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Aberrant DNA methylation at imprinted genes in testicular sperm retrieved from men with obstructive azoospermia and post vasectomy reversal.

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Methylation at imprinted genes in obstruction

Abstract

Male factor infertility has been associated with abnormal DNA methylation at imprinted genes. Little information is available on the status of imprinting in the sperm of men with azoospermia, including the association between aberrant imprinting and obstructive azoospermia (OA) or non-obstructive azoospermia (NOA). Analysis of DNA methylation at imprinted genes in the sperm of men undergoing vasectomy reversal would aid to determine whether aberrant imprinting is associated with obstruction. Testicular sperm was retrieved from testicular biopsies obtained from men with azoospermia (N=18), including OA (N=10), NOA (N=5), and unknown pathology (N=3), and from men undergoing vasectomy reversal (N=17). Sperm was also obtained from proven fertile men (N=9). DNA methylation was investigated at multiple CpG sites within the differentially methylated regions (DMRs) of three imprinted genes, *H19*, *IG-GTL2* and *MEST*, using bisulphite sequencing. Unique clones representative of single cells were analyzed. We found a significant decrease in DNA methylation at the *H19* DMR in testicular sperm of azoospermic men compared to proven fertile men. The decrease was also significant between OA and proven fertile men, and between men post vasectomy reversal and proven fertile men, suggesting that aberrant DNA methylation may be associated with obstruction. Changes in DNA methylation at *IG-GTL2* and *MEST* DMRs among groups were not significant. Our data suggest that imprinting abnormalities may be associated with obstruction and may occur in response to changes in testicular environment and not only spermatogenesis failure, as previously reported. Methylation at the *H19* DMR was particularly prone to modification in testicular sperm.

Introduction

Azoospermia is defined as the absence of sperm from the ejaculate which affects an estimated 10% to 20% of infertile men (Jarow *et al.* 1989). It results from obstruction in 40% of cases, while non-obstructive azoospermia (NOA) due to spermatogenesis failure is seen in the remaining cases (Jarow *et al.* 1989). Although in these men sperm is absent from the ejaculate, it may be retrieved from the testes and used to achieve pregnancy through intracytoplasmic sperm injection (ICSI). Recent reports have demonstrated the presence of epigenetic abnormalities in the sperm of infertile men (Marques *et al.* 2008; Kobayashi *et al.* 2007; Boissonnas *et al.* 2010), including those affected by azoospermia (Marques *et al.* 2010).

The epigenome refers to the histone and DNA methylation patterns established in a cell that control gene expression. DNA methylation is the covalent addition of a methyl group to cytosines located within CpG dinucleotides. Around 90% of DNA methylation in the genome occurs at repetitive sequences (Yoder *et al.* 1997). DNA methylation also marks the differentially methylated regions (DMRs) of imprinted genes, allowing for mono-allelic parent-specific gene expression. Proper DNA methylation is an integral part of spermatogenesis progression (Raman & Narayan 1995; Yaman & Grandjean 2006). Once DNA methylation at imprinted genes in the male gametes is established prior to entry into meiosis, it is maintained throughout development (Olek & Walter 1997; Kerjean *et al.* 2000). Abnormal DNA methylation at imprinted genes has been associated with spermatogenesis failure, primarily in men affected by moderate oligozoospermia (Kobayashi *et al.* 2007; Marques *et al.* 2008; Boissonnais *et al.* 2010), while DNA methylation at repetitive sequences appears properly set (Kobayashi *et al.* 2007; Marques *et al.* 2008). Although DNA methylation at imprinted genes in testicular sperm of men with azoospermia has been previously studied, an association between

aberrant imprinting and NOA or obstructive azoospermia (OA) has not been established (Marques *et al.* 2010). Analysis of DNA methylation at imprinted genes in the sperm retrieved from men with different pathologies, NOA and OA, would help in the understanding of factors that may disrupt DNA methylation such as spermatogenesis failure in NOA patients or obstruction in OA patients. Furthermore, obstruction may be either associated with the congenital bilateral absence of the vas deferens (CBADV), or induced by vasectomy in previously fertile men. No data is currently available on the status of DNA methylation at imprinted genes in the sperm of vasectomized men. Study of DNA methylation at imprinted genes in these men would also help to determine whether aberrant imprinting is associated with obstruction.

While most imprinted genes are methylated in the oocyte, including *MEST* (Lucifero *et al.* 2002), two imprinted genes, *H19* and the intragenic region (IG) of *GTL2*, show sperm-specific DNA methylation (Kerjean *et al.* 2000; Geuns *et al.* 2007). The *H19/IGF2*, *GTL2/DLK1* regions and *MEST* are important regulators of prenatal growth (DeChiara *et al.* 1990; Georgiades *et al.* 2000; Georgiades *et al.* 2001; Kaneko-Ishino *et al.* 1995). Abnormal methylation within the three DMRs has been associated with imprinting syndromes observed in children (Kanber *et al.* 2009; Kagami *et al.* 2007) and abortuses achieved through the use of ICSI (Kobayashi *et al.* 2009). Therefore, abnormal methylation at imprinted genes in the sperm may not only be associated with male factor infertility but may also be passed on to the progeny through the use of ICSI and affect pregnancy outcome.

