Mlh1 heterozygosity and promoter methylation associates with microsatellite instability in mouse sperm

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Abstract

DNA mismatch repair (MMR) proteins play an important role in maintaining genome stability, both in somatic and in germline cells. Loss of MLH1, a central MMR protein, leads to infertility and to microsatellite instability (MSI) in spermatocytes, however, the effect of $Mlh1$ heterozygosity in germline genome stability remains unexplored. To test the effect of $Mlh1$ heterozygosity on MSI in mature sperm, we combined mouse genetics with single-molecule PCR that detects allelic changes at unstable microsatellites. We discovered 4.5% and 5.9% MSI in sperm of 4- and 12-month old $Mlh1^{+/-}$ mice, respectively, and that $Mlh1$ promoter methylation in $Mlh1^{+/-}$ sperm correlated with higher MSI. No such elevated MSI was seen in non-proliferating somatic cells. Additionally, we show contrasting dynamics of deletions versus insertions at unstable microsatellites (mononucleotide repeats) in sperm.
Introduction

DNA mismatch repair (MMR) plays a crucial role in maintaining post-replicative genomic stability. During spermatogenesis, in pre-meiotic cells, MMR proteins MLH1, PMS2, MSH2, MSH3 and MSH6 are involved in repairing insertion-deletion (indel) mutations and single base pair mismatches, and in meiotic cells MMR proteins MLH1, MLH3, MSH4 and MSH5 are essential for ensuring meiotic crossovers (1-5). Loss-of-function of any of these genes leads to adverse consequences in genomic stability leading to various abnormalities or even infertility (1,3,6-10).

Spermatogenesis involves high levels of cell proliferation. Spermatogonial stem cells either self-renew or undergo 9–11 mitotic divisions to produce spermatocytes, which subsequently undergo meiotic cell divisions to produce haploid sperm cells (male gametes) (11). Hence, a mature sperm cell is a product of numerous rounds of DNA replication.

DNA replication is inherently mutagenic. In eukaryotes, during each round of replication, DNA polymerases α, δ and ε make on average less than $1 \times 10^{-5}$ replication errors per nucleotide (12-14). The intrinsic proofreading activity of DNA polymerase corrects most of these errors, and post-replication, nearly all remaining errors are repaired by MMR. An average of $1.8 \times 10^{-10}$ mutations per nucleotide is introduced into the mouse genome during every cell division (15). Short tandem repeat sequences in the genome called microsatellites are particularly prone to replication errors, by a process known as polymerase “slippage”. DNA polymerase often erroneously inserts or bypasses individual repeat units at microsatellites, resulting in small indel loops between the parental DNA strand and the newly replicated daughter strand. If left unrepaired, indel loops give rise to mutant alleles of novel microsatellite repeat array lengths. This molecular phenotype is known as microsatellite instability (MSI). During spermatogenesis, several rounds of DNA replication take place before cells enter the meiosis. It follows that any MSI detected in sperm is likely pre-meiotic.
that is, it originates in spermatogonia which is the only cell type in the testis to undergo extensive cell proliferation.

MMR is crucial for microsatellite stability in both somatic and germline tissues (6,7,9,16). Individuals with inherited MMR defects, in particular in MLH1 and MSH2 heterozygosity, a condition known as Lynch syndrome (LS) often develop MSI-associated colorectal cancer, endometrial cancer and various other cancers once the single functional MMR allele is lost (7,17-20). Germline and/or sporadic promoter methylation of MMR genes also leads to MSI-associated cancers (21,22). Further, MMR defects severely affect fertility and germline MSI in human (3,23,24) and in mouse (1,9). Both male and female Mlh1+/− mice are infertile, and male Mlh1+/− mice exhibit spermatocyte MSI (1,9). Despite the severity of germline phenotypes in Mlh1−/− mice, there is very limited knowledge on how heterozygosity of Mlh1 (i.e. Mlh1+/−) impacts MSI in germline cells.

Here, we investigate how Mlh1 heterozygosity affects MSI in sperm cells, and also assess spleen MSI to obtain a germline versus somatic MSI comparison. Further, by assaying Mlh1 promoter methylation status in Mlh1+/− sperm, we establish a correlation between MSI and Mlh1 promoter methylation in the germline. In addition, we establish estimates of the contribution of insertions and deletions to sperm MSI.

