Human Reproduction, Vol.36, No.1, pp. 48–60, 2021
Advance Access Publication on December 15, 2020 doi:10.1093/humrep/deaa278

human reproduction

ORIGINAL ARTICLE *Early Pregnancy*

DNA methylation defects in spermatozoa of male partners from couples experiencing recurrent pregnancy loss

Kushaan Khambata[1,*], Sanketa Raut[1], Sharvari Deshpande[1], Sweta Mohan[1], Shobha Sonawane[1], Reshma Gaonkar[1], Zakiya Ansari[1], Mamata Datar[1], Vandana Bansal[2], Anushree Patil[3], Himangi Warke[4], and Nafisa H. Balasinor[1,*]

[1]Neuroendocrinology Department, Indian Council of Medical Research-National Institute for Research in Reproductive Health, Mumbai, India [2]Department of Obstetrics and Gynecology, Nowrosjee Wadia Maternity Hospital, Mumbai, India [3]Department of Reproductive Endocrinology & Infertility, Indian Council of Medical Research-National Institute for Research in Reproductive Health (ICMR-NIRRH), Mumbai, India [4]Department of Obstetrics and Gynecology, Seth G. S. Medical College & King Edward Memorial Hospital (KEM), Mumbai, India

*Correspondence address. Neuroendocrinology Department, Indian Council of Medical Research-National Institute for Research in Reproductive Health, J. M. Street, Parel, Mumbai 40012, India. E-mail: balasinorn@nirrh.res.in; nhbalasinor@gmail.com; khambatak@nirrh.res.in (N.H.B. and K.K.)

Submitted on February 13, 2020; resubmitted on September 16, 2020; editorial decision on September 23, 2020

STUDY QUESTION: What is the sperm DNA methylation status of imprinted genes in male partners from couples experiencing recurrent pregnancy loss (RPL)?

SUMMARY ANSWER: Aberrations in sperm DNA methylation status of several imprinted genes, such as insulin like growth factor-2-H19 differentially methylated region (*IGF2-H19 DMR*), intergenic differentially methylated region (*IG-DMR*), mesoderm specific transcript (*MEST*), zinc finger protein which regulates apoptosis and cell cycle arrest (*ZAC*), DMR in intron 10 of KCNQ1 gene (*KvDMR*), paternally expressed gene 3 (*PEG3*) and paternally expressed gene 10 (*PEG10*), as well as decreased sperm global 5-methylcytosine (5mC) levels, are associated with RPL.

WHAT IS KNOWN ALREADY: RPL is defined as loss of two or more pregnancies, affecting 1–2% of couples of reproductive age. Although there are several maternal and paternal aetiological factors contributing to RPL, nearly 50% of the cases remain idiopathic. Thus, there is a need to identify putative paternal factors that could be contributing towards pregnancy loss in cases of idiopathic RPL.

STUDY DESIGN, SIZE, DURATION: In this case–control study, 112 couples undergoing RPL with no identifiable cause were recruited from September 2015 to May 2018. The control group comprised of 106 healthy proven fertile couples with no history of infertility or miscarriage.

PARTICIPANTS/MATERIALS, SETTING, METHODS: In this study, we investigated the paternal genetic and epigenetic factors that could be associated with RPL. We studied DNA methylation, by pyrosequencing, of selected imprinted genes implicated in embryo development, such as *IGF2-H19 DMR*, *IG-DMR*, *MEST*, *ZAC*, *KvDMR*, *PEG3*, *PEG10* and small nuclear ribonucleoprotein polypeptide N (*SNRPN*) in sperm of men whose partners present RPL. Global DNA methylation in sperm was evaluated by studying 5mC content and long interspersed nuclear element 1 (*LINE1*) promoter methylation. We also studied polymorphisms by pyrosequencing in the *IGF2-H19 DMR* as well in the *IGF2* promoter in both groups.

MAIN RESULTS AND THE ROLE OF CHANCE: In the RPL group, we found a significant decrease in the global sperm 5mC levels and significant decrease in DNA methylation at three CpG sites in *LINE1* promoter. For *IGF2-H19 DMR* and *IG-DMR*, a significant decrease in sperm DNA methylation at specific CpG sites was observed in RPL group. For maternally imprinted genes like *MEST*, *ZAC*, *KvDMR*, *PEG3* and *PEG10* hypermethylation was noted. Polymorphism studies for *IGF2-H19 DMR* and *IGF2* revealed significant differences in the genotypic frequencies in males.

© The Author(s) 2020. Published by Oxford University Press on behalf of European Society of Human Reproduction and Embryology. All rights reserved.
For permissions, please email: journals.permissions@oup.com

Downloaded from https://academic.oup.com/humrep/article/36/1/48/6034909 by Universita' degli Studi Teramo user on 21 March 2024

LIMITATIONS, REASONS FOR CAUTION: In this study, we analysed the methylation levels of selected candidate imprinted genes implicated in embryo development. Detection of methylation changes occurring at the genome-wide level may reveal further candidate genes having a better distinction between the control and study groups.

WIDER IMPLICATIONS OF THE FINDINGS: Our study demonstrates that certain polymorphisms and aberrant sperm methylation status in imprinted genes are associated with RPL and could contribute to the aetiology of RPL. This study suggests that investigation of paternal genetic and epigenetic factors could be useful in identification of possible causes of idiopathic RPL.

STUDY FUNDING/COMPETING INTEREST(S): This study was funded by Department of Science and Technology-Science and Engineering Research Board (EMR/2014/000145) and National Institute for Research in Reproductive Health intramural funds (RA/872/01-2020). All authors declare no conflict of interest.

TRIAL REGISTRATION NUMBER: N/A.

Key words: recurrent pregnancy loss / spermatozoa / DNA methylation / imprinted genes / polymorphism

Introduction

Recurrent pregnancy loss (RPL) is defined as loss of two or more consecutive pregnancies before the 20th week of gestation and affects globally about 1–2% of women (Atik *et al.*, 2018). In the Indian population, the prevalence of RPL has been reported to be as high as 7.46% (Ameet and Chauhan, 2016). RPL is a multifactorial condition with male and female aetiological factors. Females factors include genetic (chromosomal aberrations), anatomical (uterine abnormalities), endocrine (hyperthyroidism, hyperprolactinaemia and hyperinsulinaemia), immunological (presence of autoantibodies), infections (toxoplasma, cytomegalovirus) and haematological (thrombophilia) causes, all of which are extensively investigated and shown to be strongly associated with RPL (Meka and Reddy, 2006; Ford and Schust, 2009). Evidences suggest that RPL is also associated with certain male factors, mainly paternal age, abnormal semen parameters and sperm DNA damage (Remy *et al.*, 2005; Kazerooni *et al.*, 2009). However, 50% of the RPL cases still remain idiopathic (Ford and Schust, 2009). The role of paternal genome in mammalian embryo development and placentation was demonstrated by nuclear transplantation experiments (McGrath and Solter, 1984; Surani *et al.*, 1987). These experiments also highlighted the role of genomic imprinting in embryo development since neither gynogenetic nor androgenetic embryo reached to term. Genomic imprinting is defined as monoallelic expression of genes in parent of origin-specific manner. It is mainly brought about by DNA methylation, which differentially marks or 'imprints' the two parental alleles, thus enabling the distinction between them. Imprinted genes are characterized by the presence of differentially methylated regions (DMRs), where the repressed allele is methylated. Imprinted genes are known play an important role in embryo and placental development and growth. The imprint marks are established during gametogenesis, maintained and read during embryogenesis and throughout life of an individual. Thus, aberrations in the methylation patterns of the imprinted genes in the gametes can affect the expression of these genes in the embryo, thereby affecting its growth and development. Studies in rodents and humans have shown that abnormal genomic imprinting in the paternal genome can lead to failed fertilization, poor embryo development and implantation and foetal loss (Cattanach and Kirk, 1985; Soejima and Wagstaff, 2005). Furthermore, alterations in the methylation and hence expression pattern of imprinted genes have been associated to early pregnancy loss (Dória *et al.*, 2010; Pliushch *et al.*, 2010; Zechner *et al.*, 2010). Although aberrant methylation of imprinted genes in spermatozoa have been associated with male infertility (Marques *et al.*, 2004, 2008; Kobayashi *et al.*, 2007; Hammoud *et al.*, 2010), its association with RPL needs to be investigated. We had previously studied the methylation of a few imprinted genes, such as the insulin like growth factor-2-H19 differentially methylated region (*IGF2-H19 DMR*), intergenic differentially methylated region (*IG-DMR*), mesoderm specific transcript (*MEST*) and zinc finger protein which regulates apoptosis and cell cycle arrest (*ZAC*), in sperm in a small cohort of men whose partners have a history of RPL. However, the study was limited due to small sample size (Ankolkar *et al.*, 2012, 2013). In the present study, we investigated a larger sample size and used more sensitive detection techniques to analyse the methylation status of those imprinted genes in sperm and their association with RPL. Additionally, we have also investigated methylation levels of other imprinted loci such as the DMR in intron 10 of KCNQ1 gene (*KvDMR*), paternally expressed gene 3 (*PEG3*), paternally expressed gene 10 (*PEG10*) and small nuclear ribonucleoprotein polypeptide N (*SNRPN*). The paternally imprinted loci of *IGF2-H19 DMR* and IG-*DMR* are methylated in the sperm, while the DMRs of maternally imprinted genes of *MEST*, *ZAC*, *KvDMR*, *PEG3*, *PEG10* and *SNRPN* are unmethylated in the sperm (Tycko and Morison, 2002). We also studied the polymorphisms in *IGF2-H19 DMR* and in the *IGF2* promoter in the blood of males, as these have been reported to be associated with RPL susceptibility (Ostojic *et al.*, 2008). We also assessed the sperm global methylation status, serum folate levels, and the sperm methylation pattern in the methylenetetrahydrofolate reductase (*MTHFR*) promoter.