In this study DNA methylation at the DMRs of three imprinted genes, *H19, GTL2* and *MEST,* was evaluated in the testicular sperm retrieved from men affected by azoospermia, NOA and OA, and men undergoing vasectomy reversal, as well as in normozoospermic men of proven

fertility. DNA methylation was carried out using bisulphite sequencing so that DNA methylation at multiple CpG sites could be simultaneously analyzed and DNA methylation could be visualized at the single sperm level. We hypothesized that a higher incidence of abnormal DNA methylation at imprinted genes would be identified in testicular sperm of azoospermic men and men undergoing vasectomy reversal compared to proven fertile men. Based on the limited data available, we also hypothesized that sperm obtained from men affected by obstruction would be more prone to methylation abnormalities at imprinted genes compared to sperm retrieved from men affected by NOA.

Results

Clinical information

The vasectomy reversal men were significantly older compared to the proven fertile men ($p<0.0001$). The age difference between proven fertile men, azoospermic men, OA men, and NOA men, was not significant ($p=0.14$, $p=0.16$ and $p=0.13$, respectively). We found a significant age difference between the two groups affected by obstruction: the vasectomy reversal and OA men ($p=0.0002$).

Analysis of methylation at DMRs of imprinted genes

In total 1167 clones were analyzed with 77.3% being unique: 75.4% of clones in the vasectomy reversal group, 76.1% of clones in the azoospermia groups and 82.7% of clones in the proven fertile group were unique. An average of 4.5, 4.3 and 2.9 amplification reactions was performed per sample in the vasectomy reversal group, azoospermia group and the proven fertile group, respectively. An average of 6.7, 6.5 and 8.0 unique clones was analyzed for each sample in the vasectomy reversal group, azoospermia group and the proven fertile group, respectively. In some

cases, multiple amplification reactions failed and due to a limited amount of sample available fewer clones could be analyzed. The unique clones analyzed for the samples are indicated in Figure 1 and are shown directly in the diagram. Most unique clones originated from different amplification reaction. Based on these results, the DNA methylation level was calculated for each sample at each DMR analyzed (Figure 2). The absence of ejaculate or testicular sperm with improperly methylated *IG-GTL2* DMR, with the exception of one sample, suggests that the sperm population was not contaminated with non-germ cells, as these also carry a non-methylated imprint at the *IG-GTL2* DMR.

Methylation at the H19 DMR

In the proven fertile men, the methylation level ranged between 97.39% and 100% and hypomethylated unique clones were not detected (Figure 1A; Figure 2). The methylation level at the *H19* DMR ranged between 71.4% and 100% in the vasectomy reversal group (Figure 1B; Figure 2). Hypomethylated unique clones were found in 5 of the 17 vasectomy reversal samples: either one or two unique clones were hypomethylated or completely non-methylated in these samples (Figure 1B). In the azoospermic patient group the methylation level at the *H19* DMR ranged between 17.65% and 99.16% (Figure 2). A methylation level of 17.65% was found in a sample of unknown pathology (TP16); however, only one unique and two non-unique clones could be analyzed that may not be representative of the overall methylation level in that sample (Figure 1C). In total, hypomethylated unique clones were found in 6 of the 18 azoospermic patient samples: in 5 of the 10 OA samples (TP02, TP03, TP04, TP06, and TP07) and in one sample of unknown pathology (TP16) (Figure 1C). The incidence of abnormal methylation at the *H19* DMR was significantly higher in the azoospermia patient group compared to the proven

fertile men (7/18 vs. 0/9, Fisher's exact test, p=0.036) (Table 1). The incidence was also higher in the OA group compared to the proven fertile men (5/10 vs. 0/9, Fisher's exact test, p=0.022) (Table 1). No other significant differences were found between groups at the *H19* DMR.

We found a significant decrease in methylation at the *H19* DMR in the vasectomy reversal group compared to the proven fertile men (MW, p=0.0165). However, the significance was lost after the post hoc Dunn's correction following ANOVA (KW, p>0.05). We also found a significant decrease in methylation at the *H19* DMR in the azoospermic patient group compared to the proven fertile men (KW, p<0.01). Furthermore, there was a significant decrease in methylation at the *H19* DMR between the OA group and the proven fertile men (KW, p<0.01). The difference in the methylation level at the *H19* DMR between other groups were not significant (KW, p>0.05).

Methylation at the IG-GTL2 DMR

The methylation level at the *IG-GTL2* DMR ranged between 92.8% and 100%, 84.29% and 100%, 92.66% and 100% in the vasectomy reversal group, azoospermic group and proven fertile men, respectively (Figure 2). Hypomethylation at the *IG-GTL2* DMR affecting one of seven unique clones analyzed was found in one azoospermic patient sample of unknown pathology (TP17) (Figure 1C). Hypomethylation at the *IG-GTL2* DMR was not found in the vasectomy reversal group or the proven fertile group (Figure 1). There were no significant differences in the incidence of abnormal methylation at the *IG-GTL2* DMR between any of the groups studied (Table 1). The difference in the methylation level at the *IG-GTL2* DMR between any of the groups studied was not significant (KW, p>0.05).