Materials and Methods

Mice, genotyping and tissue collection

The mice used in this study were Mlh1 mice (B6.129- Mlh1tm1Rak, strain 01XA2, National Institutes of Health, Mouse Repository, NCI Frederick) (9). National and institutional guidelines (Animal Experiment Board in Finland and Laboratory Animal Centre of the University of Helsinki) were followed throughout. Mlh1 genotyping was performed using
genomic DNA extracted from earpieces (see Supplementary information for the genotyping protocol). Tissues were collected from 4- and 12-month old mice, snap-frozen and stored at -80°C until further use. Three Mlh1+/+ and six Mlh1+/- mice per age group were used in this study.

DNA extraction from sperm cells and spleen

Mature sperm were isolated from cauda epididymides of 4- and 12-months old mice according to a previously published protocol (25), briefly as follows. For each mouse, cauda epididymides were finely chopped using a razor blade. The chopped pieces were transferred into a microcentrifuge tube containing 1X saline-sodium citrate (SSC; 0.15 mM NaCl, 15 mM sodium citrate) and incubated for 20 minutes at room temperature. Then the cell suspension was repeatedly pipetted up and down for 2 minutes to release the sperm cells. Cells were washed twice with 1X SSC. Somatic cells were lysed with 0.15% (w/v) sodium dodecyl sulfate (SDS). Sperm cell purity was assessed under a microscope by counting the number of sperm cells versus non-sperm cells using 5 µl of the cell suspension. We obtained sperm cell purity of over 95%. Sperm cells were washed twice with 0.2X SSC, centrifuged for 3 minutes at full speed. The supernatant was removed, the sperm pellet was resuspended into 300 µl of buffer containing 100 mM Tris HCl (pH 8.0), 10 mM EDTA, 500 mM NaCl, 1% SDS and 1M β-mercaptoethanol supplemented with 100 µl of 20mg/ml proteinase K, and incubated overnight at 56 °C to lyse the sperm heads. The next day, sperm DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer’s instruction. The same kit (DNeasy Blood & Tissue Kit (Qiagen)) was used to extract DNA from the spleen of 4- and 12- month old mice. Briefly, approximately 200 mg of tissue was finely chopped with a surgical blade and transferred to the kit’s lysis buffer.
Further homogenization was done using a 20G needle and syringe. Thereafter, column-based DNA extraction was performed according to the manufacturer’s instruction.

**Single-molecule MSI analysis by PCR**

Extracted DNA was quantified using Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA), and diluted to a concentration of approximately five DNA molecules/µl (assuming 3 pg DNA per haploid mouse genome) in 5 mM Tris-HCl (pH 7.5) supplemented with 5 ng/µl carrier (sheared) herring sperm DNA (Thermo Fisher Scientific). MSI was assayed at single-DNA molecule level using single-molecule PCR (SM-PCR) (26-28). Three microsatellites were tested for MSI: two mononucleotide repeat loci A27 and A33 (29), and one dinucleotide repeat locus D14Mit15 (9). Mononucleotide tract A27 is an intergenic microsatellite located approximately 2 kb downstream of the *Epas1* gene, A33 resides within the *Epas1* gene (between exons 2 and 3), and D14Mit15 is an intergenic microsatellite at 40 kb distance from the *Ptpn20* gene.

