epigentek) in 14 samples, and DNA methylation at several repetitive sequences (LINE-1, Alu Yb8, NBL2, D4Z4) measured by bisulfite pyrosequencing in 44 sperm samples. A sperm-specific DNA methylation pattern was obtained by comparing the sperm methylomes with the DNA methylomes of differentiated somatic cells using data obtained from methylation arrays (Illumina 450K) of blood, neural and glial cells deposited in public databases.

Main results and the role of chance: In this study we conduct, for the first time, a genome-wide study to identify alterations of sperm DNA methylation in individuals with unexplained infertility that may account for the differences in their biological fertility compared to fertile individuals. We have identified 2,752 CpGs showing aberrant DNA methylation patterns, and more importantly, these differentially methylated CpGs were significantly associated with CpG sites which are specifically methylated in sperm when compared to somatic cells. We also found statistically significant ($p<0.001$) associations between DNA hypomethylation and regions corresponding to those which, in somatic cells, are enriched in the repressive histone mark H3K9me3, and between DNA hypermethylation and regions enriched in H3K4me1 and CTCF, suggesting that the relationship between chromatin context and aberrant DNA methylation of sperm in infertile men could be locus-dependent. Finally, we also show that DNA methylation patterns, not only at specific loci but also at several repetitive sequences (LINE-1, Alu

Yb8, NBL2, D4Z4), were lower in sperm than in somatic cells. Interestingly, sperm samples at Alu Yb8 repetitive sequences of infertile patients showed significantly lower DNA methylation levels than controls.

Limitations, reasons for caution: Our results are descriptive and further studies would be needed to elucidate the functional effects of aberrant DNA methylation on male fertility.

Wider implications of the findings: Overall, our data suggest that aberrant sperm DNA methylation might contribute to fertility impairment in couples with unexplained infertility and they provide a promising basis for future research.

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Key words: male infertility / DNA methylation / sperm / tissue-specific DNA methylation / repetitive sequences

Introduction

Human infertility is a disorder affecting 13-15% of couples worldwide, where in 20% of cases the male factor is fully responsible and in another 30%-40% it is contributory (Jarow, et al., 2002). In the majority of cases, male factor infertility is closely related to decreased semen quality as a consequence of urogenital abnormalities including testicular dysfunction (a large proportion being caused by genetic abnormalities such as karyotype anomalies and Y chromosome microdeletions), varicocele, infections of the genital tract, immunological problems and/or exposure to exogenous chemical or physical agents. However, in some cases the clinical evaluation of the individual is normal, thus the patient is diagnosed with infertility of unknown origin (Hamada, et al., 2011, Irvine, 1998).

Epigenetics involves the study of heritable changes affecting gene expression that are not caused by any change in DNA sequence (Holliday, 1987). The best-known epigenetic mark is DNA methylation (Esteller, 2008, Fernandez, et al., 2012), a dynamic process that takes place throughout the course of development in multicellular organisms and ensures the maintenance of normal expression patterns. Likewise, DNA methylation alterations related to different human pathologies, developmental processes and aging have been found (Fernandez, et al., 2012, Urdinguio, et al., 2009).

In particular, DNA methylation of germ cells is critically involved in many processes, including paternal genomic imprinting (Feinberg, et al., 2002), the gene-dosage reduction involved in X-chromosome inactivation in females (Payer and Lee, 2008), the silencing of transposable elements (Doerfler, 1991), and several aspects of meiosis, post-meiotic gene silencing and DNA compaction (Oakes, et al., 2007a).

The mammalian germ line undergoes extensive epigenetic reprogramming during germ cell maturation and gametogenesis. In males, widespread erasure of DNA methylation takes place in primordial germ cells (Hajkova, et al., 2002, Reik, et al., 2001) and subsequent *de novo* DNA methylation occurs during the maturation of germ cells and spermatogenesis, prior to meiosis (Oakes, et al., 2007a). As a result, the pattern of sperm DNA methylation is unique and hypomethylated compared with any other somatic cell (Eckhardt, et al., 2006, Oakes, et al., 2007b). The main targets of methylation in germ cells are non-CpG island (non-CGI) sequences in both distinct loci and repetitive sequences, but CpG islands (CGIs) can also be methylated (Oakes, et al., 2007a). Interestingly, hypomethylated promoters in the mature sperm are the promoters of developmental transcription and signalling factors. In mammals, correct sperm DNA methylation is suggested to be essential for both fertilization and early

embryo viability (Anway, et al., 2005, Bourc'his and Bestor, 2004, Carrell and Hammoud, 2010, Dada, et al., 2012, Jenkins and Carrell, 2012, Li, et al., 1992, Okano, et al., 1999, Romero, et al., 2011, Yaman and Grandjean, 2006) and therefore improved knowledge of the epigenetics of sperm is not only necessary to understand these processes, but may also provide clues to the potential causes of male infertility of unknown origin.

Early studies of sperm DNA methylation analysis were specifically performed in imprinted genes as an increased risk of congenital imprinting diseases in children conceived through assisted reproductive technologies (ART) had been suggested. These studies showed the aberrant methylation patterns of imprinted genes in poor quality sperm (Kobayashi, et al., 2007, Marques, et al., 2004, Marques, et al., 2008, Poplinski, et al., 2010). Altered sperm DNA methylation patterns have also been found in non-imprinted genes associated with spermatogenic impairment, such as Methylenetetrahydrofolate Reductase (*MTHFR*) (associated with folate metabolism and methylation reactions) (Wu, et al., 2010b), the cAMP Responsive Element Modulator (*CREM*), involved in spermatogenesis, (Nanassy and Carrell, 2011) and the Deleted in Azoospermia-like (*DAZL*) gene which is involved in germline establishment and gametogenesis (Navarro-Costa, et al., 2010).

The emergence of new technologies to analyse DNA methylation has allowed the study of alterations at the whole-genome level. In this manner, aberrant sperm DNA methylation of both imprinted and non-imprinted genes has been identified in infertile individuals with poor quality semen (Houshdaran, et al., 2007, Pacheco, et al., 2011). Furthermore, nearly 600 genes were found differentially methylated in the testes of humans with spermatogenic disorders, of which two are worth noting; Piwi-like RNA-mediated gene silencing 2 (*PIWIL2*) and Tudor domain containing 1 (*TDRD1*), two germline-specific genes involved in PIWI-interacting RNA (piRNA) processing machinery (Heyn, et al., 2012). The results of all these studies suggest that alterations of DNA methylation patterns of the germ cell could affect reproductive success.

Although defective germ cell-DNA methylation patterns have been associated with alterations in semen quality, there is a lack of such epigenetic studies in infertile men with normal sperm parameters. In this project we aim for the first time to conduct a genome-wide analysis of DNA methylation in sperm samples from normozoospermic fertile and infertile men. To address this issue, we used high-throughput 450K methylation arrays, covering the whole genome, to determine whether epigenetic changes in male germ cells could explain differences in reproductive success related to the functional quality of spermatozoa.

Materials and methods

Ethical Approval

Our study recruited semen samples from 49 selected individuals of Caucasian origin. All the participants signed an informed consent form and the project was approved by the Ethical Committee of the Puigvert Foundation.

Subjects of study

Clinical assessment of fertile and infertile individuals was conducted at the Andrology Service of the Fundació Puigvert, and included taking a full personal and family medical history to rule out heritable conditions, physical examination –with special emphasis on sexual characters, gonads and genitalia– and a minimum of two semen analyses [performed in accordance with the World Health Organization guidelines (Cooper, et al., 2010, WHO, 2010) except for motility assessments, which were done at room temperature using the WHO 1999 four-grade classification of movement]. Spermiograms included volume, pH, sperm concentration, four-category motility assessment, vitality, morphology and antisperm antibodies. Motility and sperm count were done in duplicate aliquots of ≥200 cells, and measures were adopted to control for acceptable differences between duplicates. Sperm concentration was performed on diluted, immobilized samples using haemocytometer chambers. Computer assisted sperm analysis (CASA) was performed on fresh ejaculates with the Integrated Semen Analysis System (ISAS®, version 1.01), Proiser R+D (Valencia, Spain) to obtain objective measurements of sperm kinematics (Pedigo, et al., 1989).

Semen samples from 17 fertile men (2 of whom were anonymous donors used in >10 insemination cycles per sperm donor for at least 6 female recipients; the other 15 being volunteers of proven fertility who were going to undergo vasectomy; aged 22- 49 years) with normal seminal quality, were studied as methylation controls of fertile spermatozoa. In addition, semen samples were obtained from 29 male patients (aged 30-55 years) consulting for couple infertility with no known risk factors and normal or mild defects of semen quality values that were used in husband ART (IVF-ICSI: in vitro fertilization- intracytoplasmic sperm injection technique) (**Table I**). The reference values of fertility were those most recently defined by the WHO (Cooper, et al., 2010, WHO, 2010).