Methylation at the MEST DMR

The methylation level at the *MEST* DMR ranged between 0% and 42.9%, 0% and 16.33%, 0.7% and 2.4% in the vasectomy reversal group, azoospermia group and men of proven fertility, respectively (Figure 2). Hypermethylated unique clones were found in two samples from the vasectomy reversal group (VR07 and VR09), and in one sample from the azoospermic patient group in a sample with unknown pathology (TP16) (Figure 1). Hypermethylated unique clones were not observed in any of the proven fertile group (Table 1A). Abnormal methylation at the *MEST* DMR was found in 11.8% (2/17) of vasectomy reversal samples and in 5.5% (1/18) of the azoospermia patient group (Table 1). There were no significant differences in the incidence of abnormal methylation at the *MEST* DMR between any of the groups studied. The difference in the methylation level at the *MEST* DMR between any of the groups studied was not significant (KW, $p>0.05$).

Three of the twelve samples with abnormal methylation at the *H19* DMR (VR07, VR09, TP16) also had abnormal methylation at the *MEST* DMR. The one patient with abnormal methylation at the *IG-GTL2* DMR (TP17) had normal methylation at the *H19* and the *MEST* DMRs.

Discussion

In this study testicular sperm were isolated from testicular tissue for analysis of DNA methylation at imprinted genes. Our results demonstrate a significant decrease in DNA methylation at the *H19* DMR in men with azoospermia and in men with OA compared to men of proven fertility. We also found a significant decrease in DNA methylation at the *H19* DMR in men undergoing vasectomy reversal compared to proven fertile men. The loss of methylation at

the *H19* DMR in the testicular sperm of men undergoing a vasectomy reversal and in OA men suggests that the loss of methylation may be associated with obstruction. Furthermore, we observed significant decrease in DNA methylation at the *H19* DMR in previously fertile men, those undergoing vasectomy reversals, and in infertile men, those affected by OA. The difference in DNA methylation between the two groups at the *H19* DMR was not significant, and suggests that DNA methylation at imprinted genes was not associated with the fertility status of the patients. Differences in DNA methylation at the *IG-GTL2* and *MEST* DMRs were not significant between the groups studied. The *H19* and *IG-GTL2* DMR are paternally methylated DMRs (Kerjean *et al.* 2000; Geuns *et al.* 2007); however, our results suggest the *H19* DMR is more prone to the loss of methylation compared to the *IG-GTL2* DMR. The propensity of the *H19* DMR to disturbances of DNA methylation may be related to molecular structure of the DMR or of surrounding sequences. The *H19* DMR is less repetitive compared to the *IG-GTL2* DMR (Paulsen et al. 2001) and it has been suggested that DNA methylation at more repetitive regions is more strictly conserved compared to regions that are less repetitive in nature, such as the *H19* DMR (Li et al. 2004).

Our observation of a low rate of abnormal methylation in NOA samples is consistent with the published data (Hartmann *et al.* 2006; Marques *et al.* 2010). Due to the small sample size of NOA samples analyzed, the non-significant difference in methylation at imprinted genes in NOA men needs to be confirmed in future studies. Marques et al. (2010) reported a significant difference in methylation in sub-groups of NOA and OA men at the *H19* and *MEST* DMRs. However, their analyses may have been confounded by assessing differences in methylation between groups by comparing the number of clones analyzed in each group instead of the number of samples, potentially misrepresenting the differences in methylation they observed in

their groups. In addition, amplification was limited to one reaction resulting in a lack of variability in the clones analyzed (Marques *et al.* 2010), which suggests that cells carrying the normal imprint may have been preferentially amplified misrepresenting the methylation status of different sperm cells. We performed an average of 4.4 amplification reactions and analyzed unique clones, most of which originated from different amplification reactions (Figure 1). This approach may facilitate analysis of DNA methylation in limited quantities of biological material to ensure the results are representative of different cells. It is currently unknown whether the aberrant DNA methylation observed is specific to imprinted genes or also affects other sequences, including repetitive sequences and non-imprinted genes. However, detailed analyses may be challenging since a limited quantity of sperm can be isolated from the small amount of testicular tissue biopsied that is available for research purposes.

Analysis of methylation in testicular sperm

The two published studies on DNA methylation at imprinted genes in testicular sperm of men affected by azoospermia did not include proper controls (Marques *et al.* 2010; Hartmann *et al.* 2006). We intended to use testicular sperm isolated from previously fertile men undergoing vasectomy reversal as controls for testicular sperm of azoospermic men. However, we found hypomethylated clones at the *H19* DMR and hypermethylated clones at the *MEST* DMR in the sperm of these men. More appropriate controls may have been testicular sperm obtained from men undergoing vasectomy before any changes in testicular environment may occur (Jones 2004; McVicar *et al.* 2005). However, it is uncertain whether ethical approval would be granted for the biopsy of testicular tissue in men undergoing a vasectomy. In such cases testicular biopsy

would be a clinically unnecessary procedure and the risks involved with the procedure may not be justifiable for research purposes.