PCR was performed using the Q5 High-Fidelity DNA Polymerase system (New England Biolabs, Ipswich, MA), supplemented with 1 ng/µl carrier (sheared) herring sperm DNA in a 10 µl reaction volume. To ensure that individual PCR reactions in SM-PCR are seeded with a single amplifiable DNA molecule, for each DNA sample to be analyzed we determined, using a dilution series, the DNA concentration that yielded 50% PCR success rate similarly to previous reports (27,30). This DNA concentration was determined separately for each of the three microsatellite loci assayed. By Poisson approximation, 50% PCR success rate equates to approximately one amplifiable molecule per positive reaction (27,30-32). Subsequent MSI analysis was run in 96-well PCR plates. Each PCR was seeded with approximately one amplifiable DNA molecule, and each PCR plate included four wells of PCR mix only (no template DNA added) as negative controls. Previously published primers...
(8,29) were used for PCR as follows: for A27, 0.5 µM each of primer (A27_F 5´ 6-FAM-TCCCTGTATAACCCTGGCTGACT 3´ and A27_R 5´ GCAAACCAGTTGTCTGGCGTGGA 3´), for A33, 0.2 µM each of primer (A33_F 5´ VIC-TACAGAGGATTGTCTCTTTGGAG 3´ and A33_R 5´ GCTGCTTCCTGGACATTTGGCT 3´), and for D14Mit15, 0.1 µM of each primer (D14Mit15_F 5´ NED TTGGCTGTCTACCTTGAG 3´ and D14Mit15_R 5´ TTACCCCTCATAACTCCC 3´). A33 and D14Mit15 were assayed in the same PCR, and a separate PCR was run for A27. For A33 and D14Mit15 duplexed PCR, the following PCR program was used: 30 sec at 98°C, 35 cycles of 10 sec denaturation at 98°C, 30 sec primer annealing at 66°C, 5 sec extension at 72°C, followed by 2 min final extension at 72°C. For A27, the aforementioned PCR program was used, except for the primer annealing temperature being 70°C. 1µl of each PCR product was used for fragment analysis. Fragment analysis was performed by capillary electrophoresis, with an internal size standard (GeneScan™ 500 LIZ™ dye Size Standard, Applied Biosystems, Waltham, MA), using ABI3730xl DNA Analyzer (Thermo Fisher Scientific). Between 121 and 259 amplifiable DNA molecules per sample were assayed for each microsatellite locus. Data was analyzed using Fragman R package (33). Stringent criteria were used for true microsatellite signal calling and for mutant scoring (adopted from (27,34)), and thus the mutation rates reported here are likely a conservative estimate. The criteria were as follows:

1. A true microsatellite signal should have lower-intensity stutter peaks. Stutter peaks should display the expected size difference (that is, 1 base for mononucleotide repeats, and 2 bases for dinucleotide repeats) from the dominant peak. Reactions with peaks without stutter were considered artifacts.
2. For an allele to be considered as mutant, both the highest peak and the stutter peaks should shift as a single unit. Shift of the highest peak alone was not scored as a mutant.

3. If a wildtype and (apparently) mutant allele co-occurred in a single PCR, the reaction was scored as wildtype. Non-wildtype peaks were presumed to result from replication slippage during the early rounds of PCR, and thus considered artifacts.

For each of the three microsatellite loci assayed, MSI was separately scored for insertions and deletions. MSI rate was calculated as follows:

$$\text{MSI\%} = \left(\frac{\text{total no. of single repeat-unit shifts observed}}{\text{total DNA molecules analyzed}}\right) \times 100\%$$

*MLh1* promoter methylation analysis by methylation-specific PCR (MSP)

Methylation status of the *MLh1* promoter in sperm cells and splenic cells were tested using MSP assay (35). The same DNA (undiluted stock DNA) as used in the MSI assay was used for MSP. For MSP, 200 ng of DNA was bisulfite-converted using EZ DNA Methylation-Direct Kit (Zymo Research, Irvine, CA) according to manufacturer’s instruction, and 1 µl of bisulfite-converted DNA was used for PCR. PCR was performed in 2xZymo Taq premix system (Zymo research) with previously published primers (36). Two separate PCR, one with a primer pair targeting methylated *MLh1* promoter (0.8 µM each of forward primer 5´ GAATTTGAGCGTGAGGAGTTC 3´ and reverse primer 5´ TAACCGACCGCTAAATAACTTCC 3´), and the other with primer pair targeting unmethylated *MLh1* promoter (0.8 µM each of forward primer 5´ AGAATTTGAGTGTGAGGAGTTT 3´ and reverse primer 5´ CCAACCACCTAAATAACTTCC 3´) was performed using the following PCR program: 10
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sec at 95°C, 40 cycles of 30 sec at 95°C, 30 sec at 62°C, and 60 sec at 72°C, and final extension for 7 min at 72°C. Universally methylated mouse DNA standard (cat. no. D5012, Zymo Research) and Mlh1+/+ spleen DNA (from 1-month-old mouse) was used as positive and negative controls, respectively. The PCR products were analyzed on a 1.5% agarose gel in the presence of ethidium bromide, and visualized with UV light. Methylation status of the Mlh1 promoter was scored qualitatively based on the presence or absence of the 143-bp amplification product after PCR with primer pair specific to methylated CpG site.