Semen volume, count, motility, and morphology, including the teratozoospermia index (TZI), as well as the results of the gynecological assessment of the female partner, for the samples used for the epigenetic studies are summarized in **Table I**. TZI is defined as the number of abnormalities present per abnormal spermatozoon, these being

196 defects of the head, neck/mid piece and tail defects or presence of cytoplasmic
197 droplets, thus indicating the severity of the morphology alteration (from 1 to 4 value).

198 Pregnancy outcome from IVF-ICSI treatment of infertile couples is also described for
199 patients (**Table I**).

200 **Isolation of mature germ cells and DNA extraction**

201 Semen samples were liquefacted and homogenized with a mechanical mixer at 37ºC
202 (30-60 min) and subsequently processed with a differential centrifugation technique
203 using density gradients (65-90% Puresperm®, Nidacon International AB, Mölndal,
204 Sweden) to remove somatic contaminants and to enrich the sample in terms of
205 spermatozoa. The isolated germ cells were normalized to a concentration of $1x10^6$/mL
206 and processed to obtain sperm DNA (Wizard Genomic Purification kit, Promega, USA)
207 following the manufacturer's instructions for this specific cell type. The DNA extraction
208 included RNAse A treatment of DNA samples.

209

210 **Genome-wide DNA methylation analysis with high-density arrays**

211 Microarray-based DNA methylation profiling was performed with the Illumina Infinium®
212 Human Methylation450 BeadChip (Illumina Inc., USA) (Bibikova, et al., 2011). Bisulfite
213 conversion of DNA was carried out using the EZ DNA Methylation Kit (Zymo Research,
214 Orange, CA) following the manufacturer's procedure, but with the modifications
215 described in the Infinium Assay Methylation Protocol Guide. Processed DNA samples
216 were then hybridized to the BeadChip (Illumina) following the Illumina Infinium HD
217 Methylation Protocol. Genotyping services were provided by the Spanish "Centro
218 Nacional de Genotipado" (CEGEN-ISCIII)" (www.cegen.org).
219 IDAT files from the microarray were further processed using the R/Bioconductor
220 (version 3.0; open source) package *minfi* (Hansen and Aryee). In order to adjust for the
221 different probe design types present in the 450k architecture, red and green signals
222 from the IDAT files were corrected using the SWAN algorithm (Makismovic, et al.,
223 2012). Probes with detection p-values over 0.01 in at least two samples were filtered
224 out. In accordance with Du (Du, et al., 2010), both beta values and M-values were
225 computed and employed across the analysis pipeline. M-values were used for all the
226 statistical analyses, assuming homoscedasticity, while beta values were mostly used
227 for the intuitive interpretation and visualization of results.
228 Probes that were found to co-hybridate with probes in the sexual chromosomes
229 (Lemire, et al., 2013) were removed.

Detection of differentially methylated probes

In order to identify CpG sites which were differentially methylated (dmCpGs) between the sperm samples of fertile and infertile men, a robust moderated t-test implemented in the R/Bioconductor package *limma* (Smyth, 2005) was performed. False Discovery Rate (FDR) was controlled using the Benjamini-Hochberg procedure, and a significance level of 0.05 employed. An additional threshold of effect size was applied, meaning that only those probes with the strongest differences between groups (the top 70%) were selected. The application of this threshold is essential to remove differences deriving from technical artefacts and consequently ensure a more biologically sound statistical data analysis (Pan et al. 2005). dmCpGs were defined as hypermethylated or hypomethylated when methylation values were respectively higher or lower in infertile samples compared with fertile controls.

Genomic region analysis

The probes in the microarray were assigned to a genomic region according to their position relative to the transcript information extracted from the R/Bioconductor package *TxDb.Hsapiens.UCSC.hg19.knownGene* (Carlson). A probe was said to be in a *Promoter* region when it was located inside the first exon, the 5'-UTR or a region up to 2kbp upstream of the transcription start site (TSS) of any given transcript. Similarly, a probe found inside any intron or any exon other than the first was labelled as *Intragenic*. *Intergenic* probes were determined as those which did not fall into either of the two previous categories. According to this definition, a probe could be in both a *Promoter* and an *Intragenic* region at the same time, for different transcripts. A contingency table was built for each selected subset of probes and a given genomic region, with one variable indicating whether a given probe belonged or not to the subset, and the other indicating whether a given probe was labelled with the selected region. Significance of the association was determined by a Chi-squared test. A significance level of 0.05 was used to determine if a subset was dependent with respect to a given genomic region. Odds Ratio was used as a measure of effect size.

CGI status analysis

The CGI locations used in the analyses were obtained from the R/Bioconductor package *FDb.InfiniumMethylation.hg19* (Triche). This dataset contains all the CGIs distributed along the whole genome. The generation procedure for these CGIs is described by Wu and colleagues (Wu, et al., 2010a). *CpG shores* were defined as the

2kbp regions flanking a CGI. *CpG shelves* were defined as the 2kbp region, either upstream or downstream, of each CpG shore. Probes not belonging to any of the regions thus far mentioned were assigned to the special category *Non-CGI*. Each probe was assigned to only one category. A 4x2 contingency table was constructed for every subset of probes in order to study the association between the given subset and the different CGI categories. A Chi-squared test was used to determine if any of the categories had a significant association with the given subset. For each of the CGI status levels, a 2x2 contingency table was defined and another Chi-squared test was independently used to evaluate the association of the given subset with each status level, a significance level of 0.05 being employed for all tests. Effect size was reported as the Odds Ratio for each of the individual tests.

Histone enrichment analysis

In order to analyse the enrichment of a histone mark on a given subset of probes, we used the information contained in the UCSC Browser Broad Histone track from the ENCODE project (Bernstein, et al., 2005, Bernstein, et al., 2006, Ernst, et al., 2011, Guttman, et al., 2010, Mikkelsen, et al., 2007). Histone peak data for every cell line and mark present in the ENCODE project were downloaded from the UCSC Browser (**Supplementary Table 1**). Small peaks were discarded when they were completely contained within wider peaks. For each combination of cell line and mark, a 2x2 contingency table was built to determine its association with the input subset of probes. Probes in the array were classified according to whether they belonged to the subset or not, and whether they intersected with a significant broad peak for the given combination of cell line and mark. A Fisher exact test was used to determine if the given subset of probes was significantly enriched for each combination of cell line and mark. P-values were corrected for multiple testing using FDR (using the Benjamini-Hochberg method) and a significance level of 0.05 was used to determine which probes had significant enrichment. The base-2 logarithm of the Odds Ratio was used as a measure of effect size.

Global DNA methylation analysis

Global DNA methylation status was quantified using the Methylamp global DNA methylation quantification ultra kit (Epigentek, Catalog # P-1014B, USA) following the manufacturer's instructions. Briefly, 100 or 200 ng of genomic DNA is used for a 5-methylcytosine (5-mC) quantification. The methylated fraction of DNA is recognized by a 5-methylcytosine antibody and colorimetrically quantified through an ELISA-like

reaction.

Bisulfite pyrosequencing

DNA methylation patterns of the repetitive sequences were analysed by bisulfite pyrosequencing. Bisulfite modification of DNA was performed with the EZ DNA Methylation-Gold kit (Zymo Research) following the manufacturer's instructions. Each sequence was amplified with previously described forward and reverse primers (**Supplementary Table 2**) (Bollati, et al., 2007, Choi, et al., 2009, Martinez, et al., 2012). After PCR amplification of the region of interest with the specific primers, pyrosequencing was performed using PyroMark Q24 reagents, vacuum prep workstation, equipment and software (Qiagen, Netherlands).

Identification of specific DNA methylation patterns in spermatozoa

DNA methylation data of blood (Hannum, et al., 2013) and brain (neuron and glia) (Guintivano, et al., 2013) samples produced with the Illumina Infinium Human Methylation450 were used for comparison with the sperm data in order to identify DNA methylation patterns specific to mature germ cells. DNA methylation beta value data was downloaded from GEO accession numbers GSE40279 and GSE41826**.** Methylation data for the blood dataset was adjusted for white blood cell heterogeneity using the method described in Houseman et al. 2012 (Houseman, et al., 2012). In order to feed this method, we used the original 27k database of purified white blood cell subtypes included in the authors' original implementation of the algorithm.

