Highly Sensitive Microsatellite Instability and Immunohistochemistry Assessment in Endometrial Aspirates as a Tool for Cancer Risk Individualization in Lynch Syndrome.

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ABSTRACT

Women with Lynch syndrome (LS) are at increased risk of endometrial cancer (EC), among other tumors, and are characterized by mismatch repair (MMR) deficiency and microsatellite instability (MSI). While risk-reducing gynecological surgeries are effective in decreasing EC incidence, doubts arise regarding the appropriate timing of the surgery. We explored the usefulness of highly-sensitive MSI (hs-MSI) assessment in endometrial aspirates for the individualization of gynecological surveillance in LS carriers.

Ninety-three women with LS, 25 sporadic EC patients (9 MMR-proficient and 16 MMR-deficient), and 30 women with benign gynecological disease were included in this study. Hs-MSI was assessed in prospectively collected endometrial aspirates in 67 LS carriers, EC cases, and controls. MMR, PTEN, ARID1A, and PAX2 expression patterns were evaluated in LS samples. Follow-up aspirates from eight LS carriers were also analyzed.

Elevated hs-MSI scores were detected in all aspirates from MMR-deficient EC cases (3 LS and 16 sporadic), being negative in aspirates from controls and MMR-proficient EC cases. Positive hs-MSI scores were also detected in all four LS aspirates reported as complex hyperplasia. High hs-MSI was also present in 10 of 49 aspirates (20%) from LS carriers presenting a morphologically normal endometrium, where MMR expression loss was detected in 69% of the samples. Interestingly, the hs-MSI score was positively correlated with MMR-deficient gland density and the presence of MMR-deficient clusters, colocalizing with PTEN and ARID1A expression loss. High hs-MSI scores and clonality were evidenced in two samples collected up to four months before EC diagnosis; hs-MSI scores increased over time in five LS carriers, whereas they decreased in a patient with endometrial hyperplasia after progestin therapy.

In LS carriers, elevated hs-MSI scores were detected in aspirates from premalignant and

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malignant lesions and normal endometrium, correlating with MMR protein loss. Hs-MSI

assessment and MMR immunohistochemistry may help individualize EC risk assessment

in women with LS.

INTRODUCTION

Lynch syndrome (LS) is caused by constitutional monoallelic pathogenic variants affecting DNA mismatch repair (MMR) genes: *MLH1*, *MSH2*, *MSH6*, or *PMS2*, or by *EPCAM* deletions. LS is one of the most frequent cancer predisposition syndromes, associated with an increased risk of gastrointestinal and gynecological cancers, among others. LS accounts for about 3% of colorectal and endometrial (EC) cancers.[1, 2]

EC is often the first malignancy affecting women with LS. Women carrying *MLH1*, *MSH2, MSH6*, and *PMS2* mutations have a lifetime cumulative EC risk of 37%, 48.9%, 41.1%, and 12.8%, respectively, at 75 years of age.[3] Moreover, these patients present a lifetime incidence of ovarian cancer (OC) of 10-17%.[3] Diagnosis of both cancer types is mainly premenopausal and prognosis is favorable.[3] Despite excellent survival, women remain at risk of LS-associated cancers, mainly colorectal cancer.[4]

There are no uniform clinical guidelines regarding the management of LS-associated gynecological malignancies. Recommended surveillance strategies are based on transvaginal ultrasound and, optionally, an aspiration biopsy starting at age 30-35 years.[5, 6] However, evidence that surveillance offers a survival benefit is limited[5, 7] and many women with LS will develop interval EC and OC.[8] In contrast, risk-reducing total hysterectomy and bilateral salpingo-oophorectomy, offered at 35-40 years when childbearing is complete, are effective in reducing the risk and may impact on LS women's survival.[9, 10] Nevertheless, risk-reducing surgery is associated with significant long-term side effects;[5-7, 11] hence, there is a need for a more personalized risk assessment to empower female carriers regarding the decision and timing of prophylactic surgery.

LS tumors are characterized by MMR deficiency, caused by somatic inactivation of the wild-type MMR allele and subsequent microsatellite instability (MSI). MSI has

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frequently not been considered an initiating event in LS carcinogenesis;[12] however, this traditional view is changing due to the identification of MMR deficiency and MSI in normal tissues. As in colonic tissue,[13, 14] MMR deficiency and MSI have been recently observed in single or clustered morphologically normal endometrial glands of women with LS.[15-18] Moreover, these characteristics were reported in complex atypical hyperplasia (CAH) and complex hyperplasia without atypia (CH).[15, 16, 18-22] However, the role of MMR-deficient (MMRd) endometrial glands as predictors of EC development is currently unknown.

We hypothesize that a comprehensive characterization and quantification of MSI by means of highly-sensitive approaches in endometrial biopsies of women with LS may help individualize surveillance strategies. Therefore, we aimed to preliminarily evaluate the clinical utility of highly-sensitive-MSI assessment in endometrial aspirates of LS carriers.

MATERIALS AND METHODS

Patients and samples

Patients were recruited within the Screenwide study (**Supplementary Methods**).[23] Ninety-three women with LS, 30 women with benign gynecological disease, and 25 sporadic EC patients were included in this study (**Figure S1**, **Tables 1 and S1**). Processing of endometrial aspirates and tumors (if available) and DNA isolation is detailed in **Supplementary methods**.

The 93 women with LS were stratified according to the affected MMR gene (**Tables 1 and S1**). *MSH6* and *MLH1* mutation carriers were the most prevalent (36% and 34%, respectively) followed by patients harboring *MSH2* (19%) and *PMS2* mutations (11%).

The average age was 44 (range 23-73), 69% were premenopausal, 9% perimenopausal, and 22% postmenopausal. Most were asymptomatic at the time of inclusion, whereas 5% presented abnormal bleeding. Thirty-six LS carriers underwent major gynecological surgeries: 27 (75%) prophylactic surgeries, six (17%) for malignancies, and three (8%) due to premalignant lesions.

Thirteen additional formalin-fixed paraffin-embedded (FFPE) endometrial biopsies (ten aspirates and three hysteroscopies) obtained in previous or posterior clinical follow-ups were also available in eight women (two *MLH1*, three *MSH2*, and three *MSH6* mutation carriers) from the LS cohort (**Figure S1**, **Table S2**). Four of these patients were premenopausal, three perimenopausal, and one postmenopausal. Five of them underwent hysterectomy with bilateral salpingo-oophorectomy due to malignant or premalignant lesions.

