

Changes in the Expression Profile of the Meiosis-Involved Mismatch Repair (MMR) Genes in Impaired Human Spermatogenesis

Running title: MMR mRNA in impaired sperm production

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ABSTRACT

DNA Mismatch Repair (MMR) genes have been described to participate in crossover events during meiotic recombination, which is, in turn, a key step of spermatogenesis. This evidence suggests that MMR family gene expression may be altered in infertile men with defective sperm production. In order to determine the expression profile of MMR genes in impaired human spermatogenesis, we performed transcript levels analysis of MMR genes (*MLH1*, *MLH3*, *PMS2*, *MSH4* and *MSH5*), and other meiosis-involved genes (*ATR*, *HSPA2*, *SYCP3*) as controls, by real time reverse transcription-polymerase chain reaction (RT-PCR) in testis from 13 patients with spermatogenic failure, 5 patients with primary germ-cell tumors and 10 controls with conserved spermatogenesis. Correlation of the expression values with the histological findings was also performed. The MMR gene expression values, with the exception of *PMS2*, are significantly decreased in men with spermatogenic failure. The pattern of MMR reduction correlates with the severity of damage, being maximum in maturation arrest. Specifically, expression of the testicular *MSH4* gene could be useful as a surrogate marker for the presence of intratesticular elongated spermatid in patients with non-obstructive azoospermia, contributing to predict the viability of assisted reproduction. Interestingly, a reduction in the *MSH4* and *MSH5* transcript concentration per spermatocyte was also observed. The decreased expression level of other meiosis-specific genes, such as *HSPA2* and *SYCP3*, suggests that the spermatocyte capacity to express meiosis-related genes is markedly reduced in spermatogenic failure, contributing to meiosis impairment and spermatogenic blockade.

Key Words: *MLH1*, *MLH3*, *PMS2*, *MSH4*, *MSH5*, gene expression profiling, spermatocyte, impaired sperm production

Introduction

Mammalian spermatogenesis is a developmental process in which male germ cells undergo mitotic proliferation, meiotic division and differentiation to produce a haploid gamete for sexual reproduction. One major difference between mitosis and meiosis is the formation of a proteinaceous structure called the synaptonemal complex (SC), which allows synapsis and recombination between the two homologous chromosomes during meiotic prophase I. The axial elements, the SC component also known as lateral elements after synapsis of the homologues, begin to form between the two sister chromatids of each chromosome in leptotene cells. Synapsis of homologous chromosomes usually begins even before axial element formation is complete and involves alignment of homologues, connection of the two axial elements and formation of two additional components of the SC, the central element and the transverse filaments. The process is completed at the pachynema (Schmekel and Daneholt, 1995). The SC also contributes to the crossing over at sites along the SC known as recombination nodules (Carpenter, 1987). Crossing over, crucial for homologous recombination, occurs when two nonsister chromatids of the four homologous chromatids cut and exchange equal segments, ensuring a correct segregation of homologous chromosomes. Failure to segregate the appropriate haploid complement of chromosome can have disastrous consequences by generating aneuploid gametes with the potential to cause subsequent developmental anomalies or fetal loss (Koehler et al, 1996). Alternatively, errors in recombination can activate checkpoint mechanisms resulting in meiotic arrest and sterility (Gonsalves et al, 2004; Smith and Nicolas, 1998).

DNA Mismatch Repair (MMR) family proteins, consisting of the MutS and MutL proteins in eukaryote organisms, have been evidenced to have a determinant role in DNA repair after replication errors and their malfunction can lead to cancer in mammals. Studies in yeast and mammals revealed that some members of this family participate in the meiotic recombination process either correcting the potential mismatched bases of the heteroduplex DNA molecule after recombination or promoting crossover events. Among these members there are three MutL homologues (MLH1, MLH3 and PMS2) and two MutS homologues (MSH4 and MSH5) [see (Kolas and Cohen, 2004; Surtees et al, 2004) for review]. Moreover, MSH4 and MSH5 are meiosis-specific proteins crucial for reciprocal recombination but have no apparent mismatch repair activity.

Several studies in yeast carrying disruptions in these five MMR genes showed reduced meiotic crossing over and high frequency of postmeiotic segregation, demonstrating their role in meiotic recombination [see (Kolas and Cohen, 2004; Surtees et al, 2004) for review].

To understand the role of MMR genes in DNA repair, cancer predisposition and meiosis, several knockout mouse lines have been generated demonstrating a critical role of the above mentioned MutS and MutL homologues in mammalian meiotic recombination [see (Kolas and Cohen, 2004; Surtees et al,

2004) for review]. In *Mlh1*^{-/-} and in *Mlh3*^{-/-} male mice, germinal differentiation is clearly arrested at pachytene and no mature sperm is produced. *Pms2*^{-/-} mice, however, are able to produce spermatozoa, although they are aberrant due to an abnormal chromosome synapsis in meiosis, causing sterility. Disruption of *Msh4*, as well as of *Msh5*, gene also results in sterility due to an anomalous chromosome synapsis and meiotic failure.

These MMR family members have been reported to be highly expressed in mammalian testicular tissue (Plevova et al, 2005; Santucci-Darmanin et al, 2002; Her et al, 2001; Kneitz et al, 2000; Paquis-Flucklinger et al, 1997; Bocker et al, 1999; Her and Doggett, 1998; Her et al, 1999), preferentially in spermatocytes where meiotic recombination takes place. Human *MLH1* gene is up-regulated relative to other stages in leptotene/zygotene cells, and decreases its expression in pachytene nuclei (Marcon et al, 2008). MLH1 protein localizes on the SC (Anderson et al, 1999; Oliver-Bonet et al, 2005), appearing by the early-mid pachytene transition and gradually decreasing as spermatocyte progress through late pachynema (Oliver-Bonet et al, 2005; Santucci-Darmanin et al, 2000). Mouse *Mlh3* transcripts and protein are found in zygotene and pachytene spermatocytes (Santucci-Darmanin et al, 2002). *Pms2* transcripts and protein are found elevated in mitotically proliferating spermatogonia and in leptotene and zygotene spermatocytes, declining in early-mid pachytene (Richardson et al, 2000; Santucci-Darmanin et al, 2002). Mouse *Msh4* gene is selectively expressed in spermatocytes from leptotema up to pachynema (Santucci-Darmanin et al, 2002; Santucci-Darmanin et al, 2001), and the MSH4 protein presents a similar distribution in mouse and human spermatocytes (Kneitz et al, 2000; Oliver-Bonet et al, 2005; Santucci-Darmanin et al, 2000). The expression of MSH5 protein starts after early primary spermatocytes and ends with elongated spermatids (Bocker et al, 1999).