To identify tissue-specific methylated CpG sites in a given tissue, we looked for CpGs showing mean methylation > 60% in the target tissue and < 40% in the other tissues. Similarly, to identify tissue-specific unmethylated CpG sites we looked for CpGs with methylation <40% in the target tissue and > 60% in the other tissues.

Circos data track smoothing

In order to plot the CpG information on Circos genome-wide graphs, smoothing was applied to our data. Broad Histone peak information from UCSC was averaged by partitioning the genome into intervals of 200kbp and assigning to each peak a score that corresponded to the average of the broad peak scores found within it. CpG locations were not smoothed but rather stacked on several lines. This does not mean that any CpG has a higher score than other, but simply that the higher the stack of markers, the higher the density of CpGs in the region.

338 **Statistical Analyses**

339 Statistical analyses were performed using R/Bioconductor (version 3.0; open source).
340 To identify CpG sites showing differential methylation values between the sperm
341 samples of fertile and infertile men, a robust moderated t-test implemented in the
342 R/Bioconductor package *limma* was performed. FDR was controlled using the
343 Benjamini-Hochberg procedure.
344 Significant associations between dmCpGs and specific genomic locations was
345 determined by a Chi-squared test. Odds Ratio was used as a measure of effect size.
346 A Fisher exact test was used to analyse the enrichment of dmCpGs on an specific
347 chromatin mark. P-values were corrected for multiple testing using FDR, and the base-
348 2 logarithm of the Odds Ratio was used as a measure of effect size.
349 The non-parametric Kruskal-Wallis and Wilcox tests were used to analyze differences
350 in methylation levels (global and repetitive regions) in sperm groups compared with
351 somatic cells. A value of $p<0.05$ was considered significant.
352

Results

Alterations of sperm DNA methylation are found in infertile individuals

To identify genomic regions showing differences in sperm DNA methylation patterns between fertile and infertile individuals, we performed methylation arrays (methylation assay 1 in **Table I**) of 12 sperm samples and analysed the site-specific methylation status of 485,577 CpG sites across the human genome (Bibikova, et al., 2011, Sandoval, et al., 2011). In order to analyse differential methylation patterns, we divided the samples into two groups; one composed of 5 fertile individuals (samples Control-1 to 5) and the other, 7 infertile patients (samples Patient-1 to 7). Only one of the female partners (Patient-7 partner) had a potential factor (dysovulation, corrected with medication) that may influence the fertility of the couple. The rest of the women presented no known risk factors. It is worth taking into account that we decided to establish semen diagnosis based exclusively on the first semen analysis, since fertile controls only delivered a single sample.Nevertheless, some of the subsequent semen samples collected for this study in the infertile group showed some deviation from initial values with respect to sperm morphology. It is of note, however, that abnormal sperm from infertile individuals showed a similar low severity of morphological alteration (TZI values < 1.7) as those from fertile individuals (**Table I**).

The first observation indicated that, although methylation patterns are well preserved, some CpG sites exhibited higher interindividual variability (5% showed M-values SD>0.6), irrespective of the group of samples analysed **(Fig. 1A**), which confirms variation in DNA methylation of the male germline across unrelated individuals (Flanagan, et al., 2006). Statistical analysis showed 2,752 dmCpGs between fertile and infertile men. Of these, 1,447 CpG sites were hypermethylated while 1,305 were hypomethylated in infertile patients **(Supplementary Fig. 1 and Supplementary Table 3**). Hierarchical clustering of DNA methylation data for the most variable CpG sites highlights the differences between the fertile controls and infertile patients (**Fig. 1B).**

To study, from a functional genomics point of view, the characteristics of the dmCpG sites we first determined their distribution in CGI and non-CGI regions (Wu, et al., 2010a). Interestingly, while hypermethylated CpG sites were preferentially enriched in CGI-shores ($p<0.001$; OR=1.50), hypomethylated CpG sites were enriched in CGIs ($p<0.001$, OR=1.27) (**Fig. 1C**). Intergenic regions showed a significantly increased proportion of hypomethylated CpG sites ($p<0.001$, OR=1.83) while promoter regions presented a decreased proportion of both hypermethylated ($p= 0.039$, OR=0.89) and

hypomethylated (p<0.001, OR=0.53) CpGs in infertile patients (**Fig. 1D**). Gene Ontology (GO) analysis of the dmCpGs, using DAVID gene ontology annotation groups, showed that genes around the hypermethylated CpG sites were enriched for a cell adhesion related term (GO term: homophilic cell adhesion; FDR <0.01), while genes around the hypomethylated CpG sites did not show any significant term enrichment for functional (GO) categories.

Given that the establishment of germ cell DNA methylation marks involved in paternal genomic imprinting is critical during spermatogenesis, we next compared these dmCpGs with those associated with imprinted genes (Pacheco, et al., 2011). A total of 8746 CpG sites belonging to 183 imprinted genes were present in the 450K array. We found that 54 CpG sites associated with 48 imprinted genes were aberrantly methylated in infertile patients. Specifically, 33 CpG sites (related to 28 genes) were hypermethylated and 21 CpG sites (related to 28 genes) were hypomethylated; 8 genes showed both hyper- and hypomethylation (**Supplementary Tables 4 and 5**). In addition we compared our results with those obtained by Pacheco and collaborators, who analysed 619 CpGs associated with imprinted genes from samples of sperm with low motility using 27K Illumina arrays, and found two common CpGs aberrantly methylated (associated to insulin-like growth factor 2 (*IGF2*) and heat shock 70kDa protein 6 (*HSPA6*) genes) in both studies.

To study other genomic features which may provide clues about the mechanisms underlying the aberrant methylation changes in infertile men, we investigated whether the differentially methylated regions were among those targeted by any specific histone mark in somatic cells. We compared our methylation data with previously published data on a range of histone modifications and chromatin modifiers in 10 differentiated somatic cells obtained from healthy individuals (**see Materials and Methods**). We found statistically significant (p<0.001) associations between DNA hypomethylation and the repressive histone mark H3K9me3 in most differentiated ENCODE cell lines (**Fig. 2A-B**). However, DNA hypermethylation was associated with H3K4me1 and the CCCTC-binding factor (CTCF) (p<0.001) (**Fig. 2A-B**). We also investigated whether the hyper- and hypomethylated CpGs in the sperm of infertile patients were associated with the genomic regions that are known to be enriched for nucleosomes, H2AZ or the post-translational histone marks H3K4me3 and H3K27me3 in human sperm (Hammoud, et al., 2009), and we found a significant association between hypermethylated CpGs in sperm of infertile patients and those genomic regions enriched for H3K4me3 (p= 0.036; OR:1.40).

423

424 **Differentially methylated regions are associated with sperm-specific DNA**
425 **methylation sites in infertile individuals**

426 For the further characterization of the regions associated with the dmCpG sites found
427 in our study we performed a comparative analysis between dmCpG sites and regions
428 with sperm-specific DNA methylation (**see Materials and Methods**). To identify
429 regions with tissue-specific DNA methylation we first compared the above mentioned
430 sperm methylomes of the fertile subjects (5 samples) with the DNA methylomes of
431 differentiated somatic cells. We used data obtained from methylation analysis of blood
432 (8 samples) and neural and glial cells (8 samples of each) from the same type of
433 methylation arrays (Illumina 450K) deposited in public databases (Guintivano, et al.,
434 2013, Hannum, et al., 2013). To reduce confounding factors in the blood dataset, we
435 first corrected for cellular heterogeneity (Houseman, et al., 2012).

436 The results of these comparisons showed that, as expected, the levels of DNA
437 methylation of the germ cells were lower than those of somatic cells (average M-
438 values: sperm= -0.31, blood= -0.08, neuron= 0.02, glia= 0.02) **(Supplementary Fig. 2**
439 **and Supplementary Fig. 3A),** whilst inter-individual variability of sperm was higher
440 than observed in differentiated somatic cells (mean of log (SD): sperm= -1.41; blood= -
441 1.66; glia= -1.80; neuron= -1.77) **(Supplementary Fig. 3A)**. Furthermore, we observed
442 that sperm have a large number of CpG sites showing extreme values (89.5%), both
443 unmethylated (<40 methylation %) and methylated (> 60 methylation %), while the
444 differentiated somatic cells showed a large number of probes with intermediate
445 methylation values (~33%) (**Supplementary Fig. 3A**). In addition, in sperm cells a
446 larger number of probes showed specific DNA methylation patterns (unmethylated
447 21,139; methylated 1,842) than in any of the somatic cell types (**see Materials and**
448 **Methods**) (**Supplementary Fig. 3B and Supplementary Table 6)**. Intriguingly, tissue-
449 specific unmethylated CpGs were enriched in non-CGIs in all somatic cell types, whilst
450 in sperm they were enriched in CGI-shores, and, conversely, tissue-specific methylated
451 CpGs were enriched in CGI-shores in all the somatic cells whilst being enriched in non-
452 CGIs in sperm (**Supplementary Fig. 4**), highlighting the peculiarity of this cell type in
453 terms of DNA methylation patterns.