Histopathological examination, immunohistochemical analysis, and epithelium/stroma quantification

Uterine tissue samples (endometrial aspirates and hysterectomies) were examined by experienced gynecological pathologists. The histological evaluation is detailed in **Supplementary Methods** (**Table S1**). All histological preparations were scanned using a 3D Histech P1000 digital scanner at high magnification (40x) and applying extended depth of focus. For each aspirate, a morphometric estimation of the tissue area was obtained. Digital quantification of stroma and epithelium was performed in endometrial biopsies containing MMRd clusters using an AI-based decision tree algorithm (QuPath-0.3.0 software),[24] as detailed in **Supplementary Methods**.

MMR, PTEN, ARID1A, and PAX-2 protein expression in endometrial aspirates were evaluated (as detailed in **Supplementary Methods**), providing i) the pattern of loss

classified as a) proficient (or intact expression), b) single-type loss (one isolated deficient gland), c) oligo-type loss (two to four deficient glands in the same topographic group), and d) cluster-type loss (five or more deficient glands, adjacent to each other, with no proficient gland intermixed); and ii) the area of staining loss (regarding the epithelial component exclusively). Partial losses within a gland or decreases in staining intensity were not considered. Colocalization of PTEN, ARID1A, and PAX-2 protein loss of expression was assessed using an image alignment algorithm.

Microsatellite instability analysis via a PCR-based method

MSI was assessed in a subset of samples by a pentaplex PCR-based method using the MSI Analysis System v1.2 (Promega, USA), as detailed in **Supplementary Methods**.

Highly-sensitive assessment of MSI and hotspot mutations

A custom panel targeting 192 microsatellites was reported as frequently mutated in MSI-high tumors and 39 additional hotspot substitutions were identified in EC sporadic tumors from the Cancer Genome Atlas (TCGA) dataset,[25] were designed using HaloPlex HS technology (Agilent Technologies, CA, USA) as described.[26] Most of the microsatellite markers included in our hs-MSI panel were mononucleotides (93.2%), thus avoiding the low sensitivity of dinucleotide markers to detect MSH6 deficiency-associated MSI.[26] Library preparation, and bioinformatics analysis is detailed in **Supplementary Methods**.

EC detection accuracy

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for EC detection in endometrial aspirates were calculated according to the results obtained in the histopathological evaluation, the hs-MSI metrics, or by combining both approaches as detailed in **Supplementary Methods**. Hs-MSI score and clonality values

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above 42% and 12%, respectively, were selected as the best cut-off points for EC detection accuracy (**Figure S2**).

Statistical analyses

Correlation of the hs-MSI score with age at inclusion and MMRd area was analyzed using Spearman's correlation coefficient. The median Hs-MSI score was compared between two groups using a Wilcoxon Rank Sum Test and between more than two groups using a Kruskal-Wallis test followed by a Dunn's multiple pairwise comparisons test. All tests were two-sided and *p*-values of <0.05 were considered statistically significant. All statistical analyses were conducted using R v3.6.3.[27]

RESULTS

Identification of neoplastic and preneoplastic lesions

Out of the 93 prospectively collected endometrial aspirate samples from LS women, 25 (27%) were not informative due to insufficient tissue for diagnosis. Of the 68 informative samples, three EC (3%) and four CAH/CH (4%) were identified, whereas 61 samples were classified as morphologically normal endometrium (66%) (**Figure S1**, **Tables 2 and S3**). Of note, two of the samples were collected after EC diagnosis and classified in the context of this study as non-evaluable and CAH (cases L3 and L19); one OC was identified after evaluation of the surgical specimen (case L82) (**Tables 2, S1, and S4**).

Characterization of MMR, PTEN, and ARID1A deficiency in LS endometrial aspirates

Subsequently, a subset of 67 aspirates from women with LS (3 EC, 4 CAH/CH, 50 normal endometrium, and 10 non-evaluable samples, according to the pathology report) was

selected for further evaluation (**Figure S1**). A detailed immunohistochemical characterization of MMR protein expression was only possible in 48 of the 67 (72%) aspirate samples fulfilling requirements of analyzable glands and area (2 tumors, 4 CAH/CH, and 42 morphologically normal aspirates) (see **Supplementary Methods**). In line with previous reports, MMRd glands were identified in all tumors and CAH/CH aspirates were analyzed. MMRd was also present in a relevant proportion of morphologically normal aspirates (29 of 42, 69%) (**Figure 1A**, **Table S3),** with different patterns of MMR loss of expression: 7 cases exhibited MMRd single-glands, 12 MMRd oligo-glands, and 10 MMRd gland clusters (**Figure 1B**).

Next, immunohistochemical characterization was expanded by analyzing the expression of proteins reported to play an important role in endometrial carcinogenesis.[15, 28-31] The two endometrioid carcinomas were MMRd and PTEN-deficient (PTENd), both presenting ARID1A negativity (**Figure 1C**, **Table S3)**. Regarding CAH/CH samples, three of the four showed MMRd clusters, while one sample exhibited single-type loss. In two of the three CAH/CH-containing clusters, the MMRd glandular group was negative for PTEN and ARID1A (**Tables S3 and S5**).

PTEN loss of expression in morphologically normal endometrium was observed in up to 50% of cases (**Table S3**, **Table S5, Figure 1C**). Among the 29 morphologically normal aspirates with MMRd glands, PTENd glands were observed in 15 cases (52%). PTEN loss of expression was more frequently observed in MMRd clusters (8 out of 10) as compared with other patterns (5 out of 12 in MMRd oligo-glands and 2 out of 7 in MMRd single-glands) (**Table S5, Figure 1C**). In three of the eight samples showing PTENd/MMRd colocalization, ARID1A-deficient (ARID1Ad) oligo-glands were also observed (**Table S3**), suggesting that ARID1A deficiency occurred later (**Figure 1C**).

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None of the evaluated EC, CAH/CH, or normal aspirates presented loss of PAX-2 expression (data not shown).

Evaluation of epithelium/stroma ratio in normal aspirates containing MMRd clusters

A reduction in the ratio between endometrial glands and stroma is regarded as a surrogate of clonality used for the diagnosis of endometrial hyperplasia.[32, 33] We evaluated this ratio in morphologically normal aspirates that exhibited MMRd clusters. MMRd clusters showed a modest but statistically significant increase in the epithelium/stroma ratio when compared with MMRp glands present in the same sample (n=28, 0.171 vs. 0.139, *p*-value=*0.001*) (**Figure S3**).

Quantification of MSI at high sensitivity

We used hs-MSI to quantify the accumulation of indel mutations in microsatellite markers.[26] First, aspirates from 30 controls with benign lesions and 25 sporadic EC cases were analyzed as a training series (**Figure S1**). The threshold level for hs-MSI status detection previously established in blood (4.576) proved useful for the analysis of endometrial aspirates. Hs-MSI scores of all healthy controls and MMRp EC aspirates were below the threshold (negative), whereas they were positive in all 16 sporadic MMRd EC patients (range: 11.35-80.95%) (**Figure 2A**). Of note, the lowest hs-MSI score (11.35%) corresponds to an MMRd EC showing heterogeneous loss of MMR protein expression.