We compared DNA methylation at the DMRs of imprinted genes between testicular sperm and ejaculate sperm of unobstructed proven fertile men. DNA methylation at imprinted genes is established before cells enter meiosis and is maintained throughout development (Kerjean *et al.* 2000). Further changes in DNA methylation in the sperm occur during epididymal transit; however, these changes are limited to non-imprinted genes (Ariel *et al.* 1994). While methylation at non-imprinted genes can differ between testicular and ejaculate sperm, the methylation at imprinted genes has been shown not to differ between the two cell type populations in fertile unobstructed individuals (Kerjean *et al.* 2000; Hartmann *et al.* 2006). Therefore, the observed differences in methylation at the *H19* DMR found between azoospermic men, men undergoing vasectomy reversal and men of proven fertility are unlikely to be confounded by the sperm source. The observed changes in DNA methylation were associated with obstruction as significant changes in methylation were found in OA men and in men undergoing vasectomy reversal. Previous studies have reported the presence of imprinting abnormalities or hypomethylation at imprinted genes in the sperm of unobstructed normozoospermic men used as controls (Kobayashi *et al.* 2007; Poplinski *et al.* 2009; Boissonnas *et al.* 2010). However, abnormal methylation in these men may be related to subfertility (Ludwig *et al.* 2005), as these men were not of proven fertility. Sperm DNA methylation at imprinted genes appears well conserved in unobstructed men of proven fertility status, as suggested by our and Hammoud *et al*. (2010) data.

Our groups were not age matched; however, no age-related changes in DNA methylation at the DMRs of imprinted genes have been reported in germ cells, either in spermatozoa

(Flanagan *et al.* 2006) or oocytes (Lopes *et al.* 2009). Therefore, the increased age of the vasectomy reversal men compared to the proven fertile men is unlikely to be associated with the observed changes in DNA methylation at the *H19* DMR. A significant age difference was also observed between the two groups of men affected by obstruction, both of which showed decreased DNA methylation at the *H19* DMR. This result suggests that the observed changes in DNA methylation at imprinted genes were not associated with age. Although our analysis suggests an association between obstruction and abnormal methylation at imprinted genes, the findings should be confirmed in a larger trial. Specifically, the difference in methylation at the *H19* DMR between the men undergoing vasectomy reversal and proven fertile men was statistically significant for comparisons of two independent groups but the significance was lost after correction for multiple group testing.

Etiology of abnormal DNA methylation in sperm

Loss of methylation at imprinted genes in the sperm has been associated with mutations in *Dnmt3a* and *Dnmt3l* (Kaneda *et al.* 2004; Yaman & Grandjean 2006); however, mutations in these genes could not be identified in infertile men showing aberrant imprinting (Kobayashi *et al.* 2009). Gene mutations in other enzymes involved in methylation are possible, including *DNMT1* (Li *et al.* 1992) and *MTHFR* (Kelly *et al.* 2005). Furthermore, exposure to endocrine disruptors has been associated with aberrant sperm DNA methylation at imprinted and non-imprinted genes (Stouder & Paoloni-Giacobino 2010; Anway *et al.* 2005). It is also possible that factors limited to the testicular environment may be responsible for abnormal methylation in patients with obstruction.

In our data set, aberrant imprinting was primarily observed in the testicular sperm of men affected by obstruction, OA and in those post vasectomy. The testicular environment may change as a result of obstruction in vasectomized and OA men and affect spermatogenesis (Jones 2005; McVicar *et al.* 2005). These results suggest that aberrant DNA methylation at imprinted genes may not only be associated with impaired spermatogenesis, as previously reported (Marques *et al.* 2008; Kobayashi *et al.* 2007; Poplinski *et al.* 2009; Boissonnas *et al.* 2010), but perhaps with changes in testicular environment that occur as a result of obstruction. Reduction of spermatogenesis may be associated with testicular tissue destruction related to an increase in reactive oxygen species (ROS) (Aydos *et al.* 1998), associated with DNA damage, including DNA strand breaks, as well as the generation of DNA base adducts (Franco *et al.* 2008). The DNA base adducts have been shown to impede DNA methylation by interfering with proper function of DNMTs leading to DNA hypomethylation (Weitzman *et al.* 1994; Turk *et al.* 1995; Hepburn *et al.* 1991; Tan *et al.* 1990). However, it is currently unknown whether DNA methylation at imprinted genes can also be affected by ROS induced DNA damage. Future studies should evaluate the mechanisms that lead to changes in DNA methylation.