454 Most importantly though, we found a significant association between those CpG sites
455 with specific DNA methylation in sperm and the dmCpG sites (hyper- and
456 hypomethylated) identified in infertile men ($p<0.001$; Fisher´s exact test, OR= 1.43),

457 which suggests that the alterations of sperm-specific DNA methylation patterns could
458 be associated with male infertility. A more detailed analysis showed that only
459 hypomethylated CpGs were significantly associated with both sperm-specific
460 methylated ($p=0.038$, OR: 2.00) or unmethylated ($p<0.001$; OR: 2.08) CpGs.

461

462 **Global DNA methylation patterns: comparative analysis between fertile and**
463 **infertile individuals**

464 To evaluate changes in global 5-mC levels (methylation assay 2 in **Table I**) that might
465 be associated with male infertility we analysed a different set of samples from 7 fertile
466 individuals (samples Control-6 to 12) and 7 normospermic infertile patients (samples
467 Patient-8 to 14). Only two of the female partners (those of Patient-9 and 11) had a
468 potential factor (tubal obstruction) that could influence the fertility of the couple. We
469 found no significant differences in global 5-mC levels between fertile and infertile sperm
470 samples (**Fig. 3A**).

471 In addition, since an association between DNA methylation of repetitive elements and
472 total genomic 5-methylcytosine has been described (Ehrlich, 2002, Weisenberger, et
473 al., 2005, Yang, et al., 2004), we analysed the methylation patterns of several repetitive
474 sequences (methylation assay 3 in **Table I**), both at whole genome level and at four
475 specific repetitive loci, in sperm from 17 fertile (samples Control-1 to 17) and 27 infertile
476 subjects (samples Patient-3 to 29) and compared them with differentiated somatic cells
477 (blood and brain). Successful outcome in IVF-ICSI treatment was also taken into
478 account (12 resulted in no pregnancy and 15 resulted in pregnancy) (**Table I**) as
479 success in IVF-ICSI would provide extra indications for the lack of a phenotype
480 associated with the female for the selected couples.

481 Specifically, we determined the methylation status of four repetitive sequences,
482 namely: LINE-1, an interspersed repeat found throughout the human genome and used
483 as an estimate of global methylation levels (Yang, et al., 2004); Alu Yb8, a relatively
484 young subfamily of the Alu short interspersed elements (SINEs) (Carroll, et al., 2001);
485 D4Z4, a macrosatellite found in the subtelomeric regions (Chadwick, 2009); and NBL-
486 2, a complex tandem repeat found in the centromeric regions of acrocentric
487 chromosomes (Nishiyama, et al., 2005). Our results showed no differences between
488 fertile and infertile subjects in any of the repetitive sequences analysed, with the
489 exception of Alu Yb8, where sperm of infertile individuals showed significantly lower Alu

490 methylation levels (p= 0.0011) (**Fig. 3B**). Furthermore, the comparative analysis of
491 DNA methylation of repetitive sequences (LINE-1, Alu Yb8, NBL2, D4Z4) showed no
492 statistical differences between the patient subgroups with respect to IVF-ICSI outcome.

493 However, we did find huge DNA methylation differences between germ and somatic
494 cells (blood, brain) in most of the repetitive sequences analysed, and in all cases
495 sperm samples showed lower average methylation values (**Fig. 3B**). NBL-2 showed
496 the largest differences (average methylation: sperm= 3.2%, blood= 80.2, and brain=
497 76.3; $p<0.001$). Alu (average methylation: sperm= 47.5%, blood= 90.6, and brain=
498 89.3; $p<0.001$) and D4Z4 (average methylation: sperm= 7.9%, blood= 55.6, and brain=
499 53.6; $p<0.001$) also showed great differences, and LINE-1 displayed the lowest
500 difference, with only the comparison with blood cells being statistically significant
501 (average methylation: sperm= 69.8%, blood= 74.6, and brain= 72.6) (**Fig. 3B**). These
502 results agree with the DNA methylation patterns of different repetitive regions found in
503 human sperm and human embryonic stem cells (hESCs) (Molaro, et al., 2011) as well
504 as in human sperm and B cells (Krausz, et al., 2012).

505

Discussion

Sperm and testicular DNA methylation profiles of specific genes or genomic regions from fertile and infertile human males have been compared in several studies to date (Heyn, et al., 2012, Houshdaran, et al., 2007, Kobayashi, et al., 2007, Marques, et al., 2004, Marques, et al., 2008, Pacheco, et al., 2011, Poplinski, et al., 2010). These analyses were, however, either restricted to a small number of genes or imprinted regions, or considered a larger number of genes but focused only on promoter regions. What is more, only infertile patients presenting alterations associated with either the process of spermatogenesis or semen quality were studied. In contrast, in this work we compare, for the first time, and at genome-wide level, the DNA methylation patterns of sperm from fertile individuals with the sperm of patients with unexplained infertility. Furthermore, the confounding role of the significant proportion of female causes that contribute to reproductive failure was controlled since sperm was selected from couples with no known significant female risk factor.

We analysed gene promoters and intragenic and intergenic regions, and identified alterations in the methylation of DNA in 2,752 CpGs. It is of particular note that a high level of variation in DNA methylation was observed in mature germ cells across unrelated individuals, even among fertile individuals, and may reflect differing levels of sperm function. Specifically, we found no alterations in the DNA methylation patterns of genes of the PIWI pathway, previously found to be associated with severe defects of sperm production (Heyn, et al., 2012). Neither did we find evidence for the previously described alterations in imprinted genes such as imprinted maternally expressed transcript (*H19*) or mesoderm specific transcript (*MEST*) (Kobayashi, et al., 2007, Marques, et al., 2004, Marques, et al., 2008, Pacheco, et al., 2011, Poplinski, et al., 2010) or non-imprinted genes such as *MTHFR* (Houshdaran, et al., 2007, Pacheco, et al., 2011, Wu, et al., 2010b) which have been associated with male infertility due to poor semen quality. However, we extended our study on imprinted genes and found a small number of CpGs showing aberrant DNA methylation associated with 48 imprinted genes in infertile patients. Subsequently, we compared our results with data obtained in a previous study (Pacheco, et al., 2011) where 619 CpGs associated with imprinted genes were analysed in samples from patients with low sperm motility, and, we only found two common CpG sites aberrantly methylated. Interestingly, one of the genes associated with these CpGs (*IGF2*) has been found also aberrantly methylated in abnormal sperm (Boissonnas, et al., 2010, Pacheco, et al., 2011, Poplinski, et al.,

2010). Unlike in other studies to date, here we analysed patients with normal or mild defects of semen quality, and our results show previously unidentified alterations in DNA methylation of CpG sites located at specific genes and genomic regions. This suggests that the mechanistic origins of these alterations in DNA methylation in individuals with unexplained infertility may be different from those associated with the onset of alterations in patients with low semen quality.

We used several genomic approaches to further study the peculiarities of these dmCpGs that might give some clue to their contribution to male infertility. In the case of hypomethylated CpGs, besides being preferentially located in CGI, they are more abundant in intergenic regions and depleted in promoters, all of which suggests that these CpGs are located in the clusters of CGI II and III recently described by Zeng and collaborators, which have been associated with tissue-specific DNA methylation (Zeng, et al., 2014). Intriguingly, hypermethylated related genes were also enriched for a cell adhesion ontology term, opening new avenues for investigating the functional role that aberrant DNA hypermethylation may have in cases of unexplained male infertility. This could be at least partly related to defects in functional capacity of sperm to bind the oocyte in infertile patients. It could also give evidence that some of these defects in methylation are shared with sperm morphology deficiencies, from results of other studies in which associations between teratozoospermia and deficiency in adhesion molecules were found (Glander and Schaller, 1993).