Subsequently, hs-MSI was assessed in the subset of 67 endometrial aspirates from women with LS (**Figure S1**, **Table S3**); only one aspirate from a normal endometrium was not evaluable due to low coverage. High scores were observed in all three samples diagnosed

as EC (range 43.18-64.92%) (**Figure 2A**). Hs-MSI scores of MMRd EC aspirates (both LS and sporadic) were higher (mean hs-MSI score 61.8) than those of LS normal endometrium (4.28, *p*=4.52e-10) (**Figure 2A**). Also, scores were elevated in all four aspirates diagnosed as CAH/CH (range 6.88-62.43%).

Interestingly, hs-MSI scores ranging from 5.35 to 56.08 were evidenced in 10 out of 49 (22%) LS aspirates classified as normal endometrium (**Figure 2A**). One of the negative hs-MSI scores corresponded to an OC patient (ID L82) with no malignant cells observed in the aspirate (**Table S4**). Of note, there was no correlation between hs-MSI score and age, menopausal status, menstruation stage, constitutional affected gene, or BMI in normal endometrium aspirate samples (*p*=0.2 **Figure S4**). Elevated hs-MSI scores (range 5.82-17.74%) were also observed in 3 out of 10 non-evaluable LS aspirates (**Figure 2A**).

A subset of 14 LS endometrial aspirates with different histology and variable hs-MSI scores (**Table S6**) were also analyzed with the conventional pentaplex PCR-based method. Only endometrial aspirates from two EC patients showing high hs-MSI scores (47.74 and 64.92%) were classified as MSI-high by the pentaplex method, whereas samples from patients with premalignant lesions (n=2) or normal endometrium (n=10) showing intermediate hs-MSI scores (0.53-14.06%) were classified as stable.

hs-MSI as a surrogate marker of clonality

MSI clonality, as defined by the number of markers with >10% of indel allelic frequency, was also assessed in the training series (sporadic EC and benign controls) and LS aspirates. Like hs-MSI scores, endometrial aspirates from MMRd EC cases (both sporadic and LS) showed a higher number of clonal markers than LS normal endometrium and CAH/CH samples (26.7 vs. 0.99, respectively, *p*=5.99e-10) (**Figure 2B**).

Interestingly, when hs-MSI score was represented against MSI clonality, 17 samples with a final diagnosis of MMRd EC (14 out of 16 sporadic and 3 out of 4 LS) clustered in a group with high hs-MSI scores (>42%) and a high number of clonal markers (>12%) (**Figure 3A**). The two sporadic MMRd EC cases outside the cluster corresponded to a sporadic tumor showing heterogeneous loss of MMR expression pattern, as seen via immunohistochemistry (ID S14; hs-MSI score 11.35%; clonality 0), and an aspirate (ID S2) showing high hs-MSI score (42%) but low clonality (one marker above >10%) reported as non-evaluable due to insufficient material. Of note, one LS aspirate sample reported as normal endometrium clustered within the EC group, indicating high clonality of unstable microsatellite markers in an apparently normal sample. The sample from an LS EC patient outside the cluster (ID L3) was obtained after EC diagnosis by hysteroscopy and was reported as hyperplasia by the pathologist, showing a high hs-MSI score (62%) but low clonality (one marker) (**Tables S1 and S4**).

Accuracy of hs-MSI analysis for EC detection

Next, we evaluated the capacity of hs-MSI to improve EC detection in endometrial aspirates. Histopathological assessment of the 46 samples with benign lesions and sporadic MMRd EC cases had a 100% (95%CI 76.84-100%) sensitivity and 100% (95%CI 87.23-100%) specificity for EC detection **(Table S8)**. The accuracy of the hs-MSI assessment for EC detection measured using the AUC from ROC curves was 0.997 for hs-MSI score (>42%) and 0.987 for clonality (unstable markers >12% allele frequency) (**Figure S2**). Sensitivity and specificity reached 93.3% (95%CI 68.05-99.83%) and 100% (95%CI 88.43-100%), respectively, when both metrics were combined **(Table S8)**.

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The percentage of non-evaluable LS samples was 27.2% (25 of 93) by histopathological examination and 4.4% (3 of 69) by hs-MSI analysis in their respective analyzed series. In the LS series, with informative histopathology and molecular results (n=55), sensitivity and specificity of the histopathological evaluation were 75% (95%CI 19.41-99.37%) and 100% (95%CI 93.02-100%), respectively; PPV and NPV were 100% and 98.1% (95%CI 90.33-99.64%), respectively. On the other hand, molecular analysis revealed sensitivity and specificity values of 100% (95%CI 29.24-100%) and 98% (95%CI 89.55-99.95%), respectively, while PPV and NPV were 75% (95%CI 30.11-95.43%) and 100%, respectively **(Table S8)**. When both analyses were combined, sensitivity and specificity were maintained, 100% (95%CI 29.24-100%) and 98% (95%CI 89.55-99.95%), respectively, with PPV and NPV values of 75% and 100%, respectively.

Correlation of expression patterns and hs-MSI metrics in morphologically normal endometrium

In morphologically normal endometrium, a positive correlation between hs-MSI score and MMRd area was observed (**Figure 4A**). Samples containing MMRd clusters displayed higher hs-MSI scores than MMRp and single- and oligo-MMRd glands ($p<0.05$ in all comparisons, **Figure 4B, Table S3**). Finally, the highest hs-MSI scores were observed when loss of expression of MMR colocalized with loss of PTEN (**Figure 4C, Table S3**).

Hotspot mutations detected in normal endometrium and benign lesions

In the training series, hotspot mutations in tumor driver genes were detected in 20 of 25 endometrial aspirates from sporadic EC samples but also in 20 out of 30 samples from control individuals with benign lesions (**Table S7**). No differences were observed in the number of driver mutations identified (1.96 EC vs. 1.9 controls, p=0.241, **Table S7,**

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Figure S5A), while the variant allele frequency (VAF) of the hotspots was significantly higher in EC (0.19 EC vs. 0.01 controls, *p*=2.19e-5, **Figure S5B**).

Hotspot cancer driver mutations were also identified in a high percentage of LS-associated samples (43/67, 64%) (**Figure S5A, Table S7**). Hotspot mutations in normal endometrium showed a significantly lower mean VAF compared with MMRd sporadic or LS EC cases (*p*=4.83e-5). Hotspot mutations were detected in similar proportions in LS normal endometrium (72%) and control individuals (77%), showing no differences in the number of hotspots per sample and mean VAF (**Figure S5**).