We found aberrant imprinting primarily in azoospermic men affected by OA and in vasectomy reversal cases. The OA pathology is similar to that of vasectomy reversal cases in that both types of samples came from men with normal spermatogenesis where the sperm cannot reach the ejaculate due to obstruction. Our results suggest that an altered testicular environment may disrupt DNA methylation at imprinted genes. Therefore, aberrant imprinting may not only be related to spermatogenesis failure, as previously shown, but may also be disrupted by environmental factors. Furthermore, our results also show that DNA methylation at the *H19*

317 DMR is particularly prone to methylation abnormalities in vasectomy reversal and azoospermic
318 patients.
319
320 **Materials and Methods**
321 **Sample collection**
322 Testicular biopsies were obtained from seventeen men undergoing vasectomy reversal (VR01-
323 VR17) who were previously fertile having had a child prior to undergoing vasectomy. Eighteen
324 testicular biopsies were obtained from infertile men presenting with azoospermia (AZO; TP01-
325 18). Azoospermic men were subdivided into two sub-groups: OA (OA; TP01-10) or NOA
326 (NOA; TP11-15). Patients were assigned to the OA sub-group based on the diagnosis of normal
327 spermatogenesis (TP03-TP05, TP07, TP10), the presence of CFTR gene mutations associated
328 with obstruction due to the congenital bilateral absence of the vas deferens (CBAVD) (TP01,
329 TP02, TP08; had Δ508 mutation) or both (TP06; CBAVD due to the 5T allele). Patient TP09 was
330 assigned to the OA sub-group based on the presence of epididymal head calcification. Patients
331 were assigned to the NOA sub-group based on the pathological evaluation of spermatogenesis
332 failure due to hypospermatogenesis or partial maturation arrest. The pathology results were not
333 available for three patients (TP16-18). We were unable to obtain testicular sperm from proven
334 fertile unobstructed men as biopsies from these healthy individuals may not be ethically
335 justifiable for research purposes. DNA methylation at imprinted genes in the study groups was
336 compared to that in ejaculate sperm obtained from proven fertile unobstructed men (C01-C09).
337 These men had fathered a child within two years of sample donation and showed normal semen
338 parameters according to WHO criteria (≥20 million sperm/ml, ≥ 50% motile sperm, ≥ 30%
339 normal sperm morphology) (WHO, 2010). The individuals included in this study did not have Y

chromosome microdeletions and had a normal male karyotype. They also did not have varicocele or a history of infection or trauma. Ethical approval was obtained from the University of British Columbia Ethics Committee before initiating this study.

Patient information

Age was available for 13 of the 17 vasectomy reversal cases and 12 of the 18 azoospermic patients (9 of the 10 OA, 3 of the 5 NOA). The mean age ±SD (range) for the vasectomy reversal patients was 46.2±4.0 (41-53) years. The mean age ±SD (range) for the azoospermic patients was 37.8±6.9 (28-51) years: 37.0±5.3 (28-46) years for the OA group and 40.3±11.6 (28-51) years for the NOA group. The mean age ±SD (range) for the proven fertile men was 34.1±2.4 (30-38) years.

Preparation of DNA

Ejaculate sperm were isolated by standard swim-up. Prior to digestion purity of the sample was evaluated under bright-field microscopy. DNA was digested according to Doerksen *et al.* (2000) and extracted using salt extraction. Between 200-350 testicular sperm were isolated by micromanipulation using an inverted microscope (Nikon, Tokyo, Japan) equipped with Hoffman modulating optics, a thermal stage and micromanipulators (Narishige, Tokyo, Japan).
The testicular sperm were deposited into a clean droplet of modified human tubule fluid (mHTF) (Irvine Scientific, Santa Ana, CA). Sperm were micromanipulated using custom-made micropipettes. Isolated clean sperm were transferred to a thin-walled 0.7ml microfuge tube (Sarstedt Ltd, Montreal, QC) and lysed in 20ul of the alkaline and neutralization buffers according to Manning *et al.* (2001). Experimental procedures were carried out on sperm cell populations devoid of other contaminating cells: ejaculate and testicular sperm.

Sodium bisulphite modification

Bisulphite modification is a chemical treatment of DNA that deaminates non-methylated cytosines into uracils, while methylated cytosines remains unchanged. Post amplification the original status of DNA methylation at cytosine residues can be differentiated: methylated cytosines remain as cytosines, while non-methylated cytosines are read as thymidines. Bisulphite modification was either performed on extracted DNA or on lysed sperm cells split into two aliquots of 20ul using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA) according to the manufacturer's instructions but with a shortened 2-hour incubation period to limit degradation of the small amount of DNA used. Samples with a conversion rate of or above 95% were included in the analysis: modification performed either on ejaculate and testicular sperm provided an equally high conversion rate.

Gene amplification and cloning

DNA methylation was studied at two DMRs of imprinted genes methylated in the sperm, *H19* and *IG-GTL2*, and at a DMR that is non-methylated in the sperm, *MEST*. Previously published primer sequences were used for amplification of the selected sequences (Kerjean *et al.* 2000; Geuns *et al.* 2007). Amplification was carried out as outlined by Kerjean et al. (2000). Seventeen CpG loci were analyzed within the *H19* DMR. The known C/T single nucleotide polymorphism (SNP) (SNP #1073516) at CpG 7 was excluded from methylation analysis. Fifteen CpG loci were amplified within the *IG-GTL2* DMR, but only the last ten CpG loci were analyzed due to the generation of truncated product, and 21 CpG loci were amplified and analyzed within the *MEST* DMR. PCR products of the correct size were cloned into the pGEM-T Easy Vector

System (Promega, Madison, WI) to allow for blue/white colony screening. Two to three white colonies were picked per plate so that around ten white colonies were picked for each gene.