Since the aberrant DNA methylation appeared to be related to changes in tissue-specific methylation, we aimed to identify CpGs which were specifically methylated in sperm when compared with somatic cells (brain and blood). These sperm-specific methylated regions were subsequently compared with the dmCpGs in infertile individuals. In addition to showing different DNA methylation patterns between somatic and germ cells, as previously described (Eckhardt, et al., 2006, Krausz, et al., 2012, Oakes, et al., 2007b), we found a statistically significant association between sperm dmCpGs, mainly hypomethylated CpGs in individuals with unexplained infertility and CpGs that showed sperm-specific DNA methylation, which suggests that the alterations of the mechanisms that establish the sperm-specific epigenetic program could be involved in the fertilizing quality of sperm in unexplained human male infertility.

In addition, we analysed the genomic location of these dmCpGs in the context of chromatin and found associations between aberrant DNA methylation and specific histone marks previously identified in somatic and sperm cells. Interestingly, our results

showed a significant association between hypomethylated CpGs in sperm and regions strongly enriched in repressive histone marks such as H3K9me3 in somatic cells, whereas hypermethylated regions were associated with H3K4me1 and CTCF. Since there is no reason why these chromatin marks should necessarily be the same in somatic cells and sperm cells, these associations may merely indicate that during germ cell development these dmCpGs are associated with regions with some distinct feature that leads to a differential “vulnerability” to hypo- or hyper methylation in association with infertility. On the other hand, if the chromatin marks in somatic cells associated with aberrant DNA methylation in sperm were the same in the germ cell, DNA hypermethylation of CTCF binding sites could be indicative of alterations in the architecture and function of the sperm genome of infertile patients, since CTCF binds to DNA sequences in a methylation-sensitive manner (Wang, et al., 2012), and it has been shown that CTCF appears to play a significant role in chromatin organization, as well as in the regulation of gene expression (Ong and Corces, 2014, Phillips and Corces, 2009, Wang, et al., 2012), which is especially relevant in mammalian sperm (Arpanahi, et al., 2009, Carone, et al., 2014).

In addition, we analysed associations between aberrant DNA methylation with the histone marks identified in human sperm and available in public data bases (Hammoud, et al., 2009), and found a significant association between hypermethylated CpGs in the sperm of infertile patients and genomic regions enriched for H3K4me3. Taken all together, our findings suggest that the relationship between chromatin context and the aberrant DNA methylation of sperm in infertile men could be locus-dependent. Future studies analysing the complete maps of histone posttranslational marks of sperm chromatin in normospermic infertile patients and fertile individuals will elucidate whether these alterations in DNA methylation are also associated with alterations of other specific histone marks, and whether they could affect chromatin compaction, as suggested in other studies analysing subfertile individuals (La Spina, et al., 2014, Steilmann, et al., 2010)

Apart from locus-specific DNA methylation differences, we analysed for the first time global DNA methylation changes between normospermic fertile and infertile patients. Our results showed no differences in global methylation between the groups, in contrast to the results previously found in infertile patients with poor quality of sperm (Tunc and Tremellen, 2009), suggesting that global DNA methylation changes are related to spermatogenic efficiency and the semen quality of infertile patients.

We also analysed DNA methylation changes between groups in several repetitive elements across the genome, including LINE-1, considered to represent global DNA methylation (Yang, et al., 2004). The dynamics of the DNA methylation of repetitive DNA elements during epigenetic reprogramming of primordial germ cells are gender specific (Lees-Murdock and Walsh, 2008, Sasaki and Matsui, 2008), and the functional role of the DNA methylation of retrotransposons, particularly in male germ cells, has been described (Bourc'his and Bestor, 2004). Our results related to the DNA methylation of LINE-1 retrotransposon showed no differences between groups, and agree with previous results analysing differences in infertile men exhibiting low sperm concentrations (Kobayashi, et al., 2007, Marques, et al., 2008). In contrast, LINE-1 repetitive sequences have been found to be hypomethylated in infertile patients with severe spermatogenic disorders (Heyn, et al., 2012) associated with the epigenetic inactivation of piRNA-processing genes: *PIWIL2* and *TDRD1*. Our results, however, not only failed to show differences in the DNA methylation patterns of *PIWIL2* and *TDRD1* between groups (data not shown), but neither did we find differences in methylation of LINE-1 between normospermic fertile and infertile patients.

Interestingly, we did find a significant DNA methylation decrease in another retrotransposon, AluYb8, in infertile patients. The results from previous works focused on infertile individuals showing low seminal quality had been unable to clarify the associations between male infertility and the methylation of Alu sequences (El Hajj, et al., 2011, Kobayashi, et al., 2007); Kobayashi and collaborators did not find differences between healthy controls and infertile patients (Kobayashi, et al., 2007), whereas El Hajj and collaborators, in line with the results of our study, showed that average methylation values in Alu sequences were lower in infertile men with abnormal semen parameters (El Hajj, et al., 2011). The differences between these two studies could be attributed to several causes. First different methodologies were used to measure methylation; one case used combined bisulfite restriction analysis (COBRA) assay and the other, bisulfite pyrosequencing. Secondly the difference could be the consequence of the specific Alu sequence analysed. In our work we specifically analysed the Alu Yb8 subfamily, which is relatively young and more susceptible to retrotransposon activity, thus requiring stricter control by epigenetic silencing mechanisms. The role of SINE elements in the regulation of gene expression has recently been investigated in murine models and been shown to play a role in the activity of downstream gene promoters (Estecio, et al., 2012). Methylation of SINEs in the transcriptional regulation of genes specifically expressed in testis has also been suggested to have a role (Ichiyanagi, et

al., 2011). Thus, future studies are needed to elucidate the functional effects of aberrant methylation of Alu sequences in sperm from infertile individuals.

We also analysed and compared, for the first time, the DNA methylation patterns of pericentromeric (NBL2) and subtelomeric (D4Z4) repetitive elements in both control and infertile patients, and found low methylation values in these regions, but no significant differences between groups. That said, the results of the analysis of DNA methylation in both global and repetitive sequences should be considered carefully, since the presence of mild reproductive risk factors in some females might be masking potentially fertile individuals.

Together, these results suggest that alterations of DNA methylation, both globally and at locus-specific level, and therefore of the mechanisms that produce them, are different in normospermic infertile patients compared to infertile individuals with spermatogenic impairment. As in the case of DNA methylation at specific loci, we also performed a comparative analysis of DNA methylation of the repetitive regions mentioned above in order to analyse differences between germ and somatic cells, and to ascertain the peculiarities of the former. It has previously been shown that mammalian testes have more hypomethylated loci than somatic cells (Oakes, et al., 2007b). In addition, the DNA methylation of repetitive sequences has been found to be particularly low in sperm compared to undifferentiated somatic cells (hESC) (Molaro, et al., 2011). To further characterize the methylation patterns of these repetitive sequences in sperm, we also performed methylation analysis of some of these regions in differentiated somatic cells (blood and brain). The greatest difference in methylation levels between sperm and somatic cells was observed for NBL-2. This is also the case when looking at other centromeric repeats (Molaro, et al., 2011), suggesting therefore that an overall hypomethylation of the centromeric region is characteristic of sperm. Indeed, Yamagata and collaborators have previously proposed using methylation levels of the centromeric region in order to discriminate between germ and somatic cell lineages (Yamagata, et al., 2007). Moreover, although pericentromeric regions are usually regarded as transcriptionally poor (Copenhaver, et al., 1999, Nagaki, et al., 2004), the expression of a variety of genes has been shown in the testis, with almost half the cases being unique to the tissue (She, et al., 2004). In the case of D4Z4, although to date no studies have concentrated on its methylation in sperm, Jian Li and collaborators have reported enrichment in "methylation deserts" in telomeric regions (Li, et al., 2012). Thus, the low methylation values found here for D4Z4 are probably, as in the case of NBL-2, reflective of overall hypomethylation occurring in that region,

679 and highlights the increased risk of structural mutations in germ cells due to these
680 methylation deserts (Li, et al., 2012).

681 Although Alu Yb8 methylation values in sperm were higher than those found for the
682 other repetitive elements studied, sperm and somatic cells showed large differences,
683 confirming the findings of previous studies using other analysis techniques (Hellmann-
684 Blumberg, et al., 1993, Kochanek, et al., 1993). Finally, unlike the other repetitive
685 sequences, LINE-1 showed high levels of methylation, with significant differences only
686 being found between sperm and blood cells. When comparing studies based on the
687 same technique (El Hajj, et al., 2011, Heyn, et al., 2012), the methylation values of this
688 sequence in sperm were similar. This finding is partially supported by the results of
689 Molaro and collaborators (Molaro, et al., 2011), who have studied methylation along the
690 full length of LINE-1 and discovered a higher percentage of hypomethylated regions in
691 sperm compared to hESCs. Although there are major differences between the results
692 of Molaro and collaborators and our own, these could be explained because the former
693 analysed undifferentiated somatic cells while differentiated somatic cells were
694 considered in this work, and because we only studied 3 CpGs, thereby only reflecting
695 methylation for specific locations on LINE-1.