Characterization of serial prospectively collected endometrial biopsies

To preliminarily assess the potential value of the serial analysis of hs-MSI metrics and MMRd patterns, hs-MSI and immunohistochemical analyses were performed in the follow-up endometrial biopsies available from eight LS patients (**Figures S1 and 3B, Table S2**).

Patient L3 reported intermenstrual spotting. The endometrial aspirate did not indicate any (pre)malignant lesion but an elevated hs-MSI score and clonality were detected (73.18% and 15.78, respectively) together with an extended number of clusters showing colocalized loss of expression of MMR, PTEN, and ARID1A (**Figure 3B, Table S2**). Two months later, EC was diagnosed by hysteroscopy with similar hs-MSI metrics and immunohistochemistry (**Figure 3B**, **Tables S2, and S4**). Eighteen days after hysteroscopy, a second aspirate was collected and reported as CAH (**Table S2**).

In patient L79, the first aspirate was reported as CAH (hs-MSI: 52.15%, clonality: 19.89). Four months later, the histological evaluation of a second aspirate revealed an EC with similar hs-MSI metrics (**Figure 3B**). Both aspirates showed colocalization of MMRd and

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PTENd clusters (**Table S2**). A correlation between hotspot mutations in the *PPP2R1A* and *FBXW7* genes with a VAF >5% was observed in the two aspirates and confirmed in the surgical specimen (**Table S2**).

Patients L2, L4, L75, and L78 developed CAH/CH (**Table S2**). Subsequent follow-up aspirates showed intermediate hs-MSI values between 20% and 50% (**Figure 3B**). Most samples exhibited colocalization of loss of expression of MMR and PTEN proteins and ARID1A in single- or oligo-glands (**Table S2**). Interestingly, a third aspirate from L75 presented negative hs-MSI values after CAH treatment with a levonorgestrel-releasing intrauterine device (**Figure 3B**).

Finally, serial samples from patients L80 and L81 produced hs-MSI scores above 40% (51.58% and 68.48%, respectively) (**Figure 3B**). There was no evidence of malignant or premalignant lesions in either case, but the immunohistochemistry analyses showed colocalization of MMRd and PTENd glands. Interestingly, the PTEN p.R233* hotspot mutation with a VAF of 6% was detected in both serial aspirates from L81. In addition, ARID1Ad oligo-glands were also observed in this case (**Table S2**).

DISCUSSION

Our study performs a comprehensive evaluation of MMR-deficiency and MSI status in the largest prospective series of endometrial biopsies from LS patients (**Table S9**). The results obtained confirm the presence of MSI in MMRd normal endometrium from women with LS via a highly-sensitive MSI methodology combined with MMR immunohistochemistry. The MSI levels positively correlated with the size of the groups of MMRd glands and showed frequent colocalization of loss of expression of MMR proteins, PTEN, and, less frequently, ARID1A, key events in endometrial carcinogenesis.[15, 28-31] Moreover, our results suggest that hs-MSI assessment and MMR

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immunohistochemistry in endometrial aspirates may improve the detection of endometrial preneoplastic and neoplastic lesions.

MMR deficiency in morphologically normal endometrial glands was detected in 69% of our cohort of women with LS. In most cases (22 of 29), MMR deficiency was seen in oligo or clustered patterns. This agrees with recent reports that identified MMRd morphologically normal glands in a high proportion of women with LS (47-70%), frequently seen in contiguous groups (68-87%) (**Table S9**).[16, 17] Notably, we have demonstrated, for the first time, a positive correlation between MMRd gland area and elevated hs-MSI score. This is consistent with the clonal expansion of cells harboring biallelic inactivation of an MMR gene that accumulate DNA mutations, mainly indels in microsatellite repeats. Conversely, increased MSI levels were rarely detected in previous studies using conventional MSI detection techniques (**Table S9**).[15, 18-22, 34] The presence of MMRd glands and MSI has also been observed in endometrial CAH/CH both in sporadic and LS patients, at higher proportions than in LS normal endometrium (**Table S9**).[15, 35-37]

Furthermore, samples containing MMRd clusters not only presented increased hs-MSI scores and hs-MSI clonality, but also the presence of hotspot mutations with high VAFs, frequent colocalization with PTEN deficiency, and the reduced ratio between glands and stroma as well. Therefore, our findings indicate that MMRd clusters may be an indicator of clonality and, possibly, of neoplastic potential. In fact, molecular clonality and stromal reduction, together with nuclear atypia, are the current WHO criteria for diagnosing CAH.[33] Interestingly, the presence of MMRd glands in normal endometrium was more frequently detected in EC women with LS than in healthy LS women (69% and 31%, respectively), suggesting that patients with large groups of MMRd non-neoplastic endometrial glands have a higher risk of developing EC.[17] In our cohort of LS endometrial

aspirates, loss of expression of PTEN and ARID1A co-occurring with MMRd clusters was detected in EC and CAH/CH, in agreement with previous reports describing loss of expression or mutations in driver genes in endometrial CAH in both LS and sporadic cases.[15, 35-37] Interestingly, ARID1Ad in CAH has been associated with an increased risk of progression to EC.[38] In agreement with the recent identification of low-frequency EC driver mutations in endometrial tissue from healthy individuals by highly sensitive approaches,[39, 40] our study shows, for the first time, ARID1A loss in normal endometrial glands, colocalizing with clusters of MMRd and PTENd glands. These findings indicate an early role of ARID1Ad in endometrial carcinogenesis.

EC diagnosis is based on histological examination of endometrial aspiration biopsies, which has proven accurate. However, sampling failure is one of its drawbacks.[41] Comparing hs-MSI with histopathologic diagnosis, the lower percentage of non-evaluable samples by hs-MSI, together with a high sensitivity suggests that hs-MSI assessment may complement traditional histopathological diagnosis, especially in cases where the histopathological analysis was inconclusive, avoiding the repetition of endometrial biopsies. In Lynch syndrome, MSI is the driving force of tumor carcinogenesis and all tumor cells should be MMR-deficient with no heterogeneity. Therefore, MMR-deficiency heterogeneity in tumors is not predicted to be a confounder in the analysis of LS gynecological samples by hs-MSI. Also, the high NPV of the hs-MSI approach in the LS cohort makes it potentially useful for the identification of premenopausal women at low risk of EC. Further studies including a larger number of LS-associated EC cases will be required to confirm these preliminary observations. The next steps in our research should also assess the feasibility of the hs-MSI approach to predict EC in minimally invasive samples such as cervical cytology or vaginal self-

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collected sample, which have shown promising results in gynecological surveillance of the average-risk population.[42-46]