**Statistical analysis**

Unpaired t-test was used to test the differences in MSI rates between the groups. Two-tailed P values < 0.05 were considered to be statistically significant.

**Results**

*Mlh1*+/- sperm cells display MSI at mononucleotide repeats

We assayed sperm MSI by single-molecule PCR to investigate the effects of Mlh1 heterozygosity on germline microsatellite stability. We assessed sperm DNA of 4- and 12-month-old Mlh1+/+ mice. Sperm DNA from age-matched Mlh1++/ littermates (or from closely related matings) was used as controls. We tested MSI at three microsatellites: two mononucleotide repeats A27 and A33 (**Figure 1A**) and one dinucleotide repeat D14Mit15 (**Supplementary figures 1-2**). MSI was scored separately for insertions and deletions.

While the dinucleotide D14Mit15 repeat was stable (**Supplementary figure 1**), both mononucleotide repeats displayed MSI in Mlh1+/- sperm. Mlh1+/- sperm showed substantially more 1-bp deletions than age-matched wildtype sperm (which also showed low levels of 1-bp deletions), at both 4- and 12-month time points (**Figure 1B**). Both Mlh1++/+ and Mlh1+/-
sperm showed an increase in deletions with age (Figure 1C). This increase was significant (P = 0.008) in Mlh1+/− sperm, but not in Mlh1+/- sperm. Compared to age-matched Mlh1+/- sperm, Mlh1+/- sperm had significantly more deletions (P = 0.001 and P = 0.0003 for 4- and 12-month time point, respectively), with 2.7- and 2.3-fold higher deletion rates at 4- and 12-month time points, respectively (Figure 1C). One 4-month old Mlh1+/- mouse (indicated by an arrow in Figure 1C) showed higher deletions (8%) in sperm compared to other Mlh1+/- mice in the same age group. With Grubbs’ test this mouse was categorized as an outlier (P < 0.05) and was omitted from statistical analyses.

In Mlh1+/+ sperm, insertions were more common than deletions at both time points (P = 0.002 for insertions versus deletions comparison for both 4- and 12-month time point). Compared to age-matched Mlh1+/+ mice, sperm from Mlh1+/- mice showed fewer insertions at both time points (Figure 1C). Insertions were predominantly single repeat unit (i.e. 1-bp) in size (Figure 1B), and there was no considerable change in insertion% with age in Mlh1+/- or Mlh1+/- sperm (Figure 1C).

**Mlh1 promoter methylation is frequent in sperm of Mlh1+/- mice and contributes to MSI**

We used MSP to test Mlh1 promoter methylation status in Mlh1+/- sperm, and to investigate whether germline Mlh1 promoter methylation correlates with germline MSI. The Mlh1 promoter in a given sample was scored as methylated if an amplification product (143 bp in size) was detected by PCR with methylation-specific primers. A representative gel image of the MSP assay is shown in Figure 2A.

None of Mlh1+/+ mice assayed showed Mlh1 promoter methylation in sperm (Figure 2A). In Mlh1+/- mice, Mlh1 promoter methylation was detected in 67% (4 out of 6), and 83%
(5 out of 6) sperm DNA samples at 4- and 12-month time points, respectively (Figure 2A). 

*Mlh1* promoter methylation was associated with elevated deletions (Figure 2B), but not insertions (Supplementary figure 3) in sperm.

MSP was also performed in spleen. All *Mlh1*+/− mice with *Mlh1* promoter methylation in sperm displayed *Mlh1* promoter methylation in spleen, while those without promoter methylation in sperm did not (Supplementary figure 4). *Mlh1* promoter methylation was not observed in spleen of *Mlh1*+/+ mice.

**MSI is higher in Mlh1+/− sperm than in Mlh1+/− spleen**

We also performed the SM-PCR based MSI assay in spleen, which enabled us to compare germline versus somatic MSI for each mouse. As in sperm, the dinucleotide locus D14Mit15 was also stable in spleen (Supplementary figure 5), and therefore tissue-specific MSI was compared only for mononucleotide repeats. Both sperm and spleen of wildtype mice showed only baseline levels of deletions at mononucleotide microsatellites. In *Mlh1* heterozygotes, the increase in deletions was near-exclusive to sperm, the exception being spleen DNA in the outlier mouse (Figure 3). Deletions in *Mlh1*+/− sperm were significantly higher than in spleen (2.5- and 3.2-fold at 4- and 12-month time points, respectively, Figure 3; p-values= 0.0087 and 0.0052).