696 In conclusion, DNA methylation patterns of spermatozoa are significantly different to
697 those found in other somatic cells such as blood or brain. In this work we have
698 analysed for the first time, at genome-wide resolution, the DNA methylation profiles of
699 the sperm of patients with unexplained infertility versus that of fertile individuals, and
700 we have identified almost 3,000 CpGs which display aberrant methylation. Our data
701 show that these changes are precisely associated with regions of sperm-specific
702 methylation, thereby suggesting that DNA methylation is involved in the control of the
703 functional capacity of germ cells. Further studies are necessary to elucidate the
704 mechanisms relating to the origin of these alterations, and to determine their
705 significance and functional consequences for male infertility.

706

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Authors' roles

L.B., S.L. and A.F.F. participated in the planning and design of the study. R.G.U., M.D., E.G.T. and C.B. performed the experiments and collected data. Data analysis and interpretation was performed by M.F.F., R.G.U., G.F.B. and A.F.F.. L.B., S.L. and A.F.F. wrote the manuscript. All authors revised the article and gave final approval to the submitted version.

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Conflict of interest

The authors confirm that they have no conflict of interest to declare.

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Figure legends

Figure 1. DNA methylation patterns in fertile individuals and infertile patients. (A) Deviation plot for all the CpG sites studied in sperm samples showing the variability of methylation values (grey area). Probes are ranked on the x-axis with respect to their median methylation, as visualized by a curve. Probe values represented by grey lines depicting the 5th and 95th percentile range. On the right, the distribution of standard deviation (SD) across all the probes analysed is shown and the 95th percentile is indicated. (B) Unsupervised hierarchical clustering and heatmap including the 193 most variable CpG sites (absolute M-value differences >0.2) between fertile and infertile individuals. Average methylation values are displayed from 0 (blue) to 1 (yellow). On the right, bar plot displaying the number of hyper- and hypomethylated CpG sites in infertile patients. (C) Distribution of dmCpGs relative to CGIs. (D) Relative distribution of dmCpGs across different genomic regions.
dmCpGs: differentially methylated CpGs; CGI: CpG islands

Figure 2. Chromatin signatures associated with aberrant DNA methylation in infertile patients. (A) Heatmaps showing significant enrichment of hyper- and hypomethylated CpG sites identified in infertile individuals, with different histone marks and chromatin modifiers contained in the UCSC Browser Broad Histone track from the ENCODE project. Colour code indicates significant enrichment based on log2 odds ratio (OR). (B) Circular representation of genome-wide DNA methylation changes in the infertile patients indicating whether the CpGs were hypermethylated (red) or hypomethylated (blue). Inner tracks display chromatin marks (Ctcf, H3K4me1 and H3K9me3) generated for osteoblast cells, and associated with differentially methylated regions. Broad Histone peak information is averaged in 200 kbp genomic windows and represented as histogram tracks. Two regions of chromosomes 6 and 11 are magnified (grey area) in order to detail the associations between hypo- or hypermethylated DNA regions with specific chromatin signatures.

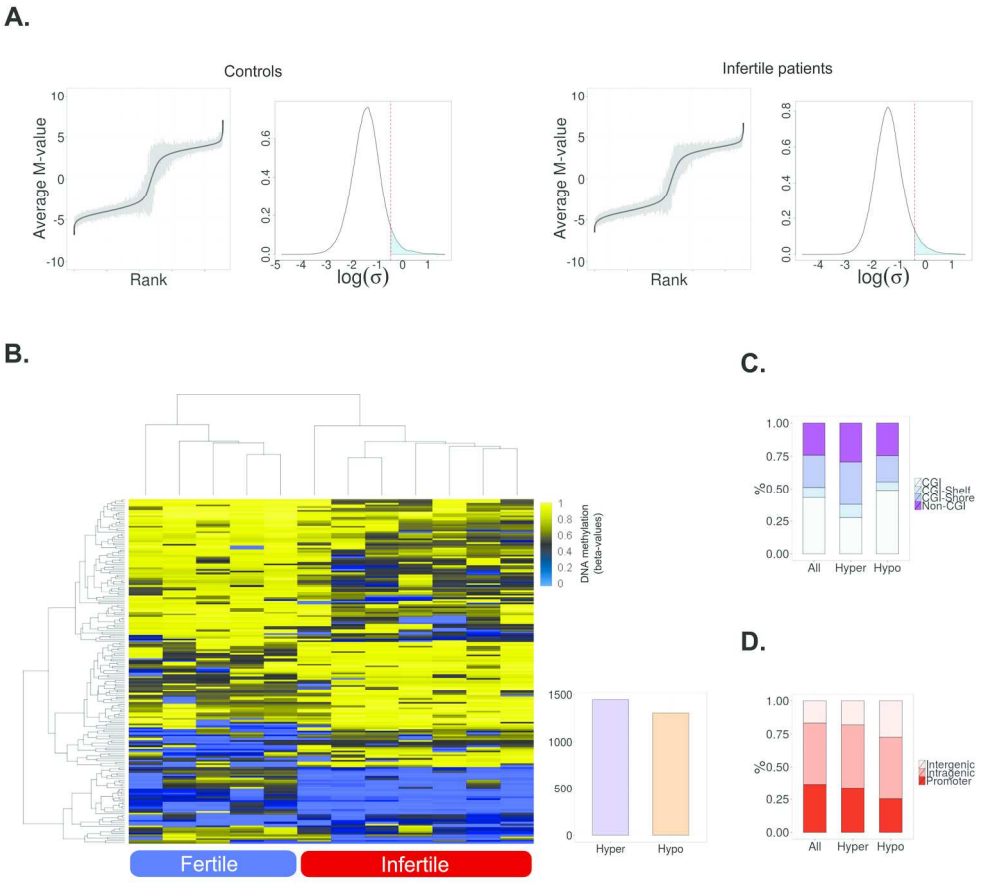
Figure 3. Global DNA methylation patterns in sperm. (A) Global DNA methylation levels of sperm from fertile individuals and normospermic infertile patients obtained in a colorimetric assay. (B) DNA methylation values of several repetitive regions (LINE-1, Alu Yb8, NBL-2, and D4Z4) measured by bisulfite pyrosequencing in sperm (controls and patients) and somatic cells (blood and brain). ***: $p<0.001$; **: $p<0.01$.

Supplementary Fig. 1. Unsupervised hierarchical clustering and heatmap including CpG sites with differential DNA methylation between fertile and infertile individuals. Average methylation values are displayed from 0 (blue) to 1 (yellow).

Supplementary Fig. 2. Quantile–quantile (QQ) plot displaying the relationship between the average beta methylation values in blood, glia and neuron (vertical axis) with the average beta methylation values in sperm (horizontal axis).

Supplementary Fig. 3. Sperm-specific DNA methylation patterns. (A) The first column depicts deviation plots for all the CpG sites in the respective cell type, showing the variability of methylation values (coloured area). The middle column shows the relationship between means and standard deviations (log) of methylation levels between samples (M-values). The red line represents the average standard deviation. The right column depicts kernel density plots showing the distribution of methylation levels for each cell type across all CpG sites analysed. The Y-axis represents M methylation values. (B) Unsupervised hierarchical clustering and heatmap including CpG sites with tissue-specific DNA methylation in sperm, blood, neuron and glial cells.

Supplementary Fig. 4. Distribution of tissue-specific (Blood, glia, neuron, and sperm) methylated and unmethylated CpGs relative to CpG Island (CGI).