This strong evidence of the requirement of *MLH1*, *MLH3*, *PMS2*, *MSH4* and *MSH5* genes for a correct recombination process during yeast and mammalian meiosis suggests that some of these genes may have a role in the regulation of spermatogenesis. Hence, we hypothesize their expression may be affected in infertile men with spermatogenic failure and/or in men diagnosed with germ-cell tumors. The aim of our study is to analyze the expression levels of *MLH1*, *MLH3*, *PMS2*, *MSH4* and *MSH5* genes in testicular tissue from infertile patients and in primary germ-cell tumors using quantitative real-time RT-PCR and evaluate the relationship between gene expression levels and patients' testicular phenotypes. The results of this study will help us to understand whether potential changes in MMR gene expression play a major role in the impairment of sperm production.

Materials and Methods

Patients and controls

Our study recruited 13 infertile patients (mean age, 33 yr; range 27-40 yr) due to severe spermatogenic failure (SpF), with a phenotype consistent with non-obstructive azoospermia or severe oligozoospermia (<5 million sperm per mL), comprising patient group 1. Six of these patients were diagnosed with maturation arrest at spermatocyte level (MA) and 7 with hypospermatogenesis (HS) phenotype. Among MA samples submitted for histological analysis (Table 1), 4 out of 5 showed arrest at the pachytene stage and 1 at the leptotene/pachytene stage. Patient group 2 was formed by 5 men diagnosed with germ-cell tumor (GCT) (30 yr; 19-44 yr). In addition, 10 infertile patients diagnosed with obstructive azoospermia (32 yr; 23-42 yr), who showed conserved spermatogenesis (CS), were studied as controls (Table 1). Both patient group 1 and controls were selected from men referred for couple infertility to the Andrology Service of the Fundació Puigvert and samples for patient group 2 were recruited from the Urology Service of the Hospital Universitari de Bellvitge. The study was approved by the Institutional Review Board of both Centers, and all the participants were informed and gave written consent to the procedures of the study.

The clinical procedures for infertile patients included anamnesis, physical examination, semen analyses [performed in accordance with World Health Organization guidelines (World Health Organization, 1999)] and hormonal study. Concentrations of FSH reflected in general the findings of testicular histology, although some patients showing blockade of primary spermatocyte or hypospermatogenesis had normal FSH (Table 1). Spermograms included volume, pH, sperm concentration, motility, vitality, morphology and fructose and citrate levels in seminal plasma. The presence of normal vas deferens was assessed by scrotal palpation. The testicular biopsy was obtained when necessary to confirm the clinical diagnosis and for sperm retrieval (TESE) and cryopreservation purposes.

The routine genetic study for all samples included karyotype and analysis of chromosome Y microdeletions, the latter performed according to the European guidelines (Simoni et al, 1999; Simoni et al, 2004). Men with a chromosomal aberration or a Y-chromosome microdeletion were not included in the study.

Testicular samples

Testicular biopsies from infertile men were obtained under local anesthesia through a small incision. Each specimen was divided into three aliquots, one piece (≈10-20 mg) was fixed in Bouin's solution and reserved for histological analysis, a second aliquot (≈100-200 mg) was processed for sperm extraction

and the third (≈ 10 mg) was immediately transferred to liquid nitrogen and stored at -80°C until analysis for gene expression experiments.

Referring to GCT, testicular samples were obtained directly after orchiectomy and macroscopic pathological evaluation. For gene expression studies, one tissue fragment was taken from the tumoral portion of the testis and was immediately frozen at -80°C .

Histological analysis

Fixed testicular biopsies were cut in $5\text{-}\mu\text{m}$ sections and stained with haematoxylin-eosin. Assessment of spermatogenic status was performed by quantification of specific germ cells, that is spermatogoniae, primary spermatocytes, round spermatids and elongated spermatids, and of Sertoli cells. The average number of cells per tubule was calculated after analysis of at least 15-20 cross-sectioned seminiferous tubules per testis. The number of elongated spermatids counted per tubule and the yield of spermatozoa extracted per 100 mg of the matched samples during the same procedure showed a Pearson's $R=0.775$ ($p<0.001$), confirming that the cell count in the tissue sections was representative of the testicular histology found in the samples used for gene expression (Silber et al, 1997). A modified Johnsen score (JS) count (Schulze et al, 1999) was calculated on the basis of the number of different cell types per tubule. The mean diameter of the seminiferous tubules was additionally evaluated (Table 1).

RNA extraction and complementary DNA synthesis

Total cellular RNA was extracted from the testicular biopsy using TriPure Isolation Reagent (Roche Applied Science, Indianapolis, IN, USA), according to manufacturer's instructions. The RNA samples were then quantified by measuring the absorbance at 260 nm using the Nanodrop spectrophotometer. The quality of RNA [28S/18S ratio and RNA Integrity Number (RIN)] was also assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Testicular RNA from the three groups of study showed similar quality values as both, 28S/18S ratio and RIN, presented no significant differences among them ($p=0.450$, $p=0.190$, respectively). Mean 28S/18S ratio value between all samples (mean \pm SD) was 1.2 ± 0.15 and mean RIN value was 7.4 ± 0.49 .

Complementary DNA (cDNA) was synthesized with $1\ \mu\text{g}$ of RNA using random primers and SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) at 42°C for 50 min.

For quantitative real-time experiments, each RNA sample was submitted to two reverse transcription (RT) reactions to minimize the variation of the experimental determination of mRNA quantities due to RT efficiency. After RT reaction, 1:4 dilutions of cDNA samples were prepared. The resulting cDNA aliquots were then stored at -20°C until use.

Primers and quantitative PCR

Hypoxanthine phosphoribosyl-transferase (*HPRT*), hydroxymethylbilane synthase (*HMBS*), peptidylprolyl isomerase A-cyclophilin A (*PPIA*) and beta-2-microglobulin (*B2M*) were selected as candidate reference genes for data normalization in quantitative PCR experiments. Ataxia telangiectasia and Rad3 related (*ATR*), heat shock 70kDa protein 2 (*HSPA2*) and synaptonemal complex protein 3 (*SYCP3*) were selected as control genes of the meiotic process. The sequences of the forward and reverse primers used to amplify the human *MLH1*, *MLH3*, *PMS2*, *MSH4*, *MSH5*, *ATR*, *HSPA2*, *SYCP3*, *HPRT* and *B2M* cDNA (Supplemental Table 1, available online at www.andrologyjournal.org) were designed using Oligo4.0 and Primer3 programs. Primers for MMR mRNA quantification were designed on coding sequences that are present in multiple testicular transcript variants of the same target gene. Primers for *HMBS* and *PPIA* cDNA amplification were previously described (Neuvians et al, 2005; Pluvinet et al, 2004). Primer specificity was first assessed prior to real-time PCR experiments by agarose gel electrophoresis and sequencing the PCR product. It was later confirmed in real-time PCR experiments by the analysis of the melting temperature, which is product-specific.

Quantitative real-time PCR reactions were performed in a LightCycler 1.5 Instrument (Roche Molecular Systems, Alameda, CA), using SYBR Green I dye and 1 µl of the diluted cDNA template in a total volume of 10 µl. The products of the two previous RT reactions were amplified twice each, to ensure best reproducibility. Negative controls without template were included in each set of PCR assays as well as a sample of known gene expression copy number, which was used as standard. In addition, patient group 1 and control group samples were always analyzed as paired samples in the same analytical run in order to exclude between-run variations. Details regarding temperatures and incubation times of amplification are shown in Supplemental Table 1.