In wildtype mice, both sperm and spleen DNA at both time points had an insertional burden (Figure 3). The substantial decrease in insertions, seen in *Mlh1*+/− sperm compared to *Mlh1*+/+ sperm, was not observed in *Mlh1*+/− spleen (Figure 3).
Discussion

There is emerging evidence that phenotypically normal somatic tissues from individuals with inherited MMR heterozygosity display MSI (37,38), raising the question whether germline cells of such individuals also exhibit MSI. Further, Mlh1 promoter methylation in sperm of LS patients has been reported (21,39,40) but little is known about how MMR heterozygosity and Mlh1 promoter methylation impact sperm MSI. We now demonstrate MSI in sperm of Mlh1 heterozygotes, and show that Mlh1 promoter methylation is frequent in Mlh1+/− sperm and may contribute to sperm MSI. Our experimental design (i.e. a highly sensitive, single-DNA molecule based MSI assay) allowed detection of MSI as low as 1%. All samples assayed showed <10% MSI; with a standard MSI assays (with a detection limit of 20-25% MSI (41)) this level of MSI would have been missed.

Interestingly, all Mlh1+/− mice harboring Mlh1 promoter methylation in sperm also showed Mlh1 promoter methylation in spleen. Our observation is in line with the human studies where LS and LS-like (individuals with germline MMR promoter methylation) patients are reported to have Mlh1 promoter methylation in multiple tissues analyzed, including sperm cells (21,40,42-44). We show, for the first time, that sperm Mlh1 promoter methylation is common in Mlh1-heterozygous mice. Further, we demonstrate that Mlh1 heterozygosity and promoter methylation associates with MSI in sperm but not in spleen of the same mouse. This result is perhaps not surprising, given that the likelihood of MSI increases with each round of DNA replication, and spermatogenesis involves sustained proliferation of spermatogonia (45) while splenocytes have a much lower proliferation rate (46).

We recently reported an insertional burden in somatic tissues with fully proficient MMR (Mlh1+/+) (47), others have shown similar insertional burden in MMR-proficient mammalian cell lines (48,49). In proliferative somatic tissues, insertions tend to substantially
decrease with decreasing MMR dosage (i.e. from $\text{Mlh1}^{+/+}$ to $\text{Mlh1}^{+/\text{--}}$ to $\text{Mlh1}^{-/-}$ tissue) with barely detectable insertions in MMR-deficient tissues and in MMR-deficient tumors, while deletions show the opposite trend (47). Insertions or deletions at microsatellites are thought originate from DNA polymerase slippage that results in the formation of a small loop on the newly synthesized, or the template strand during DNA replication, respectively (50-52). Our observation of increase in deletions (and decrease in insertions) in $\text{Mlh1}$ heterozygotes implies increased DNA polymerase slippage on the template strand and/or less efficient repair of the resulting template strand loops when MMR activity is not fully proficient. A likely explanation for the apparently lower insertion rate in $\text{Mlh1}$ heterozygotes is that deletion events erase many insertions that arose during earlier cell divisions. A tug-of-war between insertions and deletions in $\text{Mlh1}$ heterozygotes could mean that we are substantially underestimating the true extent of ongoing MSI with our molecular read-out. Regardless, we now demonstrate that the differential accumulation of insertions versus deletions at mononucleotides repeats, reported before in somatic tissues, bear out also in male germline cells.

Overall, maintenance of genome stability in the germline is crucial in order to avoid passing on any de novo defects to offspring. We have demonstrated that MMR heterozygosity provokes elevated MSI in male gametes. Further, our study highlights the utility of $\text{Mlh1}$ mice to study MMR-associated epigenetic phenotypes and MMR epimutation.
Author contributions

K.S.S. designed and carried out the experiments, performed data analysis, interpreted the data, and wrote the manuscript. M.M.T. assisted in tissue collection and MSI assay, and edited the manuscript. L.K. conceptualized and designed the study, interpreted the data, supervised K.S.S., reviewed and edited the manuscript, and acquired funding.