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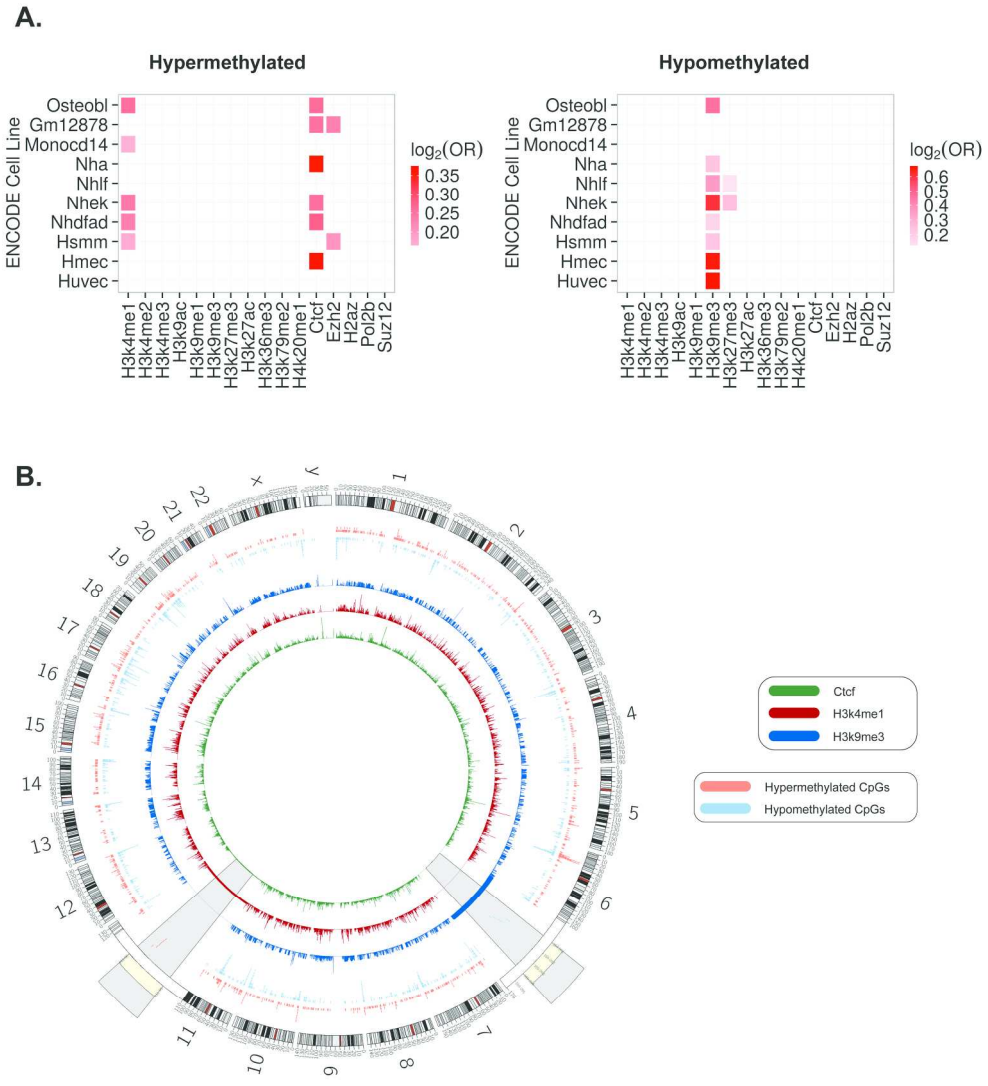

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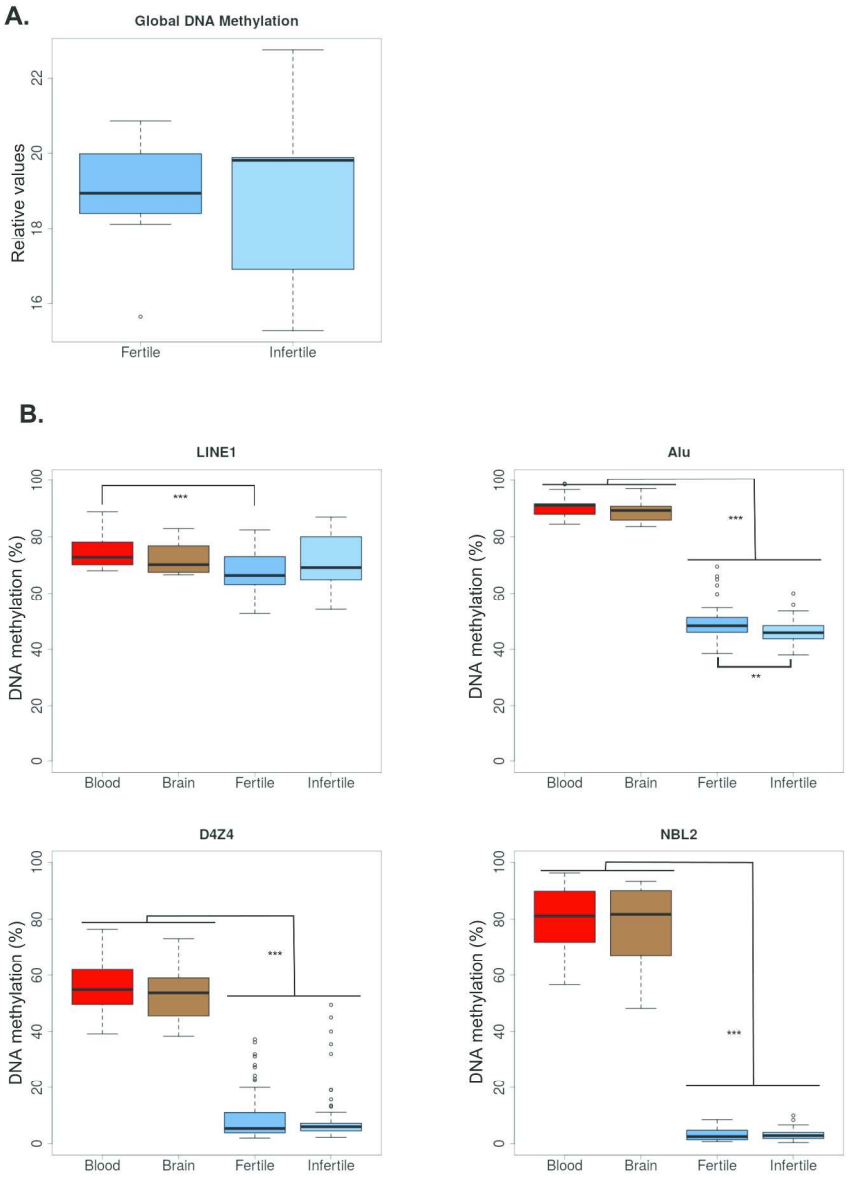

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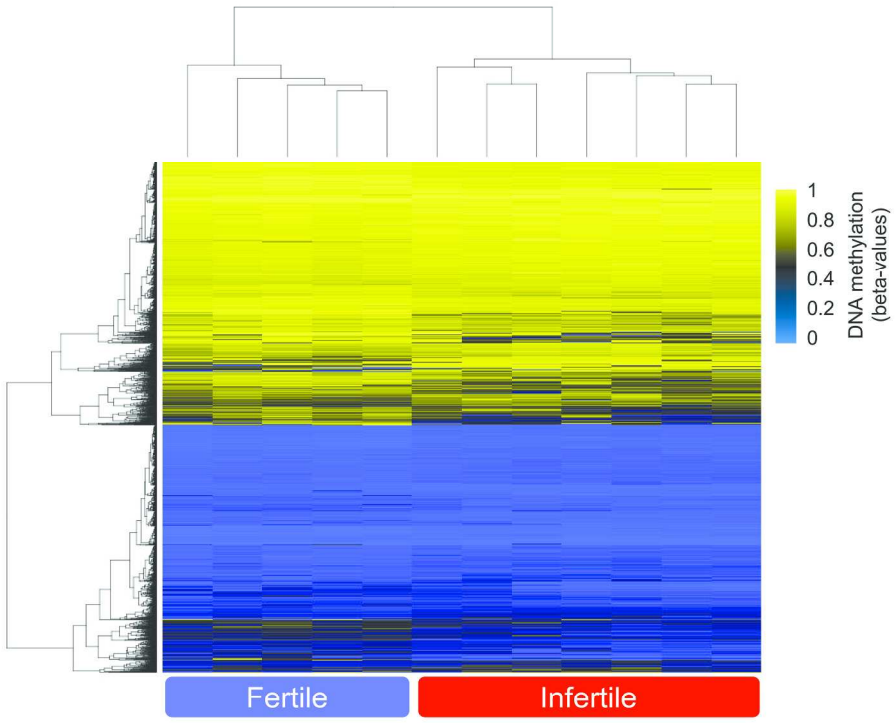
Table 1. Clinical characteristics of semen samples from fertile controls and infertile patients included in the study