The Second Derivative Maximum Method for crossing point (C_p) determination from the LightCycler Software 4.0 (Roche Diagnostics GmbH, Mannheim, Germany) and the standard curve quantification method were used to calculate the raw expression values in all PCR samples. Prior to data submission for gene expression normalization and for every gene, both target and reference genes raw expression values were standardized, aiming for an accurate comparison of the data and to avoid abundance differences between the genes. The gene expression ratio was then calculated for each duplicated cDNA sample dividing the mean expression value of the target gene by the mean expression value of the reference gene (both coming from the same RT reaction) to normalize their expression for sample-to-sample differences in RNA input, RNA quality and RT efficiency. Thus, two target/reference gene expression ratios were obtained for each individual testicular sample, one of each coming from each RT reaction. The mean value between these two ratios was submitted to statistical analysis.

Real-time PCR efficiencies for each gene of study were determined by measuring serial 1:2 dilutions of a cDNA sample in triplicate. Efficiencies were then calculated with LightCycler Software 4.0 (Roche Diagnostics GmbH, Mannheim, Germany) according to the equation: $E = 10^{(-1/\text{slope})}$. Efficiency values ranged from 1.66 to 2.30 (Supplemental Table 1). To confirm reproducibility and precision of real-time PCR experiments, intra-assay and inter-assay variation were determined. Variation was measured as the coefficient of variation (CV) of C_p from the C_p mean value. In the above mentioned RT-PCR runs, intra-assay variation ranged from 0.18% to 0.81%, confirming high reproducibility and precision. Inter-assay variation ranged from 0.65% to 3.08%.

Data analysis

Statistical analyses were performed using SPSS 12.0 software (LEAD Technologies, Inc, NJ, USA). The nonparametric Mann-Whitney U test was used to analyze differences in absolute expression levels of reference genes in both patient groups 1 and 2 compared to controls. The Mann-Whitney U test was also used to evaluate differences in relative expression of target genes in patient groups compared to controls. The nonparametric Kruskal-Wallis test was used to assess differences in RNA quality between the three groups.

The gene-stability measure M was used to select the most stable reference genes and improve the normalization of target genes. It is defined as the average pair wise variation of a particular gene with all other genes, and for a given reference gene it is calculated by determining the pair wise variation with all other reference genes as the standard deviation of the logarithmically transformed expression ratios (Vandesompele et al, 2002).

Pearson product moment correlation coefficients were calculated to determine the correlation between the expression ratios of the target genes and the different histological parameters in patient group 1 and controls. Receiver operating characteristic (ROC) curve analysis of the *MSH4* transcript was used for distinguishing between individuals with testicular spermatids and those without. Accuracy was measured as the area under the ROC curve. The threshold value was determined by Youden's index, calculated as sensitivity plus specificity – 1 (Skendzel and Youden, 1970). A p -value <0.05 was considered significant.

Results

Assessment of alternative MMR transcripts in human testis

Prior to primer design for gene expression quantification, we assessed the presence of previously described alternatively spliced transcript variants in several testicular samples by conventional RT-PCR and sequence analysis with the primers listed in Supplemental Table 1.

As regards the *MLH1* gene, three alternatively spliced transcript variants encoding different isoforms have been previously described in several somatic tissues, although their full-length nature have not been fully determined (Charbonnier et al, 1995). We found that a low proportion of testicular transcripts lacked exons 10 and 11 (in CS as well as in MA and HS tissues), while the other two *MLH1* isoforms were negatively expressed (Supplemental Figure 1, available online at www.andrologyjournal.org).

Several full-length alternative MMR mRNAs have been described in somatic and testicular tissues. We confirmed the testicular expression of the two *MLH3* mRNA isoforms, *MLH3* variant 1 (NCBI RefSeq NM_001040108), and variant 2 (NM_014381), showing the latter to contain an in-frame deletion of exon 7 that results in a shorter protein (Lipkin et al, 2000; Santucci-Darmanin et al, 2002). Both the longest (variant 1, NM_000535) and shortest (variant 2, NR_003085) *PMS2* transcripts were also found in testis. Expression of testicular *MSH4* isoform, $\Delta hMSH4$, lacking exon 6 (Santucci-Darmanin et al, 1999), was also corroborated. Referring to *MSH5*, several mRNA splice variants have been described, such as the one containing an in-frame insertion of the last 51 bp of intron 6 resulting in the longest isoform (transcript variant 1, NM_025259), a transcript variant containing three extra base pairs between exons 20 and 21 (variant 2, NM_172165) and two transcript variants resulting from the use of an alternative in-frame splice donor site in exon 1, compared to variant 1 (variant 3, NM_002441 and variant 4, NM_172166). We determined that *MSH5* variants 1, 2 and 4 were expressed, while variant 3 was absent in testis. The presence of these alternative forms was additionally determined in the infertile testicular tissues (Supplemental Figure 1).

Moreover, while assessing primer specificity, we detected six additional alternatively spliced MMR mRNA forms expressed in testis although their full-length sequence was not fully determined. These new spliced forms were a *MLH3* variant lacking exon 5, a *MLH3* variant lacking both exons 5 and 7, an alternative *MSH4* mRNA containing an in-frame deletion (the first 97 pb of exon 6), an alternatively spliced *MSH4* mRNA lacking exon 19, a variant of *MSH5* mRNA including intron 6 and a variant of *MSH5* mRNA lacking exon 9. Both *MLH3* isoforms contained a deletion of the coding region that preserves the open reading frame and, consequently, translation of these transcripts would result in shorter proteins. The deletion observed in *MSH4* and *MSH5* isoforms, however, would result in a reading frameshift with a new stop codon downstream, thus translation of these transcripts are predicted to result in truncated proteins. All these new variants were confirmed to be present in lymphocytes [with the exception of *MSH4* (-Exon 19)] as well as in both MA and HS testicular tissue (Supplemental Figure 1).

Based on these results, primers for MMR mRNA quantification were designed so as to be able to amplify different testicular transcript variants of the same target gene, and thus global differences in gene expression, affecting multiple splice variants, could be evaluated.

Expression levels of candidate reference genes. Selection of a suitable normalizer

Measurement of gene expression by real-time RT-PCR requires at least one proper internal control gene for normalization purposes in order to achieve precise and reliable quantitative expression results of the genes under study. We have assessed the suitability of four housekeeping genes widely used as normalizers in the literature: *HPRT* (purine nucleotide biosynthesis), *HMBS* (porphyrin metabolism), *PPIA* (protein folding) and *B2M* (immune response) as candidate genes for normalization in both pathological and normal testicular tissue.