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Figure legends

**Fig 1. Sperm MSI at mononucleotide repeats.** (A) Representative capillary electropherograms of most commonly observed alleles at A27 and A33. Shown are a single repeat unit insertion (top panel), the wild-type allele (middle panel) and a single repeat unit deletion (bottom panel). Highlighted with the shaded rectangle are the allelic peaks which were scored; smaller peaks flanking this highest peak are stutter peaks (a typical PCR artifact for microsatellites). (B) Various alleles observed at mononucleotide repeat markers A27 and A33, expressed as % amplifiable DNA molecules assayed. On the y-axis “+” indicates gains, i.e. insertions, and “-” indicates losses, i.e. deletions of repeat units. (C) Mononucleotide repeats display more deletions and fewer insertions in $Mlh1^{+/−}$ sperm compared to age-matched $Mlh1^{+/+}$ sperm. The data points in boxplot represent weighted average of MSI at A27 and A33. Indicated are the P-values for wildtype and $Mlh1^{+/−}$ sperm MSI rate comparisons using unpaired t-test. Arrow indicates the outlier $Mlh1^{+/−}$ mouse. n= 3 and 6 for $Mlh1^{+/+}$ mice and $Mlh1^{+/−}$ mice, respectively, for each time point.

**Fig 2. Mlh1 promoter methylation in sperm correlates with germline MSI.** (A) Representative gel image of methylation-specific PCR (MSP) for $Mlh1$ promoter. Upper and lower gel images show products for MSP-PCRs using primers specific to methylated and unmethylated $Mlh1$ promoter, respectively. (B) Deletions (% of total molecules assayed) in $Mlh1^{+/−}$ sperm (same data as in Figure 1C), with $Mlh1$ promoter methylation status indicated for each sperm sample with “M”. Samples are in the same of order as in gel image above (A). Arrow indicates the outlier $Mlh1^{+/−}$ sperm sample.
**Fig 3. Germline versus somatic MSI.** The boxplots show weighted average of MSI at mononucleotide repeats A27 and A33 in sperm and spleen of 4- and 12-month *Mlh1*+/+ and *Mlh1*+/− mice (sperm MSI data is the same as in Figure 1C). Indicated are the P-values for wildtype and *Mlh1*+/− sperm MSI rate comparisons using unpaired t-test (abbreviation: ns = non-significant). Arrow indicates the outlier *Mlh1*+/− mouse which was excluded from statistical analysis.
Figure 1

A

1 repeat-unit insertion

A27

139 bp

147 bp

A33

138 bp

146 bp

wild-type

1 repeat-unit deletion

size in bp

138 135 140 148 137 142 147 152

B

4 months

12 months

allele frequency (%)

Mlh1

+/+

+/-

+/-

+/-

C

4 months

12 months

MSI rate (deletion)

Mlh1

+/+

+/-

+/-

+/-

average deletion (%) 1.7 4.5 2.6 5.9

average insertion (%) 6 4 6 4.2

P=0.001

P=0.0003

P=0.03

P=0.02
Figure 2

A

DNA ladder
not template DNA
methylated DNA
unmethylated DNA

control

4 months

12 months

200 bp
100 bp

Mlh1

+/+

+/-

+/+

+/-

methylated
(143 bp)

unmethylated
(141 bp)

B

4 months

12 months

MSI rate (deletion)

Mlh1

+/+

+/-

+/+

+/-

M

M

M

M
Figure 3

![Figure 3: Graph showing MSI rates in sperm and spleen for different genotypes at 4 and 12 months.](https://academic.oup.com/mutage/advance-article/doi/10.1093/mutage/geab010/6178928)

- **Average deletion (%)**
  - 4 months:
    - +/+: 1.7%
    - +/-: 4.5%
    - +/+: 1.8%
    - +/-: 1.4%
    - P = 0.001
  - 12 months:
    - +/+: 2.6%
    - +/-: 5.9%
    - +/+: 1.7%
    - +/-: 1.8%
    - P = 0.0003

- **Average insertion (%)**
  - 4 months:
    - +/+: 6%
    - +/-: 4%
    - +/+: 6.6%
    - +/-: 6.3%
    - P = 0.03
  - 12 months:
    - +/+: 6%
    - +/-: 4.2%
    - +/+: 5.2%
    - +/-: 5.6%
    - P = 0.02

- **Number of mice (n)**
  - 4 months:
    - +/+: 3
    - +/-: 6
    - +/+: 3
    - +/-: 4
  - 12 months:
    - +/+: 3
    - +/-: 6
    - +/+: 3
    - +/-: 3