Furthermore, an increase in hs-MSI scores over time was observed in serial samples of five women with LS. This increase, together with the persistence of driver mutations, is consistent with the existence of a progenitor cell, which survives through menstrual cycles, and has the potential to acquire additional mutations to develop the full malignant phenotype. Indeed, elevated hs-MSI values and colocalized MMRd and PTENd clusters were detected in samples from two patients (L3 and L79) collected up to four months prior to diagnosis, in agreement with a previous report in which MSI was found in aspirates several years before the development of EC.[15] Prospective studies in larger series of sequential aspirates are needed to confirm whether deficient MMR protein expression and/or elevated hs-MSI in non-neoplastic endometrium is associated with increased probability of malignant transformation. In addition to the mutational processes, further studies will be required to elucidate the role of the immune phenotype in modulating EC risk in LS individuals,[47] since enhanced immune responses have been reported in MMRd/MSI precancerous lesions and tumors, together with systemic immune responses against neoantigens detected in cancer-free LS individuals.[48, 49]

Currently, due to later childbearing and the negative long-term side effects of risk-reducing surgeries in premenopausal women, the need to identify EC risk biomarkers that help empower women with LS in their decision-making regarding surgery timing becomes more evident. The results obtained suggest that highly sensitive MSI assessment may refine the risk assessment of EC development in LS female carriers. The high negative predictive value of the hs-MSI approach in the LS cohort makes it potentially useful for the identification of premenopausal women at low risk of EC, in whom

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hysterectomy could be postponed. If confirmed, the hs-MSI approach may represent a suitable tool for improving the clinical follow-up of women with LS.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

AUTHOR CONTRIBUTIONS

JB, XMG, GC, and MP conceived, designed, and supervised the study and drafted the manuscript. JC, FM, ELB, and ED conceived the experiments and analysis, generated the figures and tables, and drafted the manuscript. ND, LC, JMM, MS, AV, PPT, SP, JP, SF, LC, AT, LA, CM, GO, and AV recruited cases and collected and reviewed clinical and molecular data. All authors were involved in data interpretation and writing. All authors reviewed and approved the final version of the manuscript.

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DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials. Raw data were generated at the CNAG and IDIBELL. Derived data supporting the findings of this study are available from the corresponding author on request.

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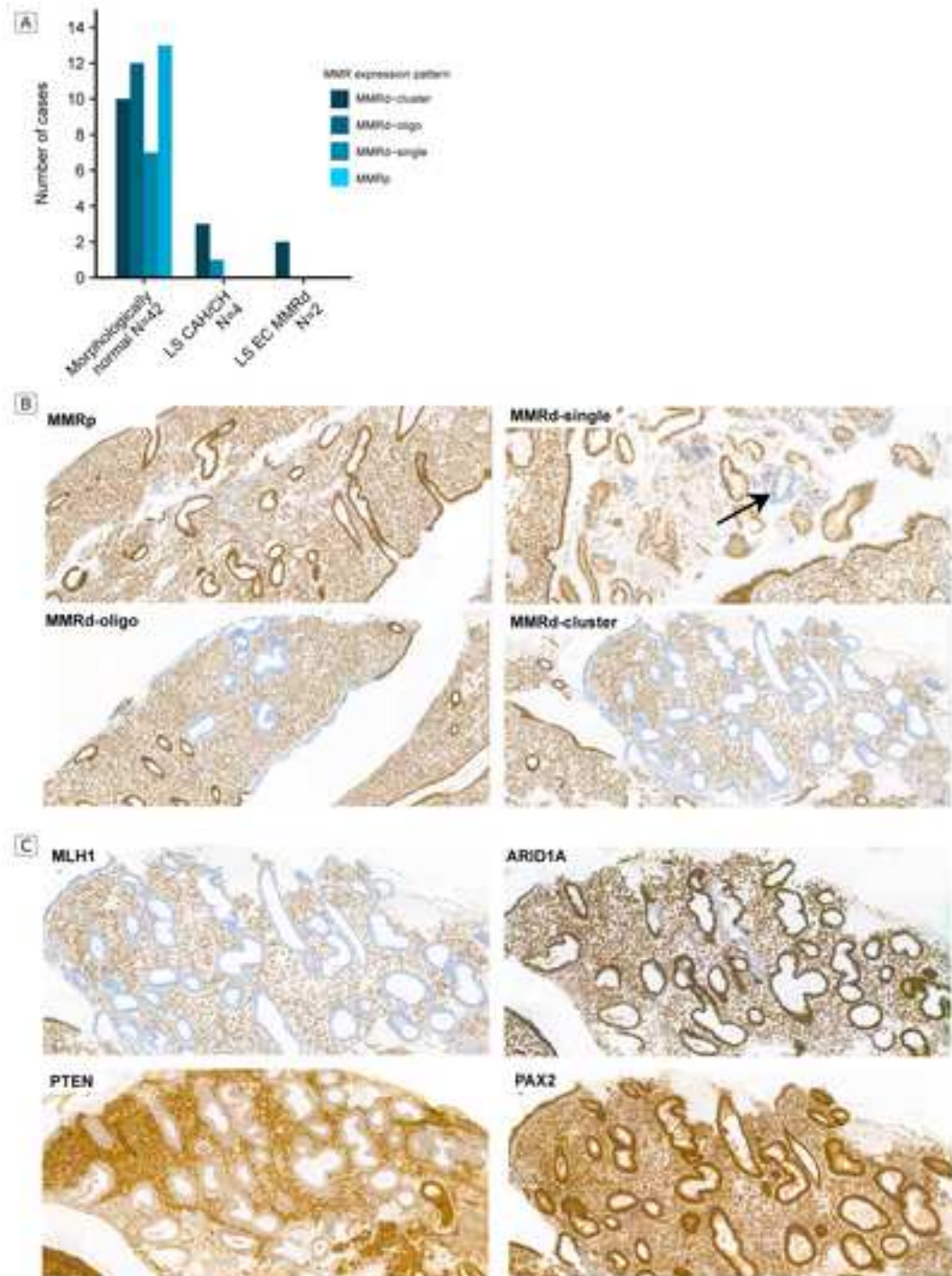
FIGURE LEGENDS