Analysis of sequencing data

Samples were submitted for sequencing to the McGill University and Génome Québec Innovation Centre (Montreal, QC). Products were sequenced with the SP6 sequencing primer using the Applied Biosystems 3730xl technology (Applied Biosystems Inc., Foster City, CA). Sequences were aligned using ClustalW2 (Larkin *et al.* 2007). DNA methylation within sequences was analyzed manually. Differences among sequences were used to determine the unique status of each clone analyzed and included different methylation patterns at CpG loci, single nucleotide changes within the sequences, and having originated from a different amplification reaction. Unique clones originate from different sperm cells and may be more representative of the methylation status of different sperm cells and the sample. Preferential amplification may occur when small quantities of starting material are used (Walsh *et al.* 1992; Findlay *et al.* 1995), therefore to prevent this from occurring multiple amplification reactions were set up per gene for each sample and unique clones were analyzed.

Data analysis

To compare DNA methylation at imprinted genes between groups the methylation level for each sample was calculated based on the number of methylated CpGs in proportion to the total number of CpGs analyzed at unique clones within each DMR analyzed. This analysis provided a percent methylation value. The methylation level has been previously used as a quantifiable measure of DNA methylation assessed by bisulphite sequencing and used to compare differences

in sperm DNA methylation between groups (Kobayashi *et al.* 2007; Kobayashi *et al.* 2009). Differences in the methylation level among groups were determined using the non-parametric Mann-Whitney test or the Kruskal-Wallis test with Dunn's post hoc test for comparisons among more than two groups, as described by Poplonski *et al.* (2009). One-tailed p-values <0.05 were considered significant. One-tailed p-values were used as per our priori.

We determined the number of individuals with aberrant imprinting in each group. These men were defined as having at least one hypomethylated clone, at the *H19* or *IG-GTL2* DMRs, or hypermethylated clone at the MEST DMR. Hypomethylated clones were identified as having less than 50% methylated CpGs, at the *H19* DMR and the *IG-GTL2* DMR, while hypermethylated clones were identified as having more than 50% of methylated CpGs, at the *MEST* DMR, as defined by Marques et al (2010). Differences in the number of individuals with abnormal methylation between groups were determined using Fisher's exact test. One-tailed p-values <0.05 were considered significant. One-tailed p-values were used as per our priori.

The unpaired two-tailed t test was used to determine significant age differences between groups. Statistical analysis was performed using GraphPad Prism (version 5.02) (GraphPad Software, San Diego, CA).

Declaration of interest

The authors declare no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure 1. Bead diagrams representing methylation at CpG sites studied at *H19*, *IG-GTL2* and *MEST* differentially methylated regions (DMRs). Methylated (black bead) and unmethylated (open bead) status of each CpG site is indicated within the studied sequences in (A) proven fertile men (C01-C09), (B) vasectomy reversal men (VR01-VR17) and in (C) men affected by azoospermia (TP01-TP18). Each strand shown indicates the methylation profile of a sperm. Unique clones analyzed at each DMR are shown directly in the diagram, and are coded on the right-hand side with the first number designating the number of non-unique clones that were analyzed for each sequence followed by the amplification reaction each clone came from. As it can be observed, most unique clones originated from different amplification reactions. Missing beads represent CpG sites that could not be analyzed. The amplification reactions are not necessarily labeled in consecutive order.

Figure 2. DNA methylation level at imprinted genes in vasectomy reversal and azoospermia groups. The methylation level (% methylation) is shown for each sample analyzed within the (A) *H19* DMR, (B) *IG-GTL2* DMR and (C) *MEST* DMR. Methylation level was analyzed in proven fertile men (C), in men undergoing vasectomy reversal (VR), and in men affected by azoospermia (AZO). The AZO group was further subdivided into three sub-groups: obstructive and non-obstructive azoospermia (OA and NOA, respectively) and of unknown pathology (UP). The horizontal lines indicate the group means and the whiskers indicate standard deviation of the group means. * indicates the median.