Materials and methods

Ethics approval and consent to participate

Prior to patient recruitment, ethical permissions were obtained from Institutional Ethics Committees of all the institutions. Written informed consent was obtained from all study participants before collection of blood and semen samples, in accordance with current edition of the NIRRH Ethics Committee for Clinical Studies (Ref: D/ICEC/Sci-54/56/2018 dated May 29, 2018). This committee is recognized by Strategic Initiative for Developing Capacity in Ethical Review (SIDCER) and Forum for Ethics Review Committees in Asia and the Western Pacific Region (FERCAP).

Downloaded from https://academic.oup.com/humrep/article/36/1/48/6034909 by Universita' degli Studi Teramo user on 21 March 2024

Participant recruitment and sample collection

Participants were recruited as described in our previous study (Ankolkar et al., 2012, 2013). Participants were recruited from Infertility Clinic at ICMR-National Institute for Research in Reproductive Health (ICMR-NIRRH) or from Gynecology outpatient departments of KEM Hospital and Nowrosjee Wadia Maternity Hospital (Parel, Mumbai, India) after informed consent. Participants were divided into control and RPL groups. Considering the prevalence of RPL as 7.48% in the Indian population, the sample size was calculated to be 107 in each group using the online calculation software (https://select-statistics.co.uk/calculators/sample-size-calculator-population-proportion/) with a confidence level of 95%.

A total of 218 couples were recruited for the study, 106 controls and 112 with RPL, from September 2015 to May 2018. The control group included couples having a normal conception and live birth in the past 1 year without any history of infertility or miscarriage. The RPL group was comprised of couples who have had two or more consecutive spontaneous miscarriage or pregnancy loss before 20 weeks of gestation without known causes. Couples where the women had infectious, hormonal or haematologic causes of miscarriages were excluded from the study. Also, couples with karyotype abnormalities in either of the partners were excluded from the study. Semen samples were obtained by masturbation after 3–5 days of sexual abstinence. Blood samples were collected from both partners. DNA extracted from whole blood was used for genotype studies, whereas the serum was separated for estimation of folate levels.

Semen analysis

Basic semen parameters, sperm concentration, motility and morphology, and chromatin compaction were analysed according to the World Health Organization (WHO) manual for semen analysis (World Health Organization, 2010) as previously described. Sperm concentration was measured using a Neubauer hemocytometer and sperm motility was assessed by computer-assisted semen analysis after appropriately diluting the sample. For sperm morphology, air-dried sperm smears were subjected to Papanicolaou staining and the number of spermatozoa with normal head and tail morphology were counted. Chromatin compaction status was assessed by chromomycin A3 (CMA3) (Sigma-Aldrich) staining as described by Bianchi et al. (1993).

Sperm genomic DNA extraction

Semen samples with normal sperm parameters were washed and processed to obtain pure sperm sample (devoid of any round cells or leucocytes) as previously described (Ankolkar et al., 2012). Genomic DNA was extracted from 10×10^6 sperm using the HiPurA Sperm Genomic DNA Purification Kit (HiMedia) as per manufacturer's instructions. Briefly, the spermatozoa were lysed using a sperm lysis buffer containing proteinase K and 1,4-dithiothreitol and then incubated at 55°C for 2 h with intermittent vortexing. The resulting lysate was subjected to RNase A treatment and the DNA was precipitated using chilled ethanol, washed and eluted in nuclease free water. DNA concentration was determined in an UV spectrophotometer by calculating ratio of absorbance of DNA at 260 and 280 nm. Pure DNA samples (with 260/280 ratios of ~1.6–1.8) were processed further for global 5-methylcytosine (5mC) content estimation and bisulfite modification.

Sperm global 5mC estimation

The sperm global 5mC content was estimated using 5mC DNA ELISA kit from Zymo Research (Irvine, CA, USA) as per manufacturer's instructions. Briefly, 100 ng of sperm genomic DNA samples (in coating buffer) were denatured and coated onto the microtitre plate at 37°C for 1 h, followed by blocking. A standard curve ranging from 0% to 100% 5mC was generated by preparing mixtures of negative and positive DNA controls provided in the kit. Diluted anti-5mC along with secondary antibody was added and incubated at 37°C for 1 h, followed by washing. The detection reaction was performed using the HRP Developer provided and the absorbance was read at 450 nm on a plate reader and relative global methylation levels were calculated from the standard curve. The amount of methylated DNA present in the sample is proportional to the absorbance measured. Experiments were performed in duplicates.

Methylation analysis of sperm DNA by bisulfite pyrosequencing

Sperm genomic DNA was subjected to bisulfite modification using MethylCode Bisulfite Conversion Kit (Invitrogen, Carlsbad, CA, USA) as per manufacturer's protocol. Briefly, 1 µg of genomic DNA was subjected sequentially to denaturation, sulphonation, deamination and desulphonation to convert the unmethylated cytosines to uracil, while the methylated cytosines were unchanged. Modified DNA was eluted in 16 µl nuclease free water and used for PCR amplification. PCR was performed with primers specific for modified sequences of long interspersed nuclear element 1 (*LINE1*) promoter, *IGF2-H19 DMR*, *IG-DMR*, *MEST*, *ZAC*, *KvDMR*, *PEG3*, *PEG10*, *SNRPN* and *MTHFR* regions (Sigma, Bangalore, India) as described in Supplementary Table SI using Pyromark PCR Amplification Kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. All PCRs were carried out in presence of 2.5 mM $MgCl_2$ (except for *MEST* and *SNRPN*). Unmodified genomic DNA samples were used to confirm primer specificity for modified DNA during primer optimization. Amplification conditions used initial denaturation for 15 min at 95°C, followed by 45 cycles of denaturation at 95°C for 30 s, primer annealing for 30 s (at the specified temperature mentioned in Supplementary Table SI), extension at 72°C for 30 s and final extension at 72°C for 10 min. For *IGF2-H19 DMR*, a step-down PCR was performed as described by Ankolkar et al. (2012). The PCR products were then bound to streptavidin-coated Sepharose Beads (GE Healthcare, Uppsala, Sweden) in the presence of binding buffer (Qiagen). Bound PCR products were then denatured and the non-biotinylated strand was washed off. The bound biotinylated strand was then annealed to the sequencing primer and the reaction mixture was subjected to pyrosequencing in PyroMark Q96 ID (Qiagen). The sequencing results were analysed using the Pyromark Q96 software.

SNP analysis by pyrosequencing

To study the polymorphisms in *IGF2* promoter (rs680) and *IGF2-H19 DMR* (rs10732516), genomic DNA from male and female partners was extracted from whole blood using QIAamp DNA blood mini kit

Downloaded from https://academic.oup.com/humrep/article/36/1/48/6034909 by Universita' degli Studi Teramo user on 21 March 2024

(Qiagen). The DNA extracted was then amplified using primers spanning the polymorphic region (mentioned in Supplementary Table SI), the amplicon was then subjected to pyrosequencing, and the data were finally analysed in the single-nucleotide polymorphism (SNP) mode of Pyromark Q96 software.

Estimation of serum folate levels

Quantitative estimation of serum folate levels was performed by competitive ELISA assay using the Folate ELISA kit (Bio-Detect, Laguna Hills, CA, USA) as per the manufacturer's instructions. Briefly, serum samples were incubated with biotin-labelled antibody against folate along with enzyme labelled folate solution. After 60 min of incubation, microwells were washed to remove excess serum proteins and enzyme. A substrate solution was added to generate an inversely proportional colour signal. From specified calibrator concentrations plotted on a graph, the folate concentrations in the unknown samples were calculated. All samples were run in duplicates.

Statistical analysis

All statistical analysis was carried out using GraphPad Prism Software Version 8.0.2 (GraphPad Software, San Diego, USA). All methylation data are represented as means ± SEM. The level of significance was set as $P < 0.05$. The values in control and RPL groups were compared by the non-parametric Mann–Whitney *U* test. The Hardy–Weinberg equilibrium was determined using the online calculator (https://wpcalc.com/en/equilibrium-hardy-weinberg/). For genotype studies, data were analysed using the χ^2 test. The diagnostic performance of global 5mC levels and the methylation levels of imprinted genes was determined by receiver operating characteristics (ROC) curves. The average methylation of the differentially methylated sites was subjected to ROC analysis to obtain sensitivity and specificity values. Youden's index (sensitivity + specificity − 1) (Habibzadeh *et al.*, 2016) was calculated to determine the points on the ROC curve having maximum sensitivity and specificity. Diagnostic efficiency, which is a measure of the ability of the specific parameter to identify or rule out the presence of a given condition (Lippi and Mattiuzzi, 2015) was also calculated (Madan *et al.*, 2004). The threshold or cut-off values having >50% sensitivity and specificity, high Youden's index and diagnostic efficiency were selected. The *post hoc* power of each of the above parameters was calculated using the ClinCal online software (https://clincalc.com/Stats/Power.aspx).

Results

Semen analysis

Semen parameters showed a wide variation in both groups and were within the normal limits according to the WHO reference values (Cooper *et al.*, 2010). There were no significant differences in semen parameters between the groups (Table I). Other semen parameters such as semen volume, pH, and colour were also within the normal range and comparable between groups. There were also no significant differences between groups regarding the mean male age (RPL group: 30.26 ± 0.55 years; Control group: 30.67 ± 0.47 years) and the mean female age (RPL group: 25.70 ± 0.45; Control group: 26.89 ± 0.43) at first conception.