Spermatogenic failure

Figure 1a shows the mRNA levels of the four candidate reference genes in patient group 1 and controls. When we first looked for differences in gene expression between both groups, non-significant differences in absolute gene expression levels were observed for the four genes analyzed (*HPRT*, $p=0.522$; *HMBS*, $p=0.077$; *PPIA*, $p=0.410$; *B2M*, $p=0.648$), suggesting that all four genes were potentially suitable reference genes for expression normalization in testis tissue from SpF and CS samples. However, in order to select the most stable control genes and improve the normalization, the gene-stability measure M (Vandesompele et al, 2002), was calculated for every reference gene. Lower M values indicate genes with less expression variation among samples. M values for our reference genes resulted in 0.82 for *HPRT*, 0.72 for *HMBS*, 0.65 for *PPIA* and 1.11 for *B2M*. Because *B2M* showed the highest M value and it was much more elevated than the M value of the other three genes, it was excluded as a reference gene for further analysis. Then M values were recalculated for the remaining genes, resulting in 0.80 for *HPRT*, 0.67 for *HMBS* and 0.69 for *PPIA*. Since all three reference genes displayed a similar M value, a normalization factor (NF), based on the average of the expression levels of the most stable reference genes – *HPRT*, *HMBS*, and *PPIA* –, was calculated for each sample as the arithmetic mean of the selected reference gene expression values. This NF was selected as the suitable value for normalization in order to calculate the relative expression of our target genes in SpF samples.

Germ-cell tumors

As regards the absolute expression levels of the reference genes in patient group 2 and control samples (Figure 1b), significant differences in expression were found for *HPRT* ($p=0.001$), *PPIA* ($p=0.019$) and *B2M* ($p=0.001$) when both groups were compared. *HMBS* expression showed non-significant differences between tumoral and normal control testicular tissue ($p=0.953$), hence, it was later used as the normalizer gene when studying GCT samples.

Relative expression of MMR genes in patients and controls

Spermatogenic failure

When studying patient group 1, normalized expression levels of MMR genes were calculated as the ratio ‘target gene/NF’ for each sample (Figure 2a). Significant differences in gene expression levels were observed between patients and controls for *MLH1* ($p=0.030$), *MLH3* ($p=0.015$), *MSH4* ($p=0.000$) and *MSH5* ($p=0.004$). Non-significant differences were found for *PMS2* ($p=0.088$) (Figure 2a and Supplemental Table 2 [available online at www.andrologyjournal.org]).

In order to demonstrate the relevance of choosing an appropriate reference gene/s to obtain reliable interpretation of target gene expression data, MMR gene expression ratios were also calculated in patient group 1 and controls using each single reference gene as normalizer. The difference of expression was statistically significant for *MSH4* and *MSH5* when data were normalized with both single reference gene and NF. However, the reliability of the interpretation of MutL homologues expression data was highly dependent on the applied normalizer. In this case, similar results were obtained when using NF or *PPIA* as normalizers (Supplemental Table 2).

When the patient group 1 was divided into MA and HS subgroups, we observed that reduction of expression was more pronounced in the maturation arrest phenotype. The percentage of expression reduction of our target genes compared to controls ranged from 24% for *PMS2* to 60% for *MSH4* in MA patients and from 11% for *PMS2* to 34% for *MSH4* in HS patients (Figure 2b). When we compared the expression ratios between MA subgroup and CS controls, we observed significant differences for *MLH1* ($p=0.016$), *MLH3* ($p=0.031$), *MSH4* ($p=0.002$) and *MSH5* ($p=0.031$) and non-significant differences for *PMS2* ($p=0.118$). Interestingly, expression ratios between HS phenotype and controls were found to be differentially expressed only for *MSH4* ($p=0.005$) and *MSH5* ($p=0.014$), while MutL homologues, although their expression was reduced in the HS subgroup, presented non-significant differences when compared to CS controls: *MLH1* ($p=0.230$), *MLH3* ($p=0.070$) and *PMS2* ($p=0.230$).

Germ-cell tumors

Normalized expression levels of MMR genes in patient group 2 compared to control group were calculated as the ‘target gene/*HMBS*’ expression ratio for each sample (Figure 3). Statistically significant differences in relative gene expression levels were observed between tumoral and normal testicular tissue in each gene (*MLH1*, $p=0.001$; *MLH3*, $p=0.008$; *PMS2*, $p=0.005$; *MSH4*, $p=0.001$; *MSH5*, $p=0.001$).

Correlation study between MMR gene expression profiles and histological parameters

In order to assess whether there is an association between gene expression and tubular cell number and confirm whether the results could be of physiological relevance, we performed the correlation study

between the five normalized gene expression ratios and several histological parameters such as: seminiferous tubular diameter, number of each type of cell from the germ line, Sertoli cell number and JS count (Figure 4a) in patient 1 and control groups. When referring to the diameter of seminiferous tubules, it significantly correlated with *MLH3*, *MSH4* and *MSH5*. When the total number of samples was considered, most of the histological parameters, with the exception of Sertoli cell and spermatogonia number, positively correlate with *MLH1*, *MLH3*, *MSH4* and *MSH5* expression levels. Interestingly, significant positive correlation coefficients were found between the number of elongated spermatids and the transcription levels of the five genes, being remarkable the correlation coefficient for *MSH4* ($r=0.815$) (Figure 4a). We hypothesize that there may be a threshold level of *MSH4* transcripts related with the presence of intratesticular elongated spermatid. The ROC curve analysis of *MSH4* expression levels indicates that the threshold that gave the maximum true-positive fraction (sensitivity) and false-positive fraction (1-specificity) was 0.917. At this threshold value, the sensitivity and the specificity for predicting the presence of ≥ 1 elongated spermatid per tubule were 80 and 100%, respectively (Figure 4b). The calculated area under the curve was 0.944, with a 95% confidence interval of 0.850 to 1. As comparison, the areas under the ROC curve of testicular volume and FSH concentration were 0.726 (95% CI 0.477 – 0.976) and 0.327 (95% CI 0.028 – 0.627) respectively.

Relative expression of other meiosis- involved genes in SpF patients and controls

In order to determine whether testicular gene expression alteration in SpF patients exclusively affected MMR genes or, on the contrary, it was a generalized phenomenon affecting other meiosis-involved genes, we have performed an additional expression profile analysis of other spermatocyte preferentially-expressed genes (Chalmel et al, 2007) such as *ATR* (cell cycle arrest and DNA damage repair), *HSPA2* (male meiosis) and *SYCP3* (synaptonemal complex structure).

Normalized expression levels of meiosis-involved genes were calculated as the ratio ‘target gene/NF’ for each sample (Figure 5). Significant differences in gene expression levels were observed between patients and controls for *ATR* ($p=0.006$), *HSPA2* ($p=0.018$), and *SYCP3* ($p=0.026$). When considering SpF subgroups, significant differences in gene expression levels were observed for *ATR* in both MA and HS subgroups when compared to CS ($p=0.042$ and $p=0.019$ respectively) and for *HSPA2* between MA and CS ($p=0.011$), whereas non-significant differences were found for *HSPA2* between HS and CS ($p=0.161$) and for *SYCP3* in both MA and HS patients comparing to CS ($p=0.073$ and $p=0.070$ respectively).