Code ID	Methylation assay	Clinical intervention	IVF-ICSI outcome	Age (years)	Semen volume (ml)	Sperm count ($\times 10^6$/ml)	Total Sperm count ($\times 10^6$)	Progressive motility (%)	Normal morphology (%)	Teratozoospermia index (TZI)	Age female partner (years)	Female gynecological assesment
Control-1	1,3	Vasectomy	-	41	4.5	174	783.0	46	11	1.69	-	N (by default)
Control-2	1,3	Vasectomy	-	49	4.5	122	549.0	46	11	1.78	-	N (by default)
Control-3	1,3	Vasectomy	-	35	6.0	32	192.0	23	6	1.64	-	N (by default)
Control-4	1,3	Vasectomy	-	39	1.5	96	144.0	58	10	1.21	-	N (by default)
Control-5	1,3	Vasectomy	-	36	3.5	42	147.0	42	6	1.49	-	N (by default)
Control-6	2,3	Vasectomy	-	45	2.8	89	244.8	67	10	1.52	-	N (by default)
Control-7	2,3	Vasectomy	-	39	3.3	81	267.3	32	6	1.50	-	N (by default)
Control-8	2,3	Vasectomy	-	48	5.5	89	489.5	67	10	1.56	-	N (by default)
Control-9	2,3	Vasectomy	-	39	4.0	65	260.0	69	5	1.45	-	N (by default)
Control-10	2,3	Vasectomy	-	42	5.8	29	168.2	71	5	1.56	-	N (by default)
Control-11	2,3	Vasectomy	-	44	3.0	250	750.0	60	8	1.65	-	N (by default)
Control-12	2,3	Vasectomy	-	38	4.6	70	322.0	54	16	1.33	-	N (by default)
Control-13	3	Vasectomy	-	34	2.0	29	58.0	24	0	1.44	-	N (by default)
Control-14	3	Vasectomy	-	35	4.0	75	300.0	46	8	1.39	-	N (by default)
Control-15	3	Vasectomy	-	44	1.0	120	120.0	68	9	1.45	-	N (by default)
Control-16	3	Semen donor	-	25	3.3	80	264.0	58	24	1.30	35,2[a]	-
Control-17	3	Semen donor	-	22	4.8	78	370.5	39	14	1.32	34,1[a]	-
Patient-1	1	IVF-ICSI	No pregnancy	42	2.0	36	72.0	50	1	1.58	41	N
Patient-2	1	IVF-ICSI	regnancy-birth (twi	37	4.8	43	204.3	42	9	1.51	37	N
Patient-3	1,3	IVF-ICSI	No pregnancy	40	6.1	10	61.0	37	2	1.49	39	N
Patient-4	1,3	IVF-ICSI	No pregnancy	36	6.6	18	118.8	52	1	1.63	35	N
Patient-5	1,3	IVF-ICSI	No pregnancy	36	5.5	91	500.5	48	5	1.67	37	N
Patient-6	1,3	IVF-ICSI	Pregnancy-birth	54	3.7	25	92.5	18	1	1.57	38	N
Patient-7	1,3	IVF-ICSI	·egnancy-miscarria	39	3.9	17	66.3	46	1	1.59	35	Treated disovulation
Patient-8	2,3	IVF-ICSI	No pregnancy	45	5.0	95	475.0	40	7	1.52	40	N
Patient-9	2,3	IVF-ICSI	No pregnancy	31	2.5	98	245.0	62	7	1.43	26	Tubal obstruction
Patient-10	2,3	IVF-ICSI	No pregnancy	33	3.0	123	369.0	69	7	1.48	35	N
Patient-11	2,3	IVF-ICSI	No pregnancy	37	2.5	171	427.5	71	8	1.61	36	Tubal obstruction
Patient-12	2,3	IVF-ICSI	No pregnancy	55	2.8	92	257.6	36	6	1.52	38	N
Patient-13	2,3	IVF-ICSI	No pregnancy	34	3.6	203	722.7	55	6	1.46	34	N
Patient-14	2,3	IVF-ICSI	No pregnancy	36	4.5	61	274.5	53	8	1.57	37	N
Patient-15	3	IVF-ICSI	Pregnancy-birth	45	2.5	56	140.0	50	6	1.46	39	Mild endometriosis
Patient-16	3	IVF-ICSI	regnancy-birth (twi	39	4.0	90	360.0	60	4	1.60	37	N
Patient-17	3	IVF-ICSI	Pregnancy-birth	39	2.8	206	576.8	40	15	1.32	35	N
Patient-18	3	IVF-ICSI	regnancy-birth (twi	36	2.8	105	288.8	58	15	1.63	33	N
Patient-19	3	IVF-ICSI	·egnancy-miscarria	40	1.8	75	131.3	51	12	1.33	35	Mild endometriosis
Patient-20	3	IVF-ICSI	·egnancy-miscarria	37	3.5	202	707.0	61	12	1.44	38	Mild endometriosis
Patient-21	3	IVF-ICSI	·egnancy-miscarria	36	1.8	42	75.6	59	6	1.34	40	N
Patient-22	3	IVF-ICSI	Pregnancy-birth	32	4.8	196	931.0	48	14	1.35	27	Tubal obstruction
Patient-23	3	IVF-ICSI	No pregnancy	30	5.3	75	393.8	50	9	1.45	38	Tubal obstruction
Patient-24	3	IVF-ICSI	regnancy-birth (twi	37	2.5	87	217.5	54	11	1.54	38	Mild endometriosis
Patient-25	3	IVF-ICSI	·egnancy-miscarria	44	4.9	119	583.1	60	12	1.42	39	Disovulation
Patient-26	3	IVF-ICSI	·egnancy-miscarria	48	1.8	164	287.0	55	7	1.62	39	Mild endometriosis
Patient-27	3	IVF-ICSI	Pregnancy-birth	35	1.7	206	342.0	58	12	1.49	32	Disovulation
Patient-28	3	IVF-ICSI	No pregnancy	41	3.6	71	255.6	47	6	1.46	38	Disovulation
Patient-29	3	IVF-ICSI	Pregnancy-birth	36	7.3	92	671.6	46	8	1.52	35	Tubal obstruction
Average Controls	1			40.0	4.0	93.2	363	43.0	8.8	1.56		
Average Patients	1			40.6	4.7	34.3	159.3	41.9	2.9	1.6		
P-value	1			*0.872*	*0.514*	*0.088*	*0.205*	*0.877*	*0.005*	*0.874*		
Average Controls	2			42.1	4.1	96.1	357.4	60.0	8.6	1.5		
Average Patients	2			38.7	3.4	120.4	395.9	55.1	7.0	1.5		
P-value	2			*0.354*	*0.244*	*0.474*	*0.704*	*0.516*	*0.335*	*0.956*		
Average Controls	3			38.5	3.8	89.5	319.4	51.2	9.4	1.5		
Average Patients	3			38.9	3.7	103.3	354.5	51.3	7.7	1.5		
P-value	3			*0.853*	*0.925*	*0.445*	*0.606*	*0.985*	*0.284*	*0.753*		

N: normal; a: age average of female recipients is indicated
IVF-ICSI: in vitro fertilization-intracytoplasmic sperm injection
Methylation assay 1: Methylation array
Methylation assay 2: Global DNA methylation
Methylation assay 3: Pyrosequencing of repetitive sequences



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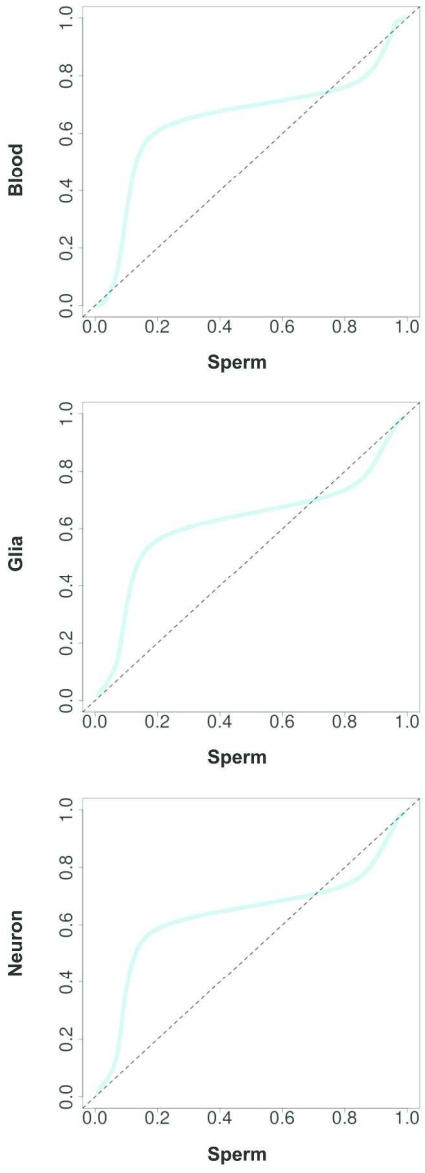

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