Global and imprinted genes methylation analysis

There was a significant reduction in sperm global 5mC levels in the RPL group (Control: 0.47 ± 0.01 vs RPL: 0.38 ± 0.01; $P < 0.0001$) (Fig. 1A).

LINE1 promoter methylation was also assessed as a surrogate marker for global DNA methylation (Yang *et al.*, 2004). A total of six CpG sites were analysed, of which three sites at CpG positions 3, 4 and 5 showed significant hypomethylation in the RPL group (Fig. 1B). However, the total methylation in the *LINE1* promoter was not significantly different between the two groups.

In the *IGF2-H19 DMR*, of the 15 CpG sites in the amplified region, the 8th CpG site harboured the rs10732516 (C/T) polymorphism was thus excluded from methylation analysis as it would become uninformative after bisulfite conversion. Of the remaining 14 CpG sites, five sites, at CpG positions 1, 2, 11, 13 and 14 were found to be significantly hypomethylated in the RPL group. However, no significant differences were observed in total methylation status of *IGF2-H19 DMR* between the groups (Fig. 1C).

For *IG-DMR*, of the seven CpG sites analysed, four sites, at CpG positions 1, 2, 4 and 5, showed significant hypomethylation in the RPL group. However, the total methylation of *IG-DMR* was not significantly different between the two groups (Fig. 1D).

Among the maternally imprinted genes, methylation status of DMRs of *MEST*, *ZAC*, *KvDMR*, *PEG10*, *PEG3* and *SNRPN* were studied. These genes are commonly unmethylated in the sperm.

In *MEST*, six CpG sites were analysed and three sites, at CpG positions 1, 2 and 6, were found to be significantly hypermethylated in the RPL group. The total methylation level of *MEST* was also significantly increased in the RPL group (Fig. 2A).

For *ZAC*, five CpG sites were analysed and three sites, at CpG positions 1, 2 and 3, were found to be significantly hypermethylated in RPL group. The total methylation level of *ZAC* was also significantly increased in the RPL group (Fig. 2B).

In *KvDMR*, nine CpG sites were evaluated and all of them appeared significantly hypermethylated in the RPL group. The total methylation levels in RPL were also significantly increased in the RPL group (Fig. 2C).

For *PEG10*, five CpG sites were studied. Although hypermethylation was noted at all the five sites, it was significant only at the CpG position 5 in the RPL group. The total methylation levels of *PEG10* were also significantly increased in the RPL group (Fig. 2D).

In case of *PEG3*, 11 CpG sites were evaluated, and significant hypermethylation was noted at CpG positions 1, 2, 4, 5, 6, 9, 10 and 11 in the RPL group. The total methylation levels were also significantly increased in the RPL group (Fig. 2E).

For *SNRPN*, we evaluated 11 CpG sites and no significant differences in methylation were observed at any of the CpG positions between the groups. The total methylation levels were also comparable between the groups (Fig. 2F).

Downloaded from https://academic.oup.com/humrep/article/36/1/48/6034909 by Universita' degli Studi Teramo user on 21 March 2024

Table I Summary of semen characteristics in control and recurrent pregnancy loss (RPL) groups.

Parameter	Control	RPL	*P*-value
Sperm concentration (millions/ml)	61.3 (22.85–330)	76.75 (23.52–292)	0.868
Total sperm motility (%)	75 (35–86.5)	71 (30.3–81.5)	0.946
Sperm morphology (%)	25.6 (8.39–47.26)	24.19 (8.26–47.32)	0.336
Percentage of sperm with normal chromatin compaction (chromomycin A3 analysis)	32.37 (21.94–61.13)	28.16 (17.19–57.22)	0.11

The values for all parameters are represented as median and the numbers in parenthesis denote the range from 5th to 95th percentile.

Genotype analysis for SNPs

The genotype frequency refers to the frequency of a particular genotype (homozygous or heterozygous) frequency in the population; whereas the allelic frequency denotes the occurrence of a specific allele (A/G or C/T). The genotype distribution for rs680 (in the *IGF2* promoter) (χ^2 = 3.51; P = 0.06) and rs10732516 (in the *IGF2-H19* DMR) (χ^2 = 1.05; P = 0.305) polymorphisms followed the Hardy–Weinberg equilibrium. There were significant differences in the genotype frequencies for rs680 and rs10732516 polymorphisms in the males in the RPL group (Table II). No significant differences were observed in allelic frequency for both the SNPs in males of both groups. There were also no significant differences in the genotype or allelic frequencies for both SNPs in the females of both groups (Table II).

Serum folate levels and methylation analysis of *MTHFR* promoter

There were no significant differences in the serum folate levels in male and female partners in both the groups (Fig. 3A). Upon methylation analysis of the *MTHFR* promoter in sperm, we did not observe any difference in the total and site-specific methylation status of the six CpG sites analysed (Fig. 3B).

ROC analysis

In order to evaluate whether sperm DNA methylation levels of imprinted genes could be used as a diagnostic tool to determine if aberrant methylation pattern can be a factor contributing to RPL, a ROC analysis was carried out.

The average methylation of the differentially methylated sites in the imprinted genes was calculated for control and RPL groups, i.e. sites 1, 2, 11, 13 and 14 for *IGF2-H19 DMR*; sites 1, 2, 4 and 5 for *IG-DMR*; sites 1, 2 and 6 for *MEST*; sites 1, 2 and 3 for *ZAC*; and sites 1, 2, 4, 5, 6, 9, 10 and 11 for *PEG3*. For *KvDMR* and *PEG10*, total methylation of all the sites was used for ROC analysis.

Results from ROC analysis are summarised in Table III and the ROC curves are depicted in Supplementary Fig. S1. The area under the curve (AUC) for all the parameters was significant. ROC analysis of global 5mC levels revealed the highest AUC (0.69) and diagnostic efficiency (65.6). ROC analysis for the average methylation of the differentially methylated sites revealed highest AUC values for *IG-DMR* and for the *IGF2-H19 DMR*. Amongst the imprinted genes, *IGF2-H19 DMR* had the highest diagnostic efficiency of 61.47. *Post hoc* calculation of the power of the study parameters revealed high power values (>95) for 5mC levels, *IGF2-H19 DMR*, *ZAC* and *KvDMR*.

Discussion

At present, there are very few male factors known to contribute to the aetiology of RPL and even fewer diagnostic or therapeutic interventions available for unexplained RPL (Atik *et al.*, 2018). Thus, there is a need to identify the factors determining the quality of male gametes, which may be contributing to RPL. Our study is an attempt in this direction, where we analysed DNA methylation pattern of various imprinted genes involved in embryo development in sperm samples from couples experiencing idiopathic RPL compared to control couples. In a previous pilot study, we have investigated the methylation status of selected imprinted genes using bisulfite sequencing and Epityper techniques (Ankolkar *et al.*, 2012, 2013). While we noted changes in the methylation levels of *IGF2-H19 DMR* (Ankolkar *et al.*, 2012), we could not detect changes in *IG-DMR*, *MEST* and *ZAC* (Ankolkar *et al.*, 2013). In the present study, we employed a more sensitive detection method, pyrosequencing, which can analyse the methylation levels in site-specific manner as compared to the Epityper technique (Busato *et al.*, 2018). For this, we also recruited a larger sample size, which is reflected in the higher statistical power values for the parameters studied. Our study suggests that decreased sperm global methylation levels and aberrant sperm methylation patterns in the imprinted genes like the *IGF2-H19 DMR*, *IG-DMR*, *MEST*, *ZAC*, *KvDMR*, *PEG10* and *PEG3* are associated with RPL. Several studies have evaluated the association of semen parameters with RPL and have presented conflicting findings. While the sperm concentrations are largely comparable between control and RPL groups, several studies have reported a decrease in sperm motility and percentage of sperm having normal morphology (Bhattacharya, 2008; Kazerooni *et al.*, 2009; Imam *et al.*, 2011; Zhang *et al.*, 2012; Ruixue *et al.*, 2013; Zidi-Jrah *et al.*, 2016; Cao *et al.*, 2017). On the other hand, some studies have reported no difference in the general semen parameters of sperm concentration, motility and morphology (Hill *et al.*, 1994; Sbracia *et al.*, 1996; Eisenberg *et al.*, 2017). In our present study, we have not found any statistically significant difference in the sperm concentration, motility and morphology, which is similar to the results of our earlier study (Ankolkar *et al.*, 2012). Some studies have reported an increase in abnormal sperm chromatin integrity and compaction in RPL groups either via aniline blue or chromomycin A3 (CMA3) staining, both of which detect defects in chromatin compaction (Kazerooni *et al.*, 2009; Zhang *et al.*, 2012; Ruixue *et al.*, 2013; Zidi-Jrah *et al.*, 2016). We found a non-significant reduction in the percentage of sperm with normal chromatin compaction in the RPL group by CMA3 staining. Although increasing paternal age is known to increase the risk of first trimester abortions (Remy *et al.*, 2005), several studies have

Downloaded from https://academic.oup.com/humrep/article/36/1/48/6034909 by Universita' degli Studi Teramo user on 21 March 2024