Figure 1: **MMR expression patterns in Lynch syndrome endometrium**. **A.** MMR expression pattern according to the aspirate report. **B.** Examples of MMRd patterns observed in normal endometrium. **C.** Examples of samples showing colocalization of MMR, PTEN, and ARID1A deficiency. MMRp: mismatch repair proficient; MMRd: mismatch repair deficient; EC: endometrial cancer; CAH/CH: complex atypical hyperplasia/complex hyperplasia without atypia.
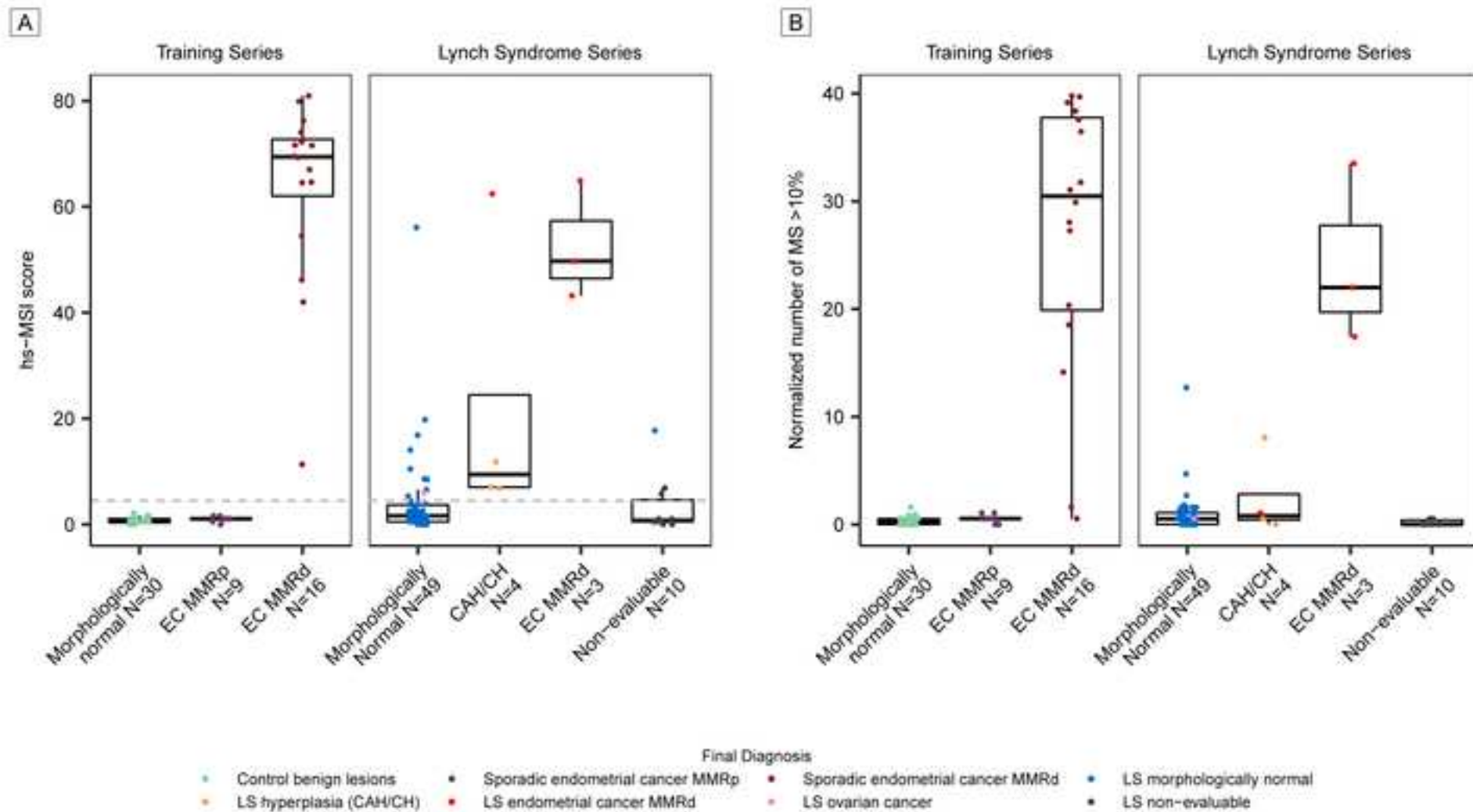
Figure 2: **Hs-MSI metrics in endometrial aspirates in the training (controls with benign lesions and sporadic EC cases) and Lynch syndrome series. A.** Hs-MSI score in endometrial aspirates, representing the percentage of unstable markers. Dashed lines represent the hs-MSI score threshold for MSI status (4.576, gray). **B.** Number of markers with an indel frequency above 10% as a measure of clonality. Samples are grouped according to the final clinical diagnosis in the training series and the result of the pathological aspirate report in the Lynch syndrome series. Points are colored according to the final diagnosis of the patient (**Table S1**). LS: Lynch syndrome; MMRp: mismatch repair proficient; MMRd: mismatch repair deficient; EC: endometrial cancer; CAH/CH: complex atypical hyperplasia/complex hyperplasia without atypia.