Expression levels of germ cell-specific genes per cell

We additionally analyzed the germ cell-specific transcript levels per spermatocyte cell in both MA and HS subgroups compared to CS controls in order to obviate the differences in gene expression due to changes in testicular cellularity and to determine whether spermatocyte gene expression is altered in spermatogenic failure. Selective germ cell expression of *MSH4* and *MSH5*, but not of *MLH1*, *MLH3* and *PMS2*, was previously confirmed as negligible transcript level values were found in three complete Sertoli Cell-Only (SCO) samples (data not shown). Values of transcript amount per cell, in arbitrary units, were obtained for each testicular sample by dividing the expression ratio value by the proportion of primary spermatocytes [known to be the germ cell stage that predominantly expresses *MSH4* and *MSH5* in the testis (Chalmel et al, 2007)] present in a seminiferous tubule of the sample (Figure 6a, 6b). Significant differences in cellular transcript levels were found for *MSH4* between SpF patients and controls ($p=0.000$), MA subgroup and controls ($p=0.001$) and HS subgroup and controls ($p=0.000$) and for *MSH5* between SpF patients and controls ($p=0.000$), MA subgroup and controls ($p=0.002$) and HS subgroup and controls ($p=0.001$).

In order to determine whether differences of expression per cell affected other germ cell-specific genes involved in meiosis, *HSPA2* and *SYCP3* transcript levels per cell were also determined in the groups of study, as these genes have been previously described to be predominantly expressed in primary spermatocytes (Chalmel et al, 2007). Selective germ cell expression of *HSPA2* and *SYCP3* was confirmed as negligible transcript level values were found in three complete Sertoli Cell-Only (SCO) samples (data not shown). Values of transcript amount per cell were determined as described for *MSH4* and *MSH5* (Figure 6c and 6d). Significant differences were found for *HSPA2* and *SYCP3* between SpF patients and controls ($p=0.001$; $p=0.000$ respectively), MA subgroup and controls ($p=0.001$; $p=0.002$ respectively) and HS subgroup and controls ($p=0.023$; $p=0.005$ respectively).

Discussion

The formation of a mature sperm is a very complex process involving the transcription of many genes. Defects in essential genes can result in impaired sperm or no sperm at all. Defective meiosis during spermatogenesis is one of the critical causes of severe sperm impairment, although the details still remain unknown. In mammals, meiosis is a fundamental process that allows a genetic exchange between maternal and paternal genomes (Nasmyth, 2002). To shed some light on the expression behavior of the meiosis-involved MMR genes in the impairment of sperm production, we evaluated testicular *MLH1*, *MLH3*, *PMS2*, *MSH4* and *MSH5* gene expression in relation to meiosis alterations.

The present analytical strategy for gene expression quantification was very carefully designed to minimize both external and internal influences on expression data and improve experimental accuracy.

Several factors were considered, including an acceptable RNA quality without statistical differences between the groups under study, duplicates of RT and PCR reactions, and low intra-assay and inter-assay variation values of PCR runs. As alternative splicing, as well as, alternative transcriptional initiation and polyadenylation are the main mechanisms for generating germ cell-specific and stage-dependent mRNAs (Eddy and O'Brien, 1998), multiple testicular transcript variants of the same target gene were considered for primer design. Transcript amounts were measured by real-time RT-PCR analysis and data normalized to suitable reference genes, which should show constitutive and stable expression levels in the samples investigated. An appropriate reference gene/s was chosen for each experimental condition affecting testicular tissue, being determinant especially when studying the biological significance of small expression differences between groups (Supplemental Table 2).

Our data indicate that testicular expression levels of meiosis-involved MMR genes (with the exception of *PMS2*) are significantly reduced in SpF patients compared to CS men. Moreover the reduction is much more significant in the MA phenotype than in HS. The MMR gene transcription efficiencies are even more reduced in GCT infertile individuals and specifically MutS homologues mRNA expression levels were very low or almost negligible in these patients, possibly related to the fact that the germ-line in testicular tumor has undergone a dedifferentiation process.

Interestingly, a remarkable significant positive correlation coefficient was found between the number of elongated spermatids and the transcription levels of *MSH4*. The testicular *MSH4* expression ratio was able to accurately predict the presence of intratesticular elongated spermatid. This could be potentially used as a surrogate marker for the presence of full spermatogenesis in patients with non-obstructive azoospermia, especially those considering further attempts of invasive testicular extraction after a first negative biopsy with fine needle sperm aspiration.

Previous data evidenced that these four MMR proteins (Mlh1, Mlh3, Msh4 and Msh5) collaborate with each other as a complex in promoting meiotic recombination and crossing over, initiated by the association of meiosis-specific MutS γ heterodimer (Msh4-Msh5) with the DNA at zygonema and followed by the recruitment of the heterodimeric complex of MutL homologues Mlh1-Mlh3 (MutL γ) at pachynema, stabilizing the interaction. Pms2, however, although capable of heterodimerizing with Mlh1, does not have a direct function in crossing over (Kolas et al, 2005) but has a role in the regulation of the nuclear or cytoplasmic location of MLH3 in the cell by competing with MLH3 for the interaction with MLH1 (Korhonen et al, 2007). This crossing over-independent regulatory role of PMS2 supports the finding of the lack of expression difference for *PMS2* but not for *MLH1*, *MLH3*, *MSH4* and *MSH5* in our testicular samples.

When studying gene expression profiles in the testis, an inherent problem to be taken into account is the cellular complexity of this organ. Changes in gene expression at the tissue level can reflect changes

in the capability of transcribing the mRNA in a specific cell type as well as changes in the cell-type composition or number. The reduction of MMR gene expression in SpF patients could be partially explained by the decreased number of germ cells that specifically express MMR genes in these individuals, in fact, a positive correlation between gene expression and germ cells was determined (Figure 4a). Nevertheless, although the number of spermatocytes per tubule was decreased in infertile samples when compared to controls, non-significant differences among groups were found ($p=0.113$), thus changes in expression observed among groups could be not exclusively explained by the spermatocyte cell number. Interestingly, an additional statistically significant reduction in the expression levels of germ cell-specific genes per spermatocyte was observed in MA and HS when compared to CS samples, demonstrated for *MSH4* and *MSH5* genes (Figure 6), being more pronounced in the maturation arrest phenotype. The histological pattern of testicular hypospermatogenesis may be related to some level of maturation arrest in the tubules, which may explain the differences in MMR gene expression profiles per cell of HS with that of the CS or MA group and should contribute to the understanding of patterns of in vivo expression of MMR genes in male infertility of testicular origin.