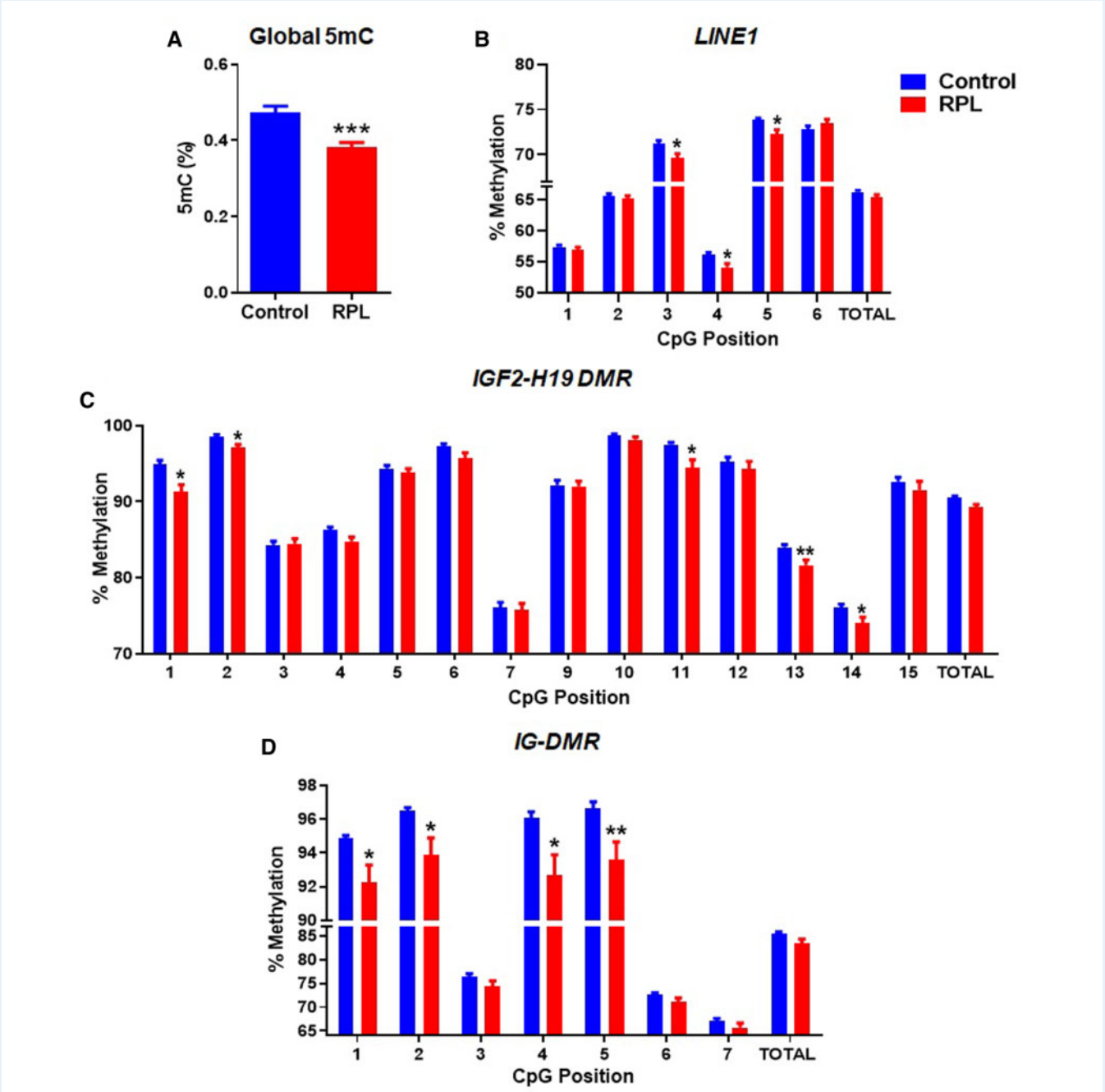


Figure 1. Global and methylation analysis of paternally imprinted DMRs. Sperm global 5mC levels (**A**) and site-wise methylation analysis of *LINE1* promoter (**B**) *IGF2-H19 DMR* (**C**) and *IG-DMR* (**D**), in control (n = 106) and RPL (n = 112) groups. All results are represented as mean ± SEM. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. IG-DMR, intergenic differentially methylated region; IGF2-H19 DMR, insulin like growth factor-2-H19 differentially methylated region; LINE1, long interspersed nuclear element 1.

found no difference in the paternal age in RPL and control groups (Sbracia *et al.*, 1996; Bhattacharya, 2008; Kazerooni *et al.*, 2009; Imam *et al.*, 2011; Zhang *et al.*, 2012; Zidi-Jrah *et al.*, 2016). Similarly, we have also noted no significant differences in the mean maternal and paternal ages at the time of first conception between RPL and control groups.

During foetal life, the methylation marks on the imprinted genes are erased in the primordial germ cells. These marks are re-established during spermatogenesis up to the primary spermatocyte stage and faithfully maintained through the cell divisions by the DNA methyltransferases (Marques *et al.*, 2011a,b). These imprint marks in the sperm are crucial for determining the allele-specific expression of these

Downloaded from https://academic.oup.com/humrep/article/36/1/48/6034909 by Universita' degli Studi Teramo user on 21 March 2024

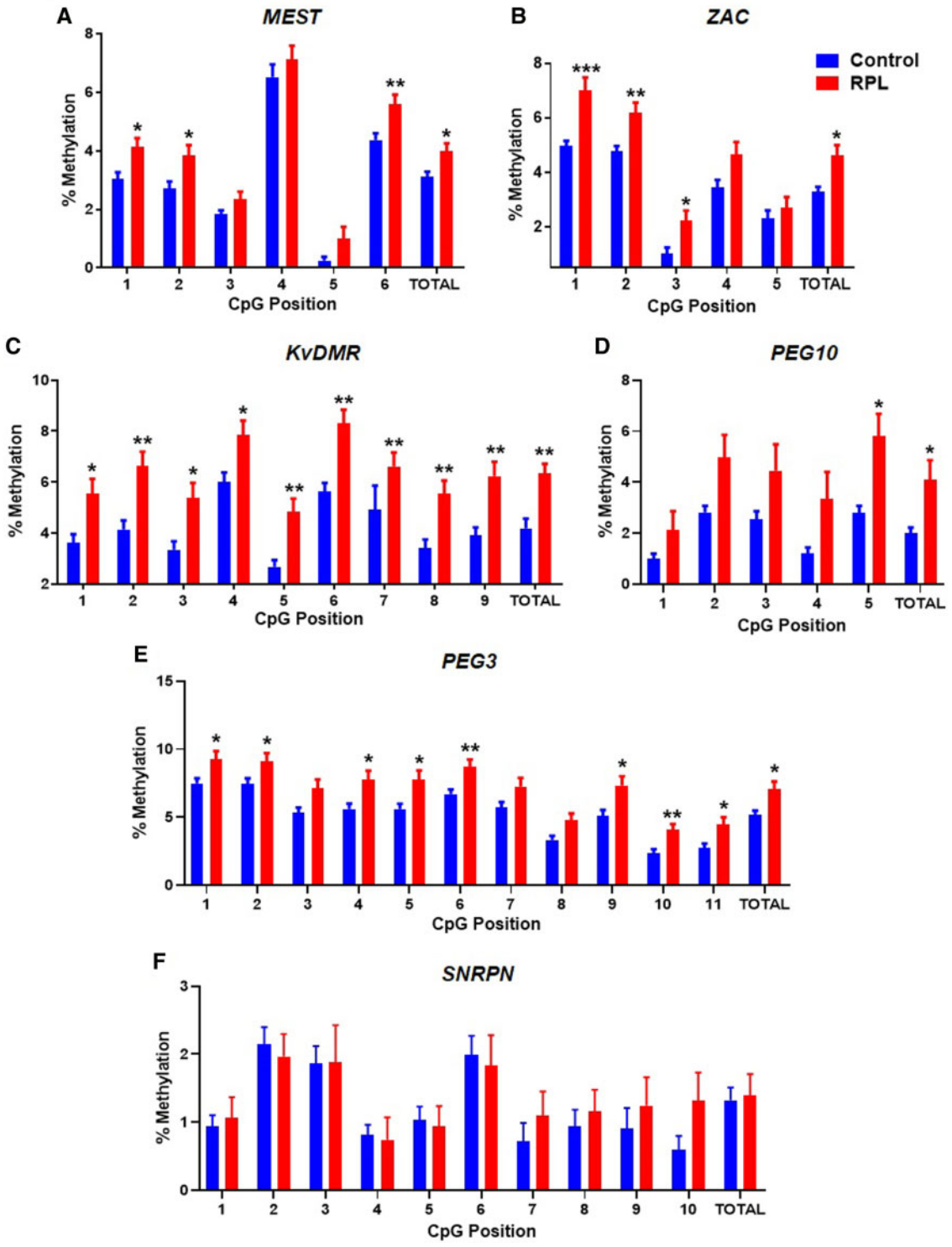


Figure 2. Methylation analysis of maternally imprinted DMRs. Site-wise methylation analysis of *MEST* (**A**), *ZAC* (**B**), *KvDMR* (**C**), *PEG10* (**D**), *PEG3* (**E**) and *SNRPN* (**F**) genes in sperm samples in control (n = 106) and RPL (n = 112) groups. All results are represented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. KvDMR, DMR in intron 10 of KCNQ1 gene; MEST, mesoderm specific transcript; PEG10, paternally expressed gene 10; PEG3, paternally expressed gene 3; SNRPN, small nuclear ribonucleoprotein polypeptide N; ZAC, zinc finger protein which regulates apoptosis and cell cycle arrest.

Downloaded from https://academic.oup.com/humrep/article/36/1/48/6034909 by Universita' degli Studi Teramo user on 21 March 2024

Table II SNP analysis: genotype and allelic distribution of rs680 and rs10732516 polymorphisms in males and females in the control and RPL groups.