Figure 3: **Hs-MSI versus clonality in training and Lynch syndrome series (A) and Lynch syndrome follow-up samples (B)**. **A.** Hs-MSI metrics in the case-control prospective series. Dashed lines represent the hs-MSI score threshold for MSI status (4.576, gray), low/intermediate hs-MSI scores (20, orange), and high hs-MSI scores (42, red). Red-colored area (hs-MSI score >42% and number of clonal markers >12%), represents high suspicion of endometrial cancer. Points are colored according to the final diagnosis of the patient (**Table S1**). **B**. Hs-MSI metrics in follow-up samples. Each plot
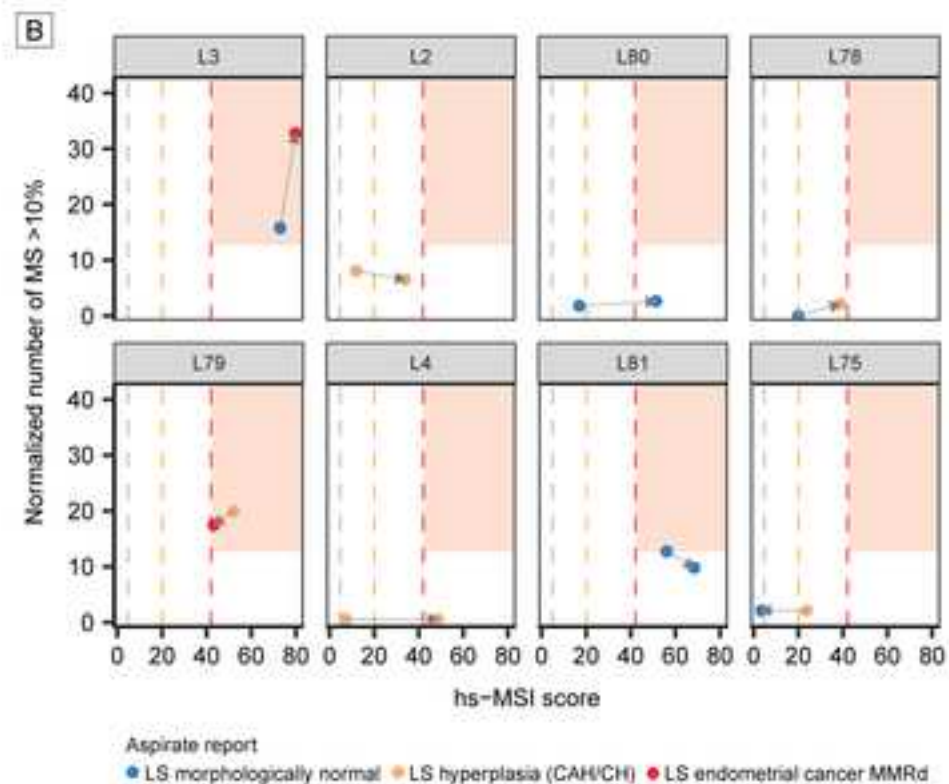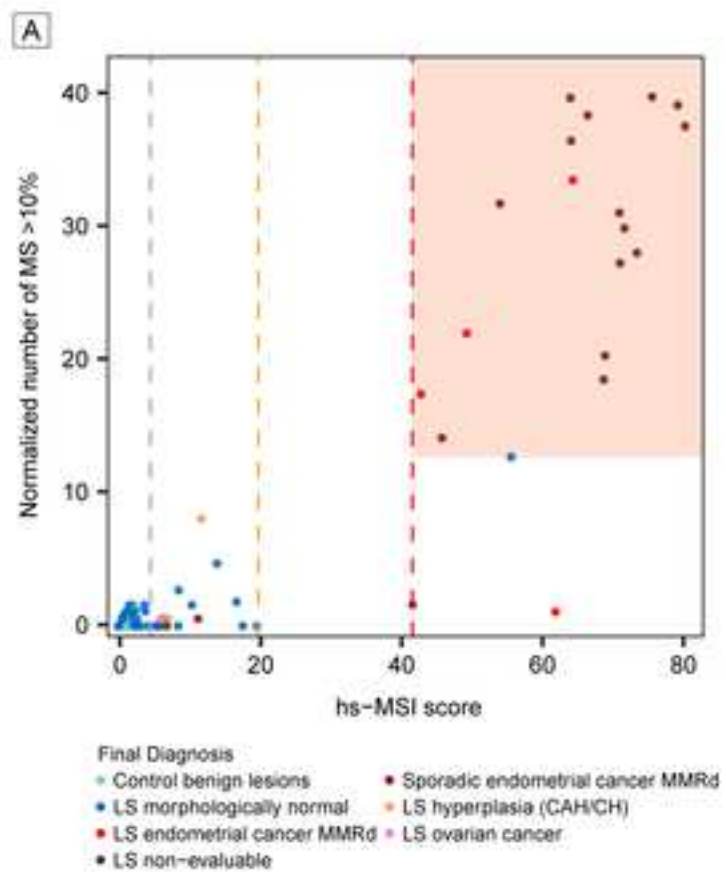
represents hs-MSI metrics in serial endometrial biopsies from the same patient (n=8). Points are colored according to the histopathological report. Samples from the same patient are connected by arrows, whose direction indicates the time progression. Dashed lines and colored area represent the same thresholds as in the main series. LS: Lynch syndrome; MMRd: mismatch repair deficient; CAH/CH: complex atypical hyperplasia/complex hyperplasia without atypia.
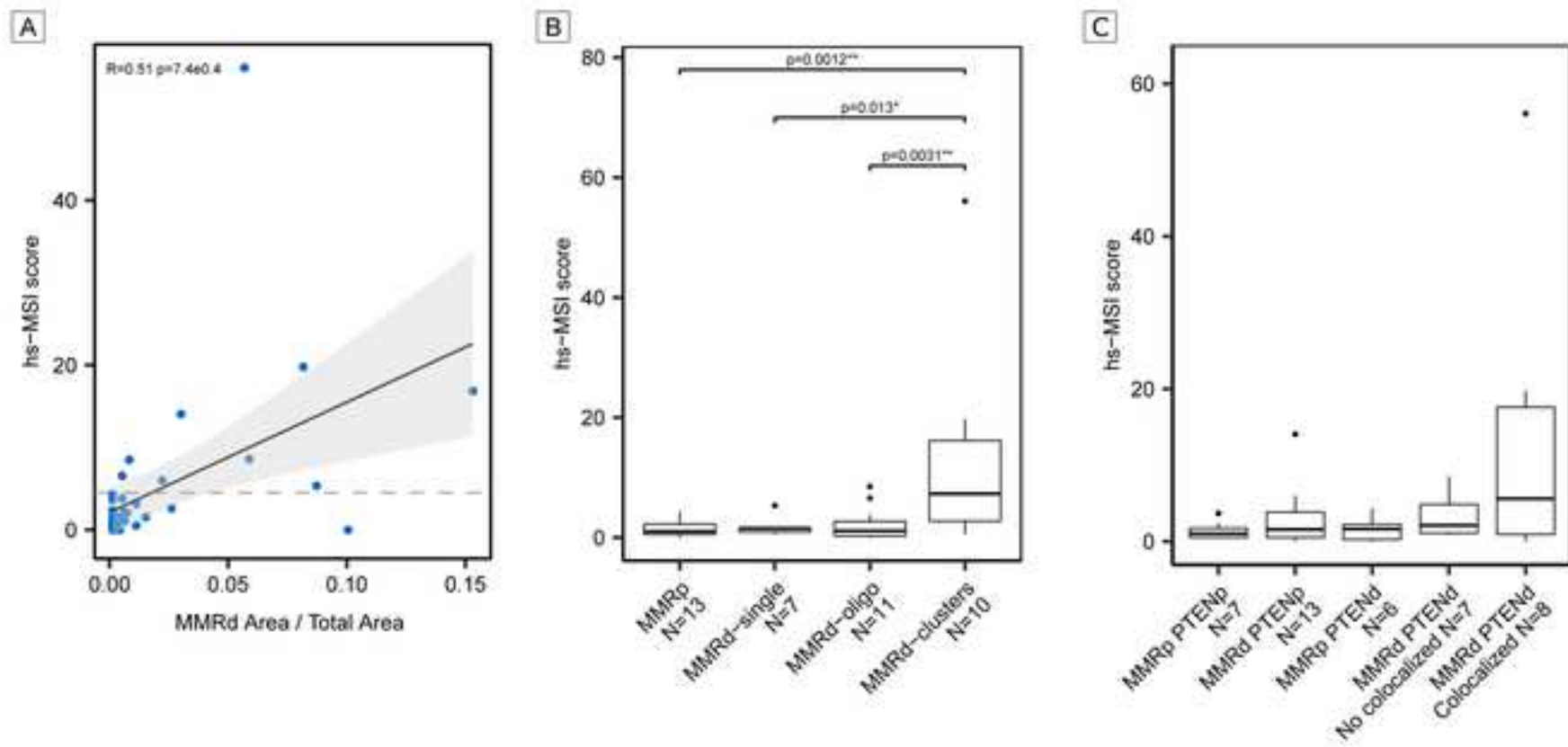
Figure 4: Correlation of hs-MSI score with MMR and PTEN immunohistochemistry analyses. A. Correlation between hs-MSI scores and area of MMR loss of expression (MMRd Area / Total Area). hs-MSI scores positively correlate with the area showing MMR loss of expression (R=0.5, *p*=7.4e-4). **B.** Correlation between hs-MSI scores and the MMR loss of expression pattern. MMRd clusters showed statistically significant higher hs-MSI scores *(p>0.05 in all comparisons)*. **C.** Correlation between hs-MSI scores and MMRd and PTEN loss of expression. Highest hs-MSI scores were observed when loss of expression of MMR colocalized with loss of PTEN. No statistically significant differences were observed between groups (*p*=0.43).