A decreased cellular expression level of other meiosis-involved genes, *HSPA2* and *SYCP3*, was also detected and correlates with the severity of testicular damage, as occurred for *MSH4* and *MSH5*. These data indicate that MMR gene expression alteration is the result of a generalized phenomenon affecting spermatocyte gene expression capacity, and support the hypothesis that the meiosis alteration may already be arising in early stages of spermatogenesis, leading to a global reduction of the meiosis-involved gene expression contributing to spermatogenic blockade. Protein data on non-obstructive testicular tissue corroborate our mRNA expression results: maturational arrest tissue showed weak HSPA2 staining within spermatocytes when compared to normal tissue by means immunofluorescence technique (Feng et al, 2001).

MSH4/MSH5 heterodimer acts locally at sites of emerging recombination events. Specifically, a role for *MSH4* in synapsis initiation and maintenance has been suggested as well as in the determination of the recombination sites by attracting *MLH3/MLH1* (Oliver-Bonet et al, 2005). Antibodies against *MLH1* are used to identify the sites of meiotic recombination on synaptonemal complex. Meiotic studies on the pachytene stage of spermatogenesis have demonstrated that non-obstructive infertile men have impaired chromosome synapsis, a significantly decreased frequency of recombination, and an increased frequency of chromosomes completely lacking a recombination site (Sun et al, 2006). It is tempting to speculate that such errors could be partially consequence of decreased expression levels of MMR genes in the spermatocytes. Moreover, the defects of germ-cell MMR expression can increase the generation of aneuploid gametes with potential consequences for fetal development, if the non-obstructive individual is included in an assisted reproduction program.

In summary, we developed a reliable approach that allows the analysis of gene expression in testicular biopsies taking into account the variability in testicular cellularity between control and pathological infertile testis. By this method we describe a reduction of transcript concentration of meiosis-involved MMR genes in patients with severe impaired sperm production, especially in those with maturation arrest. The defects of transcript levels in SpF seem to be a consequence of a global phenomenon, where the spermatocyte expression capability is affected, contributing to spermatogenic blockade. Future studies of gene expression of early cellular stages of spermatogenesis as well as the study of factors involved in regulating gene expression in the spermatogenic process may help us to understand the molecular mechanisms that regulate the correct initiation and progression of meiotic process. Moreover, these findings contribute to the search and selection of the most valuable gene markers potentially useful as additional tools for the detection of sperm production, *MSH4* as a marker of spermatogenesis, and for predicting the viability of assisted reproduction.

Acknowledgements

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Figure Legends

Figure 1. **a.)** Absolute expression levels of reference genes in patient group 1 (striated boxes) and control group (white boxes). □, outlying value (*B2M* outlying value in patient group 1, 5.87, is not represented). Non-significant differences were observed between the two groups for all genes **b.)** Absolute expression levels of reference genes in patient group 2 (striated boxes) and control group (white boxes). □, outlying values. Significant differences are Indicated by *asterisks* (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$)

Figure 2. **a.)** Expression ratios of MMR genes in patients with spermatogenic failure (black bars) and controls (white bars) using *NF* as normalizer. Significant differences are indicated by *asterisks* (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$) **b.)** Percentage of expression reduction of MMR genes in maturation arrest (black bars) and hypospermatogenesis (striated bars) subgroups compared to controls. (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$)

Figure 3. Expression ratios of MMR genes in patients diagnosed with germ-cell tumor (black bars) and controls (white bars) using *HMBS* as normalizer. Significant differences are indicated by *asterisks* (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$)

Figure 4. **a.)** Table: Pearson correlation coefficients and adjusted p -values (r ; p) between the expression ratios of the target genes and the different histological parameters for all the samples analyzed. Significant differences ($p<0.05$) are indicated in bold. sp: spermatid, JS: Johnsen score. **b.)** Testicular *MSH4* expression ratio as a marker for spermatogenesis. *Horizontal line* indicates the *MSH4* transcript ratio threshold value -0.917- that predicts the presence of testicular elongated spermatid with a sensitivity of 80% and specificity of 100% (ROC curve analysis).

Figure 5. Expression ratios of *ATR*, *HSPA2* and *SYCP3* control genes in patients with spermatogenic failure (black bars) and controls (white bars) using *NF* as normalizer. Significant differences are indicated by *asterisks* (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$)

Figure 6. *MSH4* (**a.**), *MSH5* (**b.**), *HSPA2* (**c.**) and *SYCP3* (**d.**) expression ratio per spermatocyte (x1000) of different testicular histological groups. Maturation arrest (filled triangle), hypospermatogenesis (filled rhombus) and control (filled square) samples. *Horizontal lines* indicate median values.

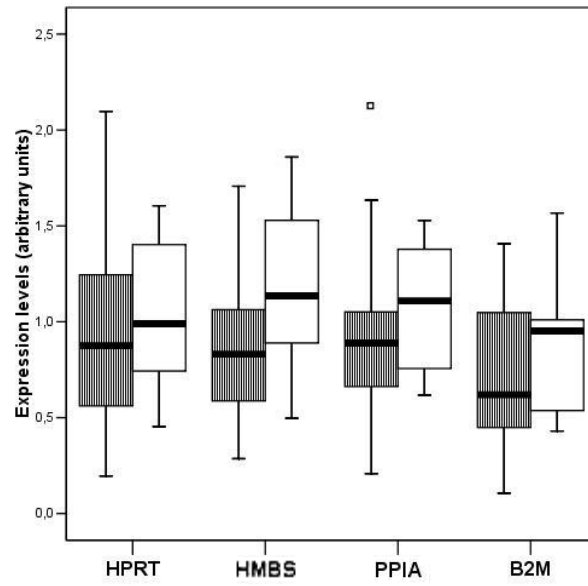
Table 1. *Phenotypical and histological description of the testicular samples of the study*