		Males						Females					
		Control (n = 89)		RPL (n = 88)				Control (n = 83)		RPL (n = 79)			
		n	%	n	%	χ^2	*P*-value	n	%	n	%	χ^2	*P*-value
rs680 (A/G)	AA	37	41.57	25	28.41	6.924	0.035	30	36.14	30	37.97	0.098	0.952
	AG	33	37.08	50	56.81			38	45.78	36	45.57		
	GG	19	21.35	13	14.77			15	18.07	13	16.46		
	A	107	60.12	100	56.82	0.3955	0.529	98	59.04	96	60.76		
	G	71	39.88	76	43.18			68	40.96	62	39.24		
rs10732516 (C/T)	CC	55	61.79	50	56.81	9.094	0.015	45	54.21	51	64.55	2.129	0.345
	CT	27	30.33	38	43.18			32	38.55	22	27.84		
	TT	7	7.86	0	0			6	7.22	6	7.59		
	C	137	76.97	138	78.4	0.1063	0.744	122	73.49	124	78.48		
	T	41	23.03	38	21.6			44	26.51	34	21.52		

$P < 0.05$ is considered significant. χ^2 values for comparing genotype frequencies were performed by 2×3 analysis; χ^2 values for comparing allelic frequencies were performed by 2×2 analysis.

Table III Receiver operating characteristics (ROC) and power analysis for average methylation of differentially methylated sites in the imprinted genes and 5-methylcytosine (5mC) levels.

	AUC	SEM	*P*-value	Threshold	Sensitivity %	Specificity %	Youden's index	Diagnostic potential	Power
5mC	0.69	0.049	0.0001	<0.3897	57.58	74.58	32.16	65.6	99.2
LINE1	0.58	0.042	0.044	<66.83	56.82	52.63	9.45	52.1	86.7
IGF2-H19 DMR	0.64	0.038	0.0002	<88.50	54.46	68.87	23.33	61.47	99.5
IG-DMR	0.65	0.038	0.0001	<96.13	56.48	63.46	19.94	60.19	78.3
MEST	0.60	0.038	0.015	>3.500	50.91	58.49	9.4	54.63	88.1
ZAC	0.63	0.039	0.0009	>3.833	50.91	70.19	21.1	60.28	96.6
KvDMR	0.62	0.042	0.008	>4.833	51.09	63.1	14.19	56.89	95.7
PEG10	0.59	0.041	0.0218	>1.900	57.95	55.1	13.05	58.06	76.4
PEG3	0.59	0.039	0.0269	>4.938	57.43	59.41	16.84	58.42	82

AUC, area under curve; H19 DMR, insulin like growth factor-2-H19 differentially methylated region; IG-DMR, intergenic differentially methylated region; LINE1, long interspersed nuclear element 1; MEST, mesoderm specific transcript; PEG10, paternally expressed gene 10; PEG3, paternally expressed gene 3; ZAC, zinc finger protein which regulates apoptosis and cell cycle arrest.

genes, which would subsequently determine the growth and development of the embryo (Trasler, 2009). Initially, to get a bird's eye view of the methylation status, we analysed sperm global methylation levels by evaluating the 5mC levels and *LINE1* promoter methylation in sperm samples. Sperm global methylation levels were found to be significantly reduced in the RPL group. Decreased global 5mC levels in sperm have been associated with poor pregnancy outcomes in patients undergoing *in-vitro* fertilization, which underlines the importance of normal DNA methylation patterns in embryo development (Benchaib *et al.*, 2005). Decreased global DNA methylation levels in the embryonic villous samples, and not in maternal decidua, were associated with abnormal embryo development and early pregnancy loss (Yin *et al.*, 2012). This study demonstrated that defects in DNA methylation in the embryo, and not in the mother, are associated with early pregnancy loss. However, the global methylation levels in corresponding spermatozoa samples were not investigated (Yin *et al.*, 2012). Our earlier rodent studies have demonstrated that decreased global methylation levels in spermatozoa are associated with post-implantation embryo loss (Dumasia *et al.*, 2017). It is plausible that the decreased sperm global 5mC levels observed in this study could contribute to aberrant methylation patterns in the resulting embryo, leading to pregnancy loss. Further studies are underway to investigate methylation patterns in the chorionic villi in cases of early pregnancy loss. Comparing the global DNA methylation and *LINE-1* methylation levels of the control spermatozoa with the other studies, we observed that there were variations in the percent global DNA methylation in the control spermatozoal samples (Supplementary Table SII). These variations in the DNA methylation levels could be attributed to the difference in the species and the techniques used to assess the global DNA methylation in the control spermatozoa (El Hajj *et al.*, 2011; Smith

Downloaded from https://academic.oup.com/humrep/article/36/1/48/6034909 by Universita' degli Studi Teramo user on 21 March 2024

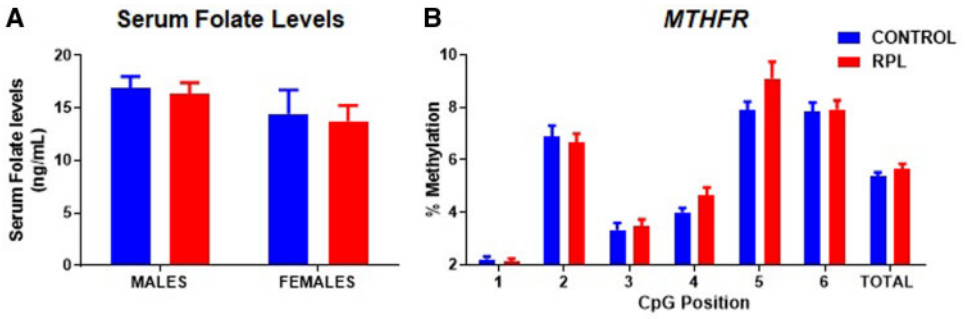


Figure 3. Serum folate levels and MTHFR methylation in control and RPL couples. Serum folate levels in male and female partners (**A**) and sperm methylation analysis of methylenetetrahydrofolate reductase (*MTHFR*) promoter (**B**) in control (n = 106) and RPL (n = 112) groups. All results are represented as mean ± SEM.

et al., 2012; Ankolkar et al., 2013; Urdinguio et al., 2015; Li et al., 2018; Fang et al., 2019; Luján et al., 2019).

We then evaluated the methylation status of certain selected imprinted genes implicated in embryo growth and development. Insulin like growth factor-2 (*IGF2*) is a potent mitogen regulating embryonic and placental growth. Mutations in *IGF2* have been known to cause intrauterine foetal growth restriction in mice (DeChiara et al., 1991). *H19* encodes for a long non-coding RNA which is known to suppress embryo growth (Gabory et al., 2009; Koukoura et al., 2011). *IGF2* is paternally expressed, whereas *H19* is maternally expressed, and together they represent one of the most widely studied imprinted loci. The expression of these genes is controlled by a common DMR, which harbours seven binding sites for an insulator protein, CCCTC-binding factor (CTCF). In paternal allele, sixth CTCF binding site is methylated, due to which CTCF protein cannot bind to the DMR and *H19* gene is silenced and *IGF2* is expressed; on the maternal allele the CTCF site is unmethylated and the expression patters are reversed (Takai et al., 2001). Although we did not observe changes in methylation at the sixth CTCF site (CpG positions 5–8 in this study), we found the CpG sites flanking this region (i.e. CpGs 1, 2, 11, 13 and 14) hypomethylated in RPL sperm samples as compared to controls. We also found decreased methylation at this DMR site in our earlier study (Ankolkar et al., 2012). Thus, hypomethylation at this locus could affect the expression of crucial imprinted genes during embryogenesis, and thus contribute to embryo loss in RPL cases. Aberrant methylation of this locus in spermatozoa is well documented in a number of studies and meta-analyses and has been associated with oligospermia and male infertility (Marques et al., 2004, 2008, 2010; Kobayashi et al., 2007; Marques and Sousa, 2013; Santi et al., 2017; Tang et al., 2018).

Along with the methylation analysis of the *IGF2-H19 DMR*, we also investigated genetic polymorphisms in the sixth CTCF binding site of this DMR (rs10732516) and in the 3′UTR of the *IGF2* gene (rs680). Interestingly, we found significant differences in the genotype frequency of these two polymorphisms in men between RPL and control groups, but not in the women. Ostojic *et al.* have reported significant differences in the *IGF2* rs680 polymorphism and not in the *H19* rs10732516 polymorphism in male partners of couples undergoing idiopathic RPL, as compared to controls, in the Slavic population, while no significant differences were noted between women of the two groups for both polymorphisms (Ostojic et al., 2008). Our results along with this study further strengthen the association of these polymorphisms in men with RPL and that the paternal transmission of the susceptible allele in these polymorphisms might contribute to RPL susceptibility.

Like *IGF2-H19 DMR*, the *IG-DMR* also represents one of the paternally imprinted loci, which controls the expression of two imprinted genes, delta like non-canonical Notch ligand 1 (*DLK1*) and *GTL2*. *DLK1* is paternally expressed gene which encodes for a transmembrane protein involved in the Notch signalling and plays a role in placental development and nutrient metabolism (Moon et al., 2002; Rocha et al., 2007). On the other hand, *GTL2*, also known as maternally expressed 3 (*MEG3*), is maternally expressed and codes for a long non-coding RNA (Takada et al., 2000), which may act as a tumour suppressor (Zhou et al., 2012). Kobayashi et al. (2007) observed aberrant methylation pattern at the *IG-DMR* in moderate or severe oligozoospermic males as compared to normal males. Although we did not observe any significant difference in the total methylation percentage at the *IG-DMR*, four CpG sites showed significant hypomethylation in RPL males. These results indicate that *IG-DMR* may not be an essential gene contributing to RPL, as loss of *DLK1* does not lead to embryo lethality but show other developmental and postnatal abnormalities, mainly defects in placentation and adipocyte differentiation (Moon et al., 2002).