Figure 1

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Figure 4

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Table 1   Click here to access/download;Table;Table 1.docx

Table 1: Summary of main characteristics of the individuals included in the study

Individual characteristics	Lynch syndrome series					Training series	
	Total	*MLH1*	*MSH2*	*MSH6*	*PMS2*	Benign lesions controls	Sporadic EC cases
Number of individuals (n)	**93**	**32 (34%)**	**18 (19%)**	**33 (36%)**	**10 (11%)**	**30**	**25**
Age (available n / total n)	93/93	93/93	93/93	93/93	93/93	30/30	25/25
Median age (y) (range)	44 y (23-73)	44 y (23-68)	42 y (27-64)	42 y (23-73)	54 y (42-66)	57 y (40-82)	65 y (48-85)
BMI (available n / total n)	89/93	30/32	18/18	31/33	10/10	(29/30)	24/25
Median BMI (kg/m^2) (range)	25 (18-38)	25 (18-36)	26 (18-34)	24 (18-38)	23 (18-32)	28 (21-41)	32 (24-47)
Menopausal status (available n / total n)	93/93	32/32	18/18	33/33	10/10	30/30	25/25
Premenopausal (%)	64 (69%)	20 (62%)	14 (78%)	26 (79%)	4 (40%)	9 (30%)	0 (0%)
Perimenopausal (%)	8 (9%)	6 (19%)	0	2 (6%)	0	3 (10%)	2 (8%)
Postmenopausal (%)	21 (22%)	6 (19%)	4 (22%)	5 (15%)	6 (60%)	18 (60%)	23 (92%)
Menstruation phase (n premenopausal /total n)	59/64	18/20	13/14	24/26	4/4	9/30	0/25
Menstruation (%)	6 (10%)	1 (6%)	2 (15%)	2 (8%)	1 (25%)	3 (33%)	-
Follicular phase (%)	26 (44%)	8 (44%)	5 (39%)	12 (50%)	1 (25%)	1 (11%)	-
Luteal phase (%)	27 (46%)	9 (50%)	6 (46%)	10 (42%)	2 (50%)	5 (56%)	-
Endometrial thickness (available n / total n)	68/93	20/32	14/18	27/33	7/10	10/30	21/25
Median (mm) (range)	7 (1-30)	7 (2-14)	7 (1-15)	7 (2-30)	5 (2-12)	7 (1-11)	19 (8-50)
Abnormal bleeding (available n / total n)	93/93	32/32	18/18	33/33	10/10	30/30	25/25
Abnormal Bleeding (%)	5 (5%)	3 (9%)	0 (0%)	2 (6%)	0 (0%)	19 (63%)	23 (92%)
Surgical intervention performed* (n /total n)	36/93	13/32	7/18	13/33	3/10	14/30	24/25
Surgery indication:							
Benign pathology	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	14 (100%)	0 (0%)
Malignant pathology	6 (17%)	3 (23%)	1 (14%)	1 (8%)	1 (33%)	0 (0%)	24 (100%)
Premalignant pathology	3 (8%)	0 (0%)	1 (14%)	2 (15%)	0 (0%)	0 (0%)	0 (0%)
Prophylactic surgery	27 (75%)	10 (77%)	5 (71%)	10 (77%)	2 (66%)	0 (0%)	0 (0%)

*Surgical interventions considered: hysterectomies, adnexectomies, and salpingectomies; BMI: body mass index.

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Table 2: Results of the histological evaluation of LS endometrial aspirate samples and final clinical diagnosis.
When a tumor was diagnosed in another collected aspirate from the same patient or in the surgical piece, the final diagnosis was updated.

Result of endometrial aspirate histological evaluation	Lynch syndrome carriers (n, % of total individuals)					Final clinical diagnosis
	MLH1 (n=32)	*MSH2* (n=18)	*MSH6* (n=33)	*PMS2* (n=10)	Total (n=93)	
Normal endometrium	20 (32%)	10 (16%)	25 (41%)	6 (10%)	61 (66%)	60 Normal endometrium **1 Ovarian cancer**
Complex hyperplasia without atypia	0 (0%)	1 (100%)	0 (0%)	0 (0%)	1 (1%)	1 Complex hyperplasia without atypia
Complex hyperplasia with atypia	1 (33%)	0 (0%)	2 (67%)	0 (0%)	3 (3%)	2 Complex hyperplasia with atypia **1 Endometrial cancer**
Endometrial cancer	2 (67%)	0 (0%)	1 (33%)	0 (0%)	3 (3%)	3 Endometrial cancer
Non-evaluable	9 (36%)	7 (28%)	5 (20%)	4 (16%)	25 (27%)	24 Non-evaluable **1 Endometrial cancer**
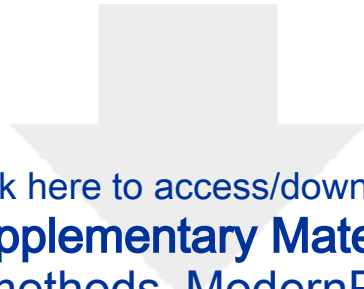
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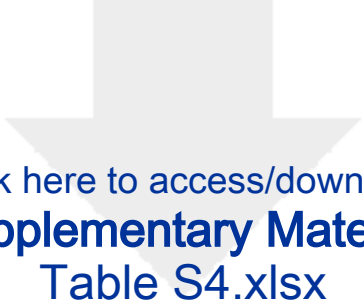Supplementary methods_ModernPathology_R.docx

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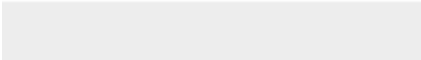
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