No. patient	Diagnosis	Histology	FSH (U/L)	Semen Sperm conc. (million/ml)	Tubular diameter	Spgonia	Spcyte I	Round Sptid	Elongated Sptid	Sertoli cells	Johnsen score
Patient group 1											
1	SpF (SA)	MA 95%	20.40	0.0	154.10	17.30	8.50	1.00	0.0	8.90	4.80
2	SpF (SA)	MA 100%	8.96	0.0	145.80	16.60	15.30	0.50	0.0	15.00	5.20
3	SpF (SSO)	MA 95%	15.30	0.005	179.50	27.85	35.10	6.65	0.40	13.75	6.20
4	SpF (SSO)	MA >80%	3.60	0.4	196.80	24.80	21.10	1.90	0.50	11.80	5.20
5	SpF (SSO)	MA>80%	n/a	0.004	184.25	21.20	26.15	19.10	0.35	17.05	6.85
6	SpF (SSO)	MA>80%	13.30	3.5	n/a	n/a	n/a	n/a	n/a	n/a	n/a
7	SpF (SSO)	HS hom	n/a	0.5	184.50	14.50	21.20	10.40	5.90	9.20	7.80
8	SpF (SSO)	HS hom	15.60	0.009	156.70	26.00	45.90	28.20	1.50	18.90	7.70
9	SpF (SSO)	HS hom	3.98	3.0	158.70	20.20	31.10	22.50	5.10	17.00	8.10
10	SpF (SSO)	HS hom	3.00	5.0	205.00	20.20	28.60	15.70	0.10	23.30	6.60
11	SpF (SSO)	HS hom	7.18	0.08	188.00	18.50	33.50	20.40	6.70	19.00	8.30
12	SpF (SSO)	HS mix	14.20	0.5	190.95	18.80	6.60	2.00	1.30	12.75	5.75
13	SpF (SSO)	HS hom	3.17	0.007	182.50	16.10	16.45	11.35	4.85	18.30	7.40
Patient group 2											
14	GCT	CSem	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
15	GCT	EC	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
16	GCT	EC	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
17	GCT	MX	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
18	GCT	MX	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Control group											
19	OA	CS	3.60	0.0	176.25	20.25	29.75	27.00	4.50	6.00	7.50
20	OA	CS	5.96	0.0	193.75	21.95	28.85	18.90	19.65	11.95	9.30
21	OA	CS	2.10	n/a	209.40	21.15	34.65	24.00	24.15	15.05	9.20
22	OA	CS	6.60	0.0	247.80	26.80	32.00	22.05	18.40	13.55	8.90
23	CBAVD	CS	1.89	0.0	192.50	25.55	42.25	30.75	30.30	15.75	9.85
24	CBAVD	CS	3.50	0.0	n/a	n/a	n/a	n/a	n/a	n/a	n/a
25	CBAVD	CS	5.90	0.0	197.75	20.10	27.35	29.80	19.10	11.85	9.00
26	CBAVD	CS	4.30	0.0	192.50	18.65	31.30	22.50	22.20	12.00	9.45
27	CUAVD	CS	6.40	0.0	220.15	25.70	38.15	23.30	23.95	15.60	9.30
28	CUAVD	CS	5.35	0.0	194.75	14.75	29.75	12.50	22.25	8.35	8.65

The mean number of the different type of cells per tubule is given in each group.

Abbreviations: Spgonia: spermatogonia; Spcyte: spermatocyte, Sptid: spermatid, SpF: spermatogenic failure, SA: secretory azoospermia, SSO: severe secretory oligozoospermia, GCT, germ-cell tumor; OA, obstructive azoospermia; CBAVD, congenital bilateral absence of the vas deferens; CUAVD, congenital unilateral absence of the vas deferens. MA, maturation arrest; HS hom, homogeneous hypospermatogenesis; HS mix: mixed hypospermatogenesis CSem, classic seminoma; EC, embryonic carcinoma; MX, mixed germ-cell tumor (80% embryonic carcinoma; 20% classic seminoma); CS, conserved spermatogenesis.

Figure 1
a.



b.

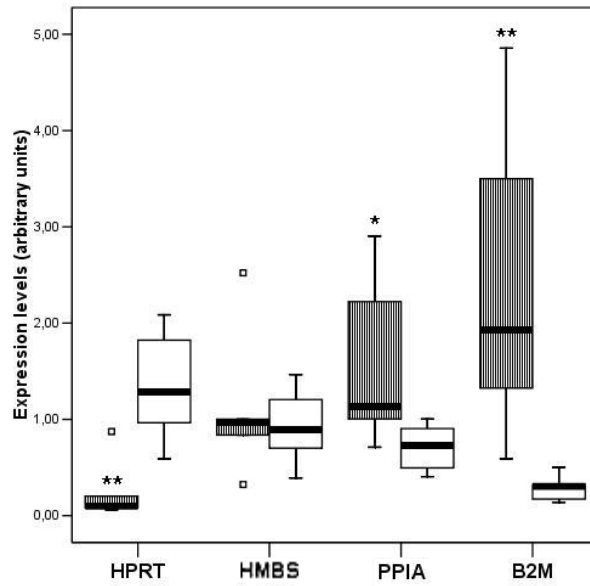
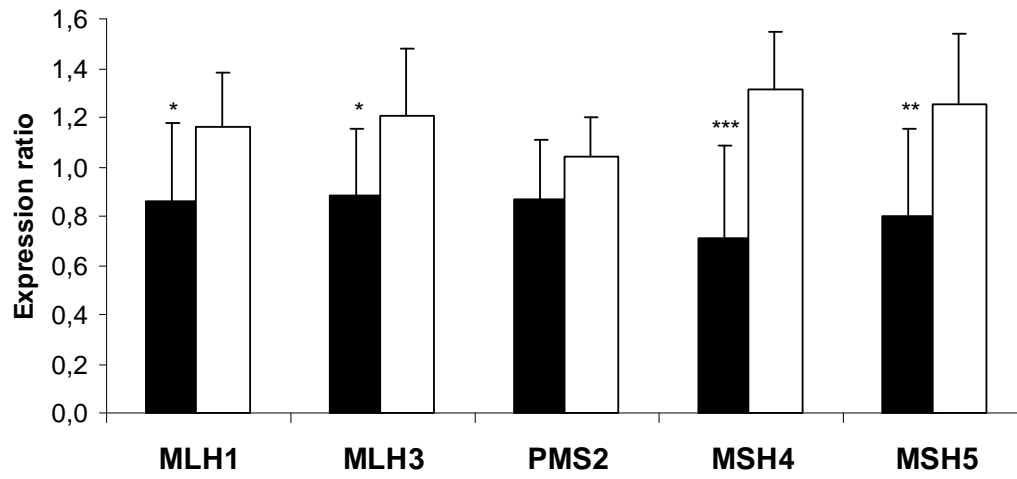


Figure 2

a.



b.