We then analysed the methylation at certain maternally imprinted genes, *ZAC*, *MEST*, *KvDMR*, *PEG3*, *PEG10* and *SNRPN*. These genes are expressed from the paternal allele and are normally unmethylated in the sperm. *ZAC* (also known as *PLAGL1*) encodes for a zinc finger transcription factor that is involved in the regulation of cell cycle and apoptosis, and also functions as a tumour-suppressor gene. As most imprinted genes, it is involved in embryo development, and loss of this gene leads to embryo growth restriction and lethality (Varrault et al., 2006). *MEST* (mesoderm-specific transcript homologue) encodes for a α/β hydrolase fold family enzyme (Kaneko-Ishino et al., 1995), is expressed in human first trimester villous and invasive cytotrophoblast, and is thought to play a functional role in angiogenesis during trophoblast invasion (Mayer et al., 2000). *KvDMR* represents the second imprinting control region in the cluster of imprinted genes on human

Downloaded from https://academic.oup.com/humrep/article/36/1/48/6034909 by Universita' degli Studi Teramo user on 21 March 2024

chromosome 11p15.5, the first one being the *IGF2-H19 DMR*. *KvDMR* controls the expression of six maternally expressed genes, potassium voltage-gated channel subfamily Q member 1 (*KCNQ1*), cyclin-dependent kinase inhibitor 1C (*CDKN1C*), achaete-scute family bHLH transcription factor 2 (*ASCL2*), pleckstrin homology like domain family A member 2 (*PHLDA2*), tumour suppressing subtransferable candidate 4 (*TSSC4*) and solute carrier family 22 member 18 (*SLC22A1L*), some of which are involved in placental growth and development, and the paternally expressed transcript KCNQ1 opposite strand/antisense transcript 1 (*KCNQ1OT1*) (Beatty *et al.*, 2006).

PEG3 encodes for a zinc finger transcription repressor, which is widely expressed during embryonic development and regulates growth and maternal behaviour (Li *et al.*, 1999). *PEG10* plays a critical role in embryo development and placentation, as the PEG10 knockout mice show early embryonic lethality due to defects in placenta (Ono *et al.*, 2006). *SNRPN* gene encodes for a component of the small nuclear ribonucleoprotein complex, which functions in pre-mRNA processing and contributes to tissue-specific alternative splicing (Glenn *et al.*, 1993). Loss of imprinting at this locus is observed in the imprinting disorder, Prader–Willi syndrome (Bervini and Herzog, 2013). Abnormal methylation patterns of these genes were associated with oligozoospermia and male infertility (Marques *et al.*, 2004; 2008; Kobayashi *et al.*, 2007; Hammoud *et al.*, 2010; El Hajj *et al.*, 2011; Marques and Sousa, 2013). Hypermethylation of these loci, other than *SNRPN*, in sperm of the RPL group indicates that correct methylation patterns are essential for ensuring normal embryo development.

It is interesting to note that *ZAC* is postulated to regulate a network of imprinted genes regulating embryo growth, as the deregulation of *ZAC* expression affects the expression of several other imprinted genes such as *IGF2*, *H19*, *DLK1* and *CDKN1C*. Further, *ZAC* also directly regulates the *IGF2-H19* locus by binding to a shared enhancer element (Varrault *et al.*, 2006). The existence of such an imprinted gene network (IGN) was further supported by the findings that the *H19* RNA also acts as a trans-regulator of IGN. Deletion of *H19* leads to up-regulation of several genes in the IGN, and this overexpression can be restored by transgenic expression of *H19* (Gabory *et al.*, 2009). These studies further highlight the crucial role of imprinted genes during embryogenesis and hint towards a cooperative regulation of their expression. Since the expression of these genes are primarily determined by DNA methylation of their respective imprinting control regions, it is plausible that deregulation of important genes, *ZAC* and *H19*, might have a cascading effect on the expression of other related genes in the IGN. Thus, the aberrant methylation patterns of these imprinted genes in the paternal allele may collectively lead to aberrant expression of imprinted genes in early embryo development and may contribute towards embryo loss. Further experimental validation of expression of these genes in extraembryonic tissues in RPL cases would be required to support this hypothesis.

We also studied the methylation status of the *MTHFR* gene promoter, which codes for an important enzyme involved in folate metabolism. Circulating folic acid (vitamin B9) is sequentially reduced to 5,10-methylenetetrahydrofolate, which enters the folate cycle and is converted to 5-methyltetrahydrofolate by MTHFR. This reaction is crucial in conversion of homocysteine to methionine, which acts as a methyl group donor for the process of DNA methylation (Ducker and Rabinowitz, 2017). Hypermethylation of the *MTHFR* promoter is associated with idiopathic male infertility (Wu *et al.*, 2010; Karaca *et al.*, 2016) and to RPL (Cao *et al.*, 2013). Additionally, hypomethylation at the *H19* locus is associated with hypermethylation of the *MTHFR* promoter in cases of male infertility (Rotondo *et al.*, 2012). Therefore, we analysed the methylation level of the *MTHFR* promoter and found no significant differences in the methylation levels of individual CpG sites as well as in the total methylation between sperm samples of RPL and control groups.

Since folate deficiency can impair the DNA methylation pathway, we also investigated serum folate levels in RPL and control groups and found no significant differences between both groups. Our results indicate that the folate metabolism pathways may be unaffected in RPL, and studies are underway to determine other specific mechanisms which may be responsible for decreased sperm global 5mC levels and aberrant methylation at imprinted gene loci.

Detection of DNA methylation levels of imprinted genes in sperm by pyrosequencing has been proposed as a prospective marker in the field of andrology since it is more stable over time as compared to the classical evaluation of semen parameters (Kläver *et al.*, 2013). There is currently a dearth of paternal factors identified for idiopathic RPL. Therefore, we tested whether the differences in methylation levels of imprinted genes observed in the RPL group could be used as diagnostic markers to detect 'epigenetically abnormal' sperm by performing ROC analysis. Our results revealed that sperm global 5mC level is the best candidate with the highest diagnostic efficiency. Also, since it can be evaluated using a basic immunoassay and does not require the initial investment of a pyrosequencing facility; it can be a good diagnostic candidate to be explored at the clinical level. Among the imprinted genes studied, *IGF2-H19 DMR* and *ZAC* (both of which regulate the IGN) were the best candidate genes. We propose that the feasibility and diagnostic potential of these markers should be further investigated in a clinical setting.

Study limitations

In this study, we have studied the methylation levels in a sample of a few CpG sites in the DMR or promoter regions of selected candidate imprinted genes implicated in embryo development from the studies reported in literature. Investigating other CpG sites or islands within these regions, which could be more susceptible to methylation aberrations, might yield better distinctions between the control and study groups. Detection of methylation changes occurring at genome-wide level may reveal additional candidate genes with better sensitivity and specificity values. Investigating the methylation and expression of these imprinted genes in embryonic chorionic villi samples in RPL cases may further help in correlating them with the aberrant methylation patterns in the spermatozoa. However, such samples were not available for this study.

Conclusion

Our study highlights the defects in DNA methylation and aberrant imprinting patterns in sperm of men whose partners have experienced idiopathic RPL. While it is not possible to attribute the RPL pathophysiology to any one particular contributing factor, it is more prudent that a combination of the genetic mutations and epi-mutations in the sperm together increases the susceptibility to RPL. Further studies exploring

Downloaded from https://academic.oup.com/humrep/article/36/1/48/6034909 by Universita' degli Studi Teramo user on 21 March 2024

the genome-wide methylation defects will assist in building a network of genes contributing towards RPL.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

Acknowledgements

The authors acknowledge help provided by Dr Anita Kumar in primer designing for pyrosequencing. The authors are thankful for the technical assistance provided by Mr Suryakant Mandavkar and Mr Deepak Shelar. The authors acknowledge the technical help provided by Mrs Tejashree Sontakke for recruitment of participants for the study. We are thankful to all of the couples participating in this study.

Authors' roles

N.H.B. conceived and designed the study. V.B., A.P. and H.W. helped in recruiting the study participants. S.M., S.S., R.G., Z.A. and M.D. assisted in recruiting the study participants and in sample collection and processing. K.K. carried out all the methylation analysis. S.R. and S.D. carried out the genotyping studies and global 5mC estimation. K.K., S.R. and S.D. wrote the manuscript. N.H.B. made critical reviews and approved the final manuscript.

Funding

This study is funded by the Department of Science and Technology-Science and Engineering Research Board (EMR/2014/000145) and ICMR-National Institute for Research in Reproductive Health intramural funds (RA/872/01-2020).

Conflict of interest

The authors declare that they have no conflict of interests and nothing to disclose.

References

Ameet P, Chauhan N. An epidemiology study to determine the prevalence and risk factors associated with recurrent spontaneous miscarriage in India. *J Obstet Gynecol India* 2016;**66**:310–315.

Ankolkar M, Patil A, Warke H, Salvi V, Mokashi N, Pathak S, Balasinor NH. Methylation analysis of idiopathic recurrent spontaneous miscarriage cases reveals aberrant imprinting at H19 ICR in normozoospermic individuals. *Fertil Steril* 2012;**98**:1186–1192.