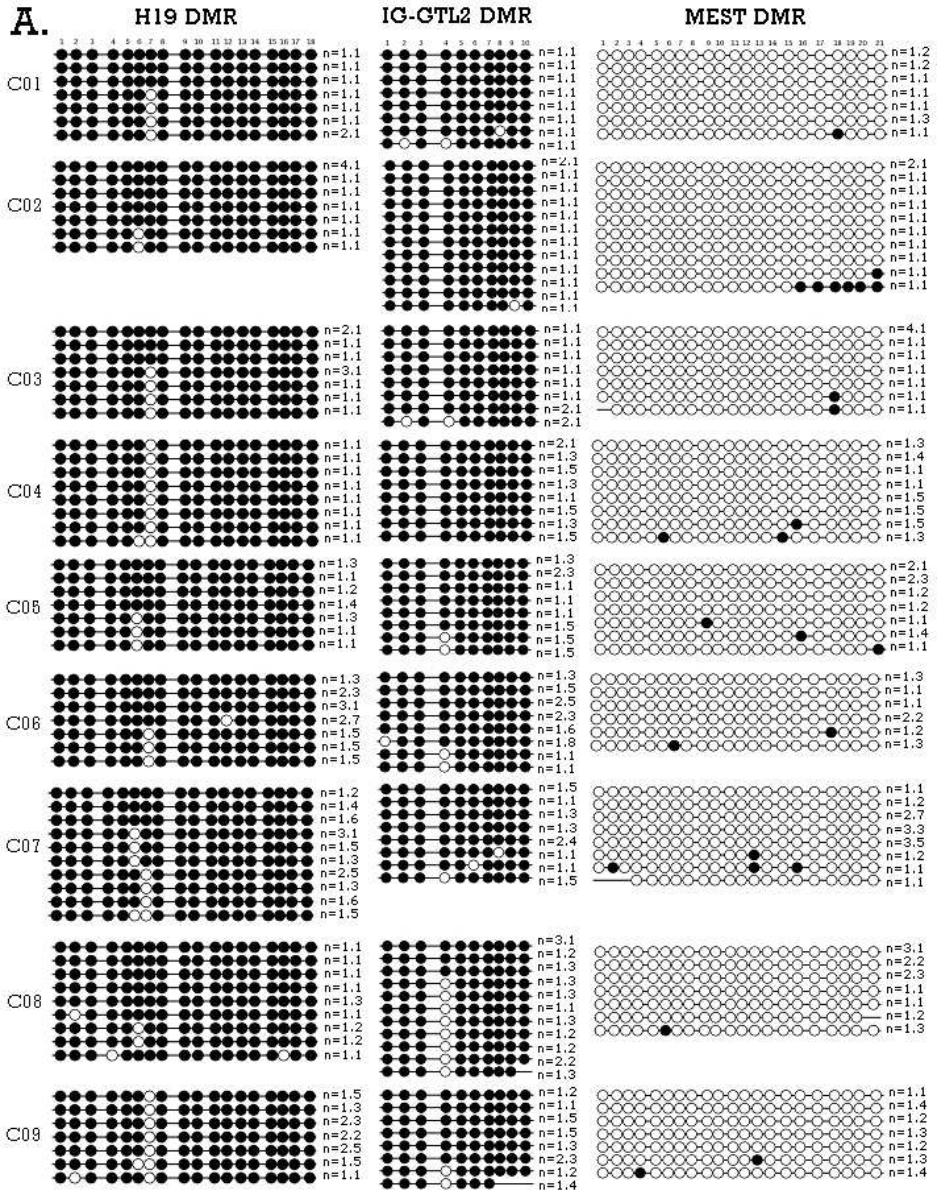
Table 1. Aberrant imprinting in the sperm of men with azoospermia and vasectomy reversal.

Study Group	DMR analyzed		
	H19	***IG-GTL2***	***MEST***
Proven fertile men	0/9	0/9	0/9
Vasectomy reversal	5/17 (29.4)	0/17	2/17 (11.8)
AZO	7/18 (38.9)*	1/18 (5.5)	1/18 (5.5)
OA	5/10 (50)*	0/10	0/10
NOA	1/5 (20)	0/5	0/5
Unknown pathology	1/3 (33.3)	1/3 (33.3)	1/3 (33.3)

Percentages shown in brackets

* significantly different compared to proven fertile men (Fisher`s exact test, $p<0.05$)

DMR: differentially methylated region, AZO: azoospermia, OA: obstructive azoospermia, NOA: non-obstructive azoospermia



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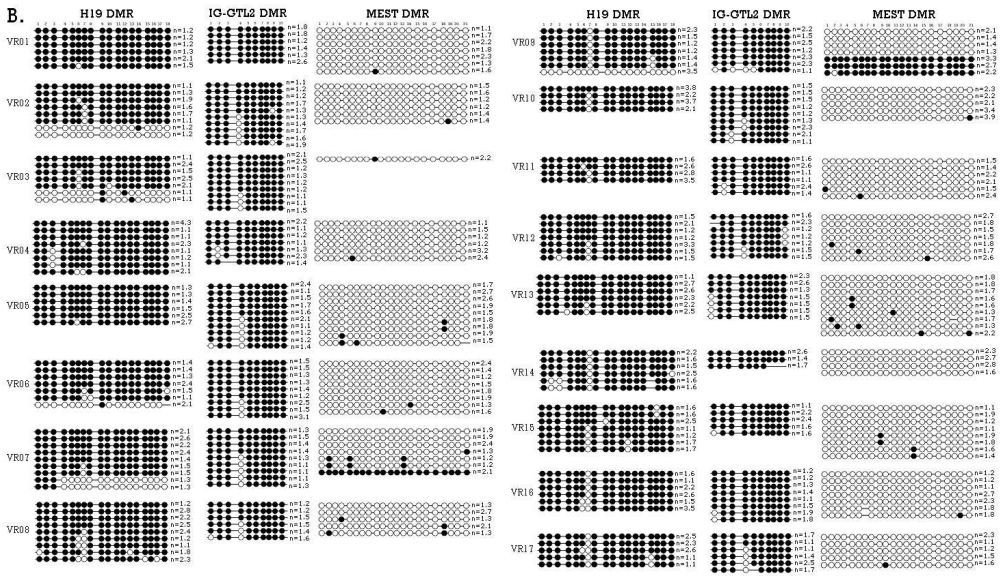