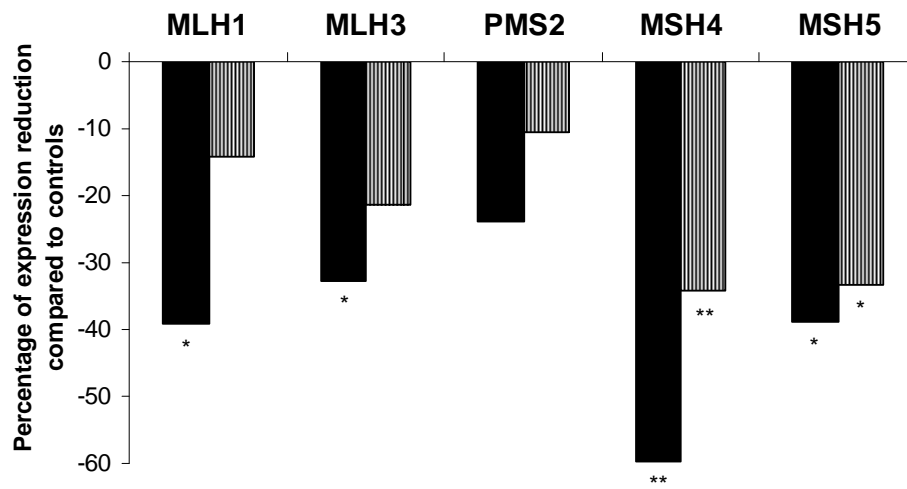


Figure 3

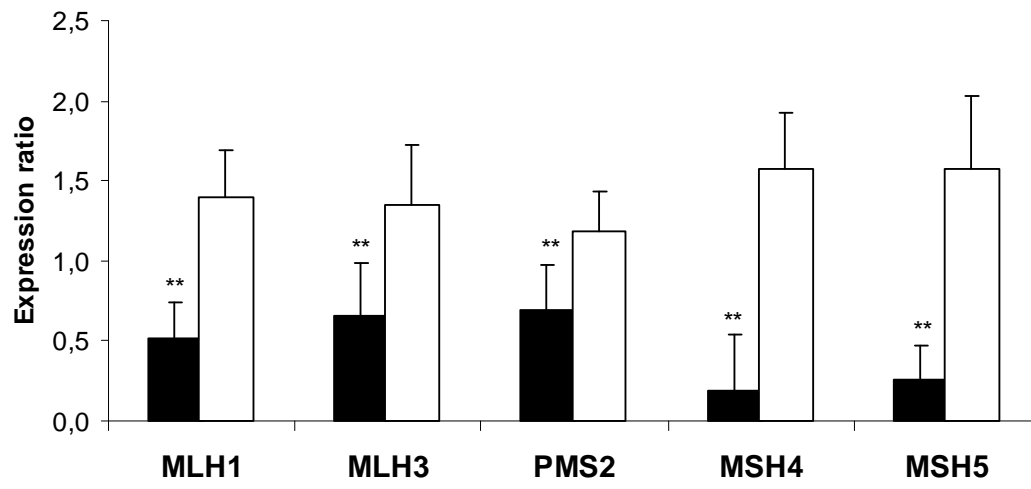


Figure 4

A.

	Tubular Diameter	Spermatogoniae	Spermatocyte I	Round sp	Elongated sp	Sertoli cells	Total cell number	JS
MLH1	0.313; <i>p</i> =0.166	0.213; <i>p</i> =0.354	0.715; <i>p</i>=0.000	0.735; <i>p</i>=0.000	0.697; <i>p</i>=0.000	0.270; <i>p</i> =0.236	0.816; <i>p</i>=0.000	0.817; <i>p</i>=0.000
MLH3	0.467; <i>p</i>=0.033	0.010; <i>p</i> =0.964	0.498; <i>p</i>=0.022	0.411; <i>p</i> =0.064	0.744; <i>p</i>=0.000	0.014; <i>p</i> =0.953	0.583; <i>p</i>=0.006	0.746; <i>p</i>=0.000
PMS2	0.419; <i>p</i> =0.059	0.108; <i>p</i> =0.643	0.186; <i>p</i> =0.420	0.379; <i>p</i> =0.091	0.467; <i>p</i>=0.033	0.122; <i>p</i> =0.598	0.363; <i>p</i> =0.078	0.461; <i>p</i>=0.035
MSH4	0.448; <i>p</i>=0.042	0.184; <i>p</i> =0.424	0.731; <i>p</i>=0.000	0.785; <i>p</i>=0.000	0.815; <i>p</i>=0.000	0.090; <i>p</i> =0.700	0.851; <i>p</i>=0.000	0.923; <i>p</i>=0.000
MSH5	0.482; <i>p</i>=0.027	0.253; <i>p</i> =0.268	0.689; <i>p</i>=0.001	0.526; <i>p</i>=0.014	0.751; <i>p</i>=0.000	-0.035; <i>p</i> =0.881	0.717; <i>p</i>=0.000	0.729; <i>p</i>=0.000

B.

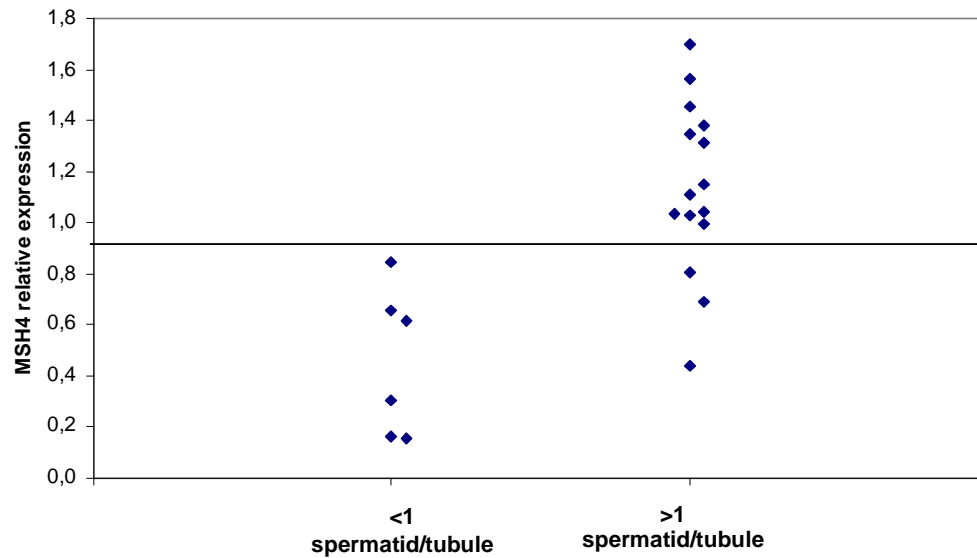


Figure 5

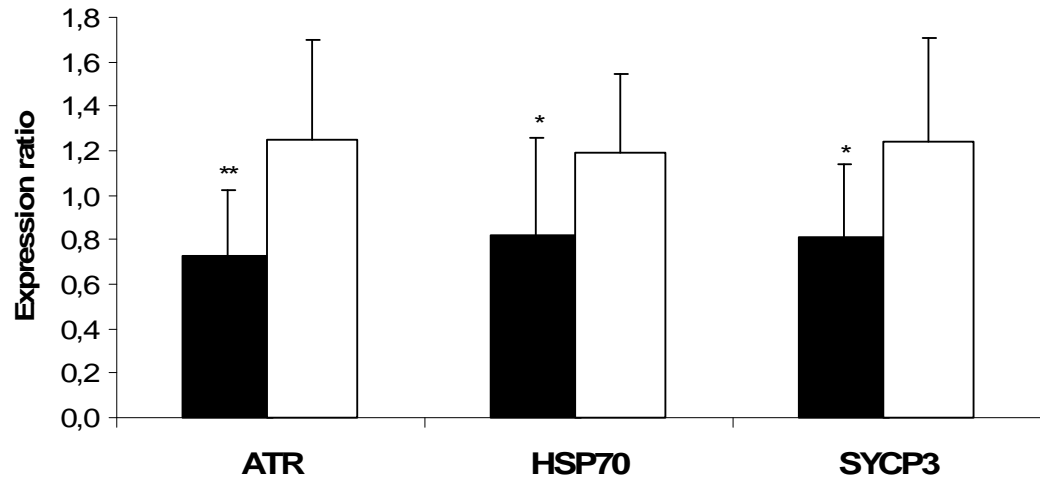


Figure 6