Ankolkar M, Salvi V, Warke H, Vundinti BR, Balasinor NH. Methylation status of imprinted genes *DLK1-GTL2*, *MEST (PEG1)*, *ZAC (PLAGL1)*, and *LINE-1* elements in spermatozoa of normozoospermic men, unlike H19 imprinting control regions, is not associated with idiopathic recurrent spontaneous miscarriages. *Fertil Steril* 2013;**99**:1668–1673.

Atik RB, Christiansen OB, Elson J, Kolte AM, Lewis S, Middeldorp S, Nelen W, Peramo B, Quenby S, Vermeulen N *et al.* ESHRE guideline: recurrent pregnancy loss. *Hum Reprod Open* 2018;**2**:1–12.

Beatty L, Weksberg R, Sadowski PD. Detailed analysis of the methylation patterns of the KvDMR1 imprinting control region of human chromosome 11. *Genomics* 2006;**87**:46–56.

Benchaib M, Braun V, Ressnikof D, Lornage J, Durand P, Niveleau A, Guerin JF. Influence of global sperm DNA methylation on IVF results. *Hum Reprod* 2005;**20**:768–773.

Bervini S, Herzog H. Mouse models of Prader–Willi syndrome: a systematic review. *Front Neuroendocrinol* 2013;**34**:107–119.

Bhattacharya SM. Association of various sperm parameters with unexplained repeated early pregnancy loss—which is most important? *Int Urol Nephrol* 2008;**40**:391–395.

Bianchi PG, Manicardi MC, Bizzaro D, Bianchi U, Sakkas D. Effect of deoxyribonucleic acid protamination on fluorochrome staining and in situ nick-translation of murine and human mature spermatozoa. *Biol Reprod* 1993;**49**:1083–1088.

Busato F, Dejeux E, El Abdalaoui H, Gut IG, Tost J. Quantitative DNA methylation analysis at single-nucleotide resolution by pyrosequencing. *Methods Mol Biol* 2018;**1708**:427–445.

Cao X, Cui Y, Zhang X, Lou J, Zhou J, Wei R. The correlation of sperm morphology with unexplained recurrent spontaneous abortion: a systematic review and meta-analysis. *Oncotarget* 2017;**8**:55646–55656.

Cao Y, Xu J, Zhang Z, Huang X, Zhang A, Wang J, Zheng Q, Fu L, Du J. Association study between methylenetetrahydrofolate reductase polymorphisms and unexplained recurrent pregnancy loss: a meta-analysis. *Gene* 2013;**514**:105–111.

Cattanach BM, Kirk M. Differential activity of maternally and paternally derived chromosome regions in mice. *Nature* 1985;**315**:496–495.

Cooper TG, Noonan E, von Eckardstein S, Auger J, Baker HWG, Behre HM, Haugen TB, Kruger T, Wang C, Mbizvo MT *et al.* World Health Organization reference values for human semen. *Hum Reprod Update* 2010;**16**:231–245.

DeChiara TM, Robertson EJ, Efstratiadis A. Parental imprinting of the mouse insulin-like growth factor II gene. *Cell* 1991;**64**:849–859.

Dória S, Sousa M, Fernandes S, Ramalho C, Brandão O, Matias A, Barros A, Carvalho F. Gene expression pattern of IGF2, PHLDA2, PEG10 and CDKN1C imprinted genes in spontaneous miscarriages or fetal deaths. *Epigenetics* 2010;**5**:444–450.

Ducker GS, Rabinowitz JD. One-carbon metabolism in health and disease. *Cell Metab* 2017;**25**:27–42.

Dumasia K, Kumar A, Deshpande S, Balasinor NH. Estrogen signaling, through estrogen receptor β, regulates DNA methylation and its machinery in male germ line in adult rats. *Epigenetics* 2017;**12**:476–483.

Eisenberg ML, Sapra KJ, Kim SD, Chen Z, Louis GMB. Semen quality and pregnancy loss in a contemporary cohort of couples recruited before conception: data from the Longitudinal Investigation of Fertility and the Environment (LIFE) study. *Fertil Steril* 2017;**108**:613–619.

El Hajj N, Zechner U, Schneider E, Tresch A, Gromoll J, Hahn T, Schorsch M, Haaf T. Methylation status of imprinted genes and

Downloaded from https://academic.oup.com/humrep/article/36/1/48/6034909 by Universita' degli Studi Teramo user on 21 March 2024

repetitive elements in sperm DNA from infertile males. *Sex Dev* 2011;**5**:60–69.

Fang L, Zhou Y, Liu S, Jiang J, Bickhart DM, Null DJ, Li B, Schroeder SG, Rosen BD, Cole JB *et al*. Integrating signals from sperm methylome analysis and genome-wide association study for a better understanding of male fertility in cattle. *Epigenomes* 2019;**3**:10.

Ford HB, Schust DJ. Recurrent pregnancy loss: etiology, diagnosis, and therapy. *Rev Obstet Gynecol* 2009;**2**:76–83.

Gabory A, Ripoche MA, Le Digarcher A, Watrin F, Ziyyat A, Forné T, Jammes H, Ainscough JFX, Surani MA, Journot L *et al*. H19 acts as a trans regulator of the imprinted gene network controlling growth in mice. *Development* 2009;**136**:3413–3421.

Glenn CC, Porter KA, Jong MTC, Nicholls RD, Driscoll DJ. Functional imprinting and epigenetic modification of the human SNRPN gene. *Hum Mol Genet* 1993;**2**:2001–2005.

Habibzadeh F, Habibzadeh P, Yadollahie M. On determining the most appropriate test cut-off value: the case of tests with continuous results. *Biochem Med* 2016;**26**:297–307.

Hammoud SS, Purwar J, Pflueger C, Cairns BR, Carrell DT. Alterations in sperm DNA methylation patterns at imprinted loci in two classes of infertility. *Fertil Steril* 2010;**94**:1728–1733.

Hill J, Abbott A, Politch J. Sperm morphology and recurrent abortion. *Fertil Steril* 1994;**61**:776–778.

Imam SN, Shamsi MB, Kumar K, Deka D, Dada R. Idiopathic recurrent pregnancy loss: role of paternal factors; a pilot study. *J Reprod Infertil* 2011;**12**:267–276.

Kaneko-Ishino T, Kuroiwa Y, Miyoshi N, Kohda T, Suzuki R, Yokoyama M, Viville S, Barton SC, Ishino F, Surani MA. Peg1/Mest imprinted gene on chromosome 6 identified by CDNA subtraction hybridization. *Nat Genet* 1995;**11**:52–59.

Karaca MZ, Konac E, Yurteri B, Bozdag G, Sogutdelen E, Bilen CY. Association between methylenetetrahydrofolate reductase (MTHFR) gene promoter hypermethylation and the risk of idiopathic male infertility. *Andrologia* 2016;**49**:2–7.

Kazerooni T, Asadi N, Jadid L, Kazerooni M, Ghanadi A, Ghaffarpasand F, Kazerooni Y, Zolghadr J. Evaluation of sperm's chromatin quality with acridine orange test, chromomycin A3 and aniline blue staining in couples with unexplained recurrent abortion. *J Assist Reprod Genet* 2009;**26**:591–596.

Kläver R, Tüttelmann F, Bleiziffer A, Haaf T, Kliesch S, Gromoll J. DNA methylation in spermatozoa as a prospective marker in andrology. *Andrology* 2013;**1**:731–740.

Kobayashi H, Sato A, Otsu E, Hiura H, Tomatsu C, Utsunomiya T, Sasaki H, Yaegashi N, Arima T. Aberrant DNA methylation of imprinted loci in sperm from oligospermic patients. *Hum Mol Genet* 2007;**16**:2542–2551.

Koukoura O, Sifakis S, Zaravinos A, Apostolidou S, Jones A, Hajiioannou J, Widschwendter M, Spandidos DA. Hypomethylation along with increased H19 expression in placentas from pregnancies complicated with fetal growth restriction. *Placenta* 2011;**32**:51–57.

Li C, Fan Y, Li G, Xu X, Duan J, Li R, Kang X, Ma X, Chen X, Ke Y *et al*. DNA methylation reprogramming of functional elements during mammalian embryonic development. *Cell Discov* 2018;**4**:1–2.

Li L, Keverne EB, Aparicio SA, Ishino F, Barton SC, Surani MA. Regulation of maternal behavior and offspring grwoth by paternally expressed Peg3. *Science* 1999;**284**:330–333.

Lippi G, Mattiuzzi C. The biomarker paradigm: between diagnostic efficiency and clinical efficacy. *Pol Arch Intern Med* 2015;**125**:282–288.

Luján S, Caroppo E, Niederberger C, Arce JC, Sadler-Riggleman I, Beck D, Nilsson E, Skinner MK. Sperm DNA methylation epimutation biomarkers for male infertility and FSH therapeutic responsiveness. *Sci Rep* 2019;**9**:1–2.

Madan T, Priyadarsiny P, Vaid M, Kamal N, Shah A, Haq W, Katti SB, Sarma PU. Use of a synthetic peptide epitope of Asp f 1, a major allergen or antigen of *Aspergillus fumigatus*, for improved immunodiagnosis of allergic bronchopulmonary aspergillosis. *Clin Diagn Lab Immunol* 2004;**11**:552–558.

Marques CJ, Barros A, Sousa M. Sperm epigenetic profile. In Zini A. and Agarwal A. (eds.), *Sperm Chromatin: Biological and Clinical Applications in Male Infertility and Assisted Reproduction*. 2011b; Springer, New York, USA, pp. 243–257.

Marques CJ, Carvalho F, Sousa M, Barros A. Genomic imprinting in disruptive spermatogenesis. *Lancet* 2004;**363**:1700–1702.