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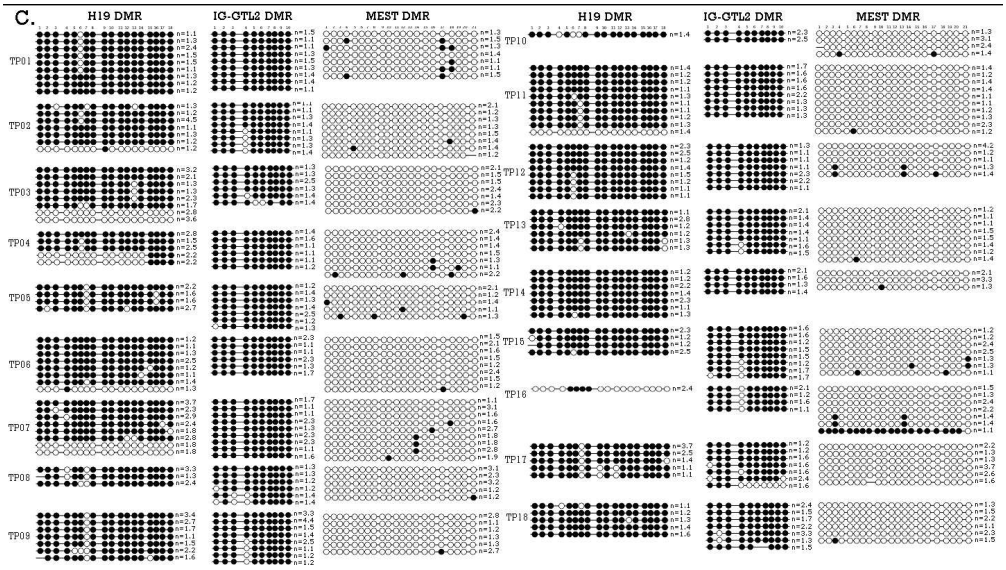

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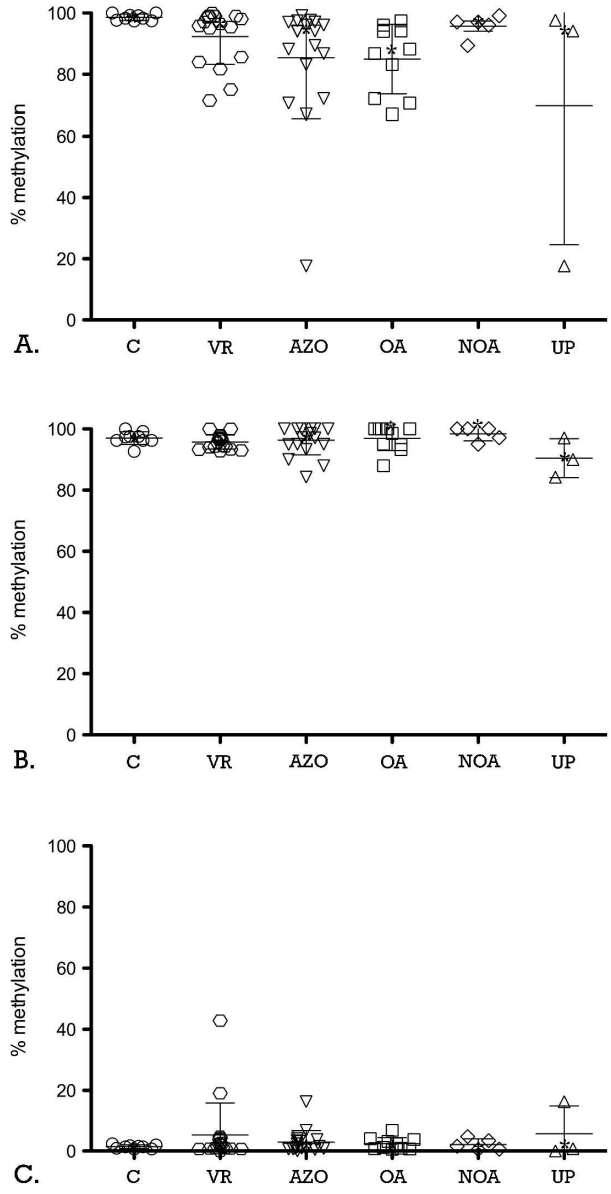

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112x221mm (500 x 500 DPI)