Marques CJ, Costa P, Vaz B, Carvalho F, Fernandes S, Barros A, Sousa M. Abnormal methylation of imprinted genes in human sperm is associated with oligozoospermia. *Mol Hum Reprod* 2008;**14**:67–73.

Marques CJ, Francisco T, Sousa S, Carvalho F, Barros A, Sousa M. Methylation defects of imprinted genes in human testicular spermatozoa. *Fertil Steril* 2010;**94**:585–594.

Marques CJ, Pinho MJ, Carvalho F, Bièche I, Barros A, Sousa M. DNA methylation imprinting marks and DNA methyltransferase expression in human spermatogenic cell stages. *Epigenetics* 2011a;**6**:1354–1361.

Marques CJ, Sousa M. Imprinted gene anomalies in sperm. In Douglas T. Carrell (ed.), *Paternal Influences on Human Reproductive Success*. 2013; Cambridge University Press, Cambridge, UK, pp. 27–37.

Mayer W, Hemberger M, Frank H-G, Grümmer R, Winterhager E, Kaufmann P, Fundele R. Expression of the imprinted genes MEST/Mest in human and murine placenta suggests a role in angiogenesis. *Dev Dyn* 2000;**217**:1–10.

McGrath J, Solter D. Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell* 1984;**37**:179–183.

Meka A, Reddy M. Recurrent spontaneous abortions: an overview of genetic and non-genetic backgrounds. *Int J Hum Genet* 2006;**6**:109–117.

Moon YS, Smas C, Lee K, Villena JA, Kim K, Yun EJ, Sul HS. Mice lacking paternally expressed Pref-1/Dlk1 display growth retardation and accelerated adiposity. *Mol Cell Biol* 2002;**22**:5585–5592.

Ono R, Nakamura K, Inoue K, Naruse M, Usami T, Wakisaka-Saito N, Hino T, Suzuki-Migishima R, Ogonuki N, Miki H *et al*. Deletion of Peg10, an imprinted gene acquired from a retrotransposon, causes early embryonic lethality. *Nat Genet* 2006;**38**:101–106.

Ostojic S, Pereza N, Volk M, Kapovic M, Peterlin B. Genetic predisposition to idiopathic recurrent spontaneous abortion: contribution of genetic variations in IGF-2 and H19 imprinted genes. *Am J Reprod Immunol* 2008;**60**:111–117.

Pliushch G, Schneider E, Weise D, El Hajj N, Tresch A, Seidmann L, Coerdt W, Müller AM, Zechner U, Haaf T. Extreme methylation values of imprinted genes in human abortions and stillbirths. *Am J Pathol* 2010;**176**:1084–1090.

Downloaded from https://academic.oup.com/humrep/article/36/1/48/6034909 by Universita' degli Studi Teramo user on 21 March 2024

Remy S, Bouyer J, Windham G, Fenster L, Werwatz A, Swan SH. Influence of paternal age on the risk of spontaneous abortion. *Am J Epidemiol* 2005;**161**:816–823.

Rocha ST, Tevendale M, Knowles E, Takada S, Watkins M, Ferguson-Smith AC. Restricted co-expression of Dlk1 and the reciprocally imprinted non-coding RNA, Gtl2: implications for Cis - acting control. *Dev Biol* 2007;**306**:810–823.

Rotondo JC, Bosi S, Bazzan E, Di Domenico M, De Mattei M, Selvatici R, Patella A, Marci R, Tognon M, Martini F. Methylenetetrahydrofolate reductase gene promoter hypermethylation in semen samples of infertile couples correlates with recurrent spontaneous abortion. *Hum Reprod* 2012;**27**:3632–3638.

Ruixue W, Hongli Z, Zhihong Z, Rulin D, Dongfeng G, Ruizhi L. The impact of semen quality, occupational exposure to environmental factors and lifestyle on recurrent pregnancy loss. *J Assist Reprod Genet* 2013;**30**:1513–1518.

Santi D, De Vincentis S, Magnani E, Spaggiari G. Impairment of sperm DNA methylation in male infertility: a meta-analytic study. *Andrology* 2017;**5**:695–703.

Sbracia M, Cozza G, Grasso JA, Mastrone M, Scarpellini F. Semen parameters and sperm morphology in men in unexplained recurrent spontaneous abortion, before and during a 3 year follow-up period. *Hum Reprod* 1996;**11**:117–120.

Smith ZD, Chan MM, Mikkelsen TS, Gu H, Gnirke A, Regev A, Meissner A. A unique regulatory phase of DNA methylation in the early mammalian embryo. *Nature* 2012;**484**:339–344.

Soejima H, Wagstaff J. Imprinting centers, chromatin structure, and disease. *J Cell Biochem* 2005;**95**:226–233.

Surani MAH, Barton SC, Norris ML. Experimental reconstruction of mouse eggs and embryos: an analysis of mammalian development. *Biol Reprod* 1987;**36**:1–16.

Takada S, Tevendale M, Baker J, Georgiades P, Campbell E, Freeman T, Johnson MH, Paulsen M, Ferguson-Smith AC. Delta-like and Gtl2 are reciprocally expressed, differentially methylated linked imprinted genes on mouse chromosome 12. *Curr Biol* 2000;**10**:1135–1138.

Takai D, Gonzales FA, Tsai YC, Thayer MJ, Jones PA. Large scale mapping of methylcytosines in CTCF-binding sites in the human H19 promoter and aberrant hypomethylation in human bladder cancer. *Hum Mol Genet* 2001;**10**:2619–2626.

Tang Q, Pan F, Yang J, Fu Z, Lu Y, Wu X, Han X, Chen M, Lu C, Xia Y *et al*. Idiopathic male infertility is strongly associated with aberrant DNA methylation of imprinted loci in sperm: a case-control study. *Clin Epigenet* 2018;**10**:1–10.

Trasler JM. Epigenetics in spermatogenesis. *Mol Cell Endocrinol* 2009;**306**:33–36.

Tycko B, Morison IM. Physiological functions of imprinted genes. *J Cell Physiol* 2002;**192**:245–258.

Urdinguio RG, Bayón GF, Dmitrijeva M, Toraño EG, Bravo C, Fraga MF, Bassas L, Larriba S, Fernández AF. Aberrant DNA methylation patterns of spermatozoa in men with unexplained infertility. *Hum Reprod* 2015;**30**:1014–1028.

Varrault A, Gueydan C, Delalbre A, Bellmann A, Houssami S, Aknin C, Severac D, Chotard L, Kahli M, Le Digarcher A *et al*. Zac1 regulates an imprinted gene network critically involved in the control of embryonic growth. *Dev Cell* 2006;**11**:711–722.

World Health Organization (2010). *WHO Laboratory manual for the examination and processing of human sperm* (5th ed.) Geneva: World Health Organization.

Wu W, Shen O, Qin Y, Niu X, Lu C, Xia Y, Song L, Wang S, Wang X. Idiopathic male infertility is strongly associated with aberrant promoter methylation of methylenetetrahydrofolate reductase (MTHFR). *Plos One* 2010;**5**:e13884.

Yang AS, Estecio MRH, Doshi K, Kondo Y, Tajara EH, Issa JPJ. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. *Nucleic Acids Res* 2004;**32**:38e.

Yin LJ, Zhang Y, Lv PP, He WH, Wu YT, Liu AX, Ding GL, Dong MY, Qu F, Xu CM *et al*. Insufficient maintenance DNA methylation is associated with abnormal embryonic development. *BMC Med* 2012;**10**:1–10.

Zechner U, Pliushch G, Schneider E, El Hajj N, Tresch A, Shufaro Y, Seidmann L, Haaf T, Coerdt W, Mu AM. Quantitative methylation analysis of developmentally important genes in human pregnancy losses after ART and spontaneous conception. *Mol Hum Reprod* 2010;**16**:704–713.

Zhang L, Wang L, Zhang X, Xu G, Zhang W, Wang K, Wang Q, Qiu Y, Li J, Gai L. Sperm chromatin integrity may predict future fertility for unexplained recurrent spontaneous abortion patients. *Int J Androl* 2012;**35**:752–757.

Zhou Y, Zhang X, Klibanski A. MEG3 noncoding RNA: a tumor suppressor. *J Mol Endocrinol* 2012;**48**:R45–R53.

Zidi-Jrah I, Hajlaoui A, Mougou-Zerelli S, Kammoun M, Meniaoui I, Sallem A, Brahem S, Fekih M, Bibi M, Saad A *et al*. Relationship between sperm aneuploidy, sperm DNA integrity, chromatin packaging, traditional semen parameters, and recurrent pregnancy loss. *Fertil Steril* 2016;**105**:58–64.

Downloaded from https://academic.oup.com/humrep/article/36/1/48/6034909 by Universita' degli Studi Teramo user on 21 March 2024



QUALITY, INNOVATION, AND SERVICE—IT'S ALL AT THE CENTER OF EVERYTHING WE DO.

From developing assisted reproductive technologies that maximize performance, like the first ART media and cultures, to expertise that streamlines productivity, FUJIFILM Irvine Scientific brings together decades of industry expertise with a powerhouse of innovation, turning opportunities into realities. Together, we're working to support healthy futures—from retrieval to realization.

ALL IN *FOR LIFE.*
irvinesci.com/*ALLIN*

FUJIFILM
Value from Innovation

IrvineScientific

FUJIFILM Irvine Scientific and its logo are registered trademarks of FUJIFILM Irvine Scientific, Inc. in various jurisdictions. ©2023 FUJIFILM Irvine Scientific. P/N 018187 Rev.00