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Original

# (DNA Mismatch Repair Deficiency in Colorectal Adenocarcinoma: an Evaluation Test for Suspecting Lynch Syndrome)

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**Abbreviations:**
DNA; deoxyribonucleic acid.
CCR; Colorectal Cancer.
dMMR, deficiency in MMR repair.
IHC; immunohistochemistry.
IMS; MMR microsatellite instability; DNA; Mismatch Repair.
SL; Lynch syndrome.

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## Abstract

**Aim:** To profile the clinical and pathological characteristics, as well as to determine the prevalence of failed repair of deoxyribonucleic acid incompatibility by immunohistochemistry, of people diagnosed with colon adenocarcinoma at an age of < 70 years and to assess the detection of suspected cases of Lynch syndrome using immunohistochemistry by comparing all cases with those that only meet criteria clinical and pathological of suspicion.

**Methods:** An observational and cross-sectional study was carried out that included 249 patients, whose demographic characteristics, personal and family history of cancer, compliance with the Amsterdam, Bethesda and PREMM5 criteria, as well as the pathological characteristics of colorectal adenocarcinoma were analyzed. The prevalence of the immunohistochemical alteration of the MLH1, MSH2, MSH6 and PMS2 proteins that was compatible with a suspected case of Lynch syndrome was determined, and the probability and agreement of finding this alteration according to the clinical-pathological variables was estimated.

**Results:** The use of immunohistochemistry showed a prevalence of 22 cases (8.8%) with a suspected alteration of Lynch syndrome. Of these cases, none met all the clinical-pathological criteria for suspicion of the syndrome. Alternatively, most of the participants who met the criteria for suspicion did not have an alteration in the tests. It was shown that there is a higher prevalence of the alteration in immunohistochemistry when the cancer is located right (proximal to the splenic angle) and when Bethesda criteria 3 or 4 are met. It was documented that there is a null to low agreement between the clinical criteria and immunohistochemistry.

**Conclusion:** These findings suggest that immunohistochemistry testing in patients with colorectal adenocarcinoma could identify more candidates for complementary studies to confirm Lynch syndrome, regardless of the clinical and pathological criteria that are usually used to select who undergoes this immunohistochemistry test.

**Keywords:** colorectal neoplasia, hereditary neoplastic syndromes, DNA incompatibility repair, microsatellite instability, Lynch syndrome

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Colorectal cancer (CCR) is one of the most common neoplasms in the world and in Costa Rica it represented the third cause of incidence and the fourth in mortality in 2020.[1] Approximately 5% of cases are related to a hereditary etiology, in which Lynch syndrome (LS) was the main cause in this group.[2]

The origin of this condition is related to germline pathogenic variants in the deoxyribonucleic acid (DNA) sequence of the *MLH1, MSH2, MSH6, PMS2* and *EPCAM genes,* with an autosomal dominant inheritance pattern, resulting in the inactivation of the DNA mismatch repair (MMR) mechanism and the accumulation of mutations in the microsatellite regions of DNA, which in turn increases the likelihood of malignant transformation.[3,4] Individuals with LS have an increased risk of developing CCR as well as extracolonic neoplasms such as endometrium, stomach, ovary, small intestine, hepatobiliary tract, renal pelvis, ureter, skin, brain, among others; the risk of onset varies according to the genetic pathway affected.[5] Therefore, the identification of suspected and eventually confirmed people with LS has a clinical impact, since early detection and risk reduction measures can be established to reduce cancer incidence, morbidity and mortality.[6,7]

Previous studies have shown that detecting a tumor with MMR repair deficiency (dMMR) or high microsatellite instability (MSI) is a valid strategy for screening cases for SL,[8–10] especially since classic clinical criteria such as Amsterdam and Bethesda have shown low sensitivity to detect and confirm the syndrome.[11,12] Computerized predictive tools ($PREMM_{1,2,6}$, $PREMM_{5,}$ MMRpro, MMRpredict) have also been validated as alternatives based on personal and family history of neoplasms.[13–15] Some pathologic features of the tumor may also help identify MMR-deficient neoplasms.[16]

In Costa Rica's social security hospitals, there is no routine detection of suspected cases of SL, nor has previous research related to this condition been documented, so it is unknown which of the available alternatives could have the greatest capacity to detect suspected cases, an initial step necessary to refer these people to a genetic counseling consultation where it can be confirmed a hereditary etiology.

The objective of this study is to profile the clinical and pathological characteristics of people diagnosed with CCR at an age of ≤ 70 years, to determine the prevalence of dMMR analyzed by immunohistochemistry (IHC) and to analyze whether more cases cataloged as candidates for a confirmatory germline genetic study would be detected confirmatory of whether this test if it is recommended in all cases. not only when there are clinical-pathological criteria of suspicion of the syndrome.

## Methods

This study was designed for a retrospective collection of information from the available medical records of individuals who were diagnosed with CCR at the age of ≤ 70 years, by collecting the history and characteristics of cancers diagnosed in the person and the description of the neoplasms of family members recorded in the participant's record.

This study was approved by the central scientific ethics committee of the Caja Costarricense de Seguro Social (Costa Rican Social Security Fund) and its development adhered to the committee´s requirements and Law 9234, the Regulatory Law on Biomedical Research and its regulations.

### Patients

Participants were selected from the physical records of the Pathology Service of the Hospital Dr. Rafael Ángel Calderón Guardia, Caja Costarricense de Seguro Social (Costa Rica Social Security Fund), in the period between January 1, 2015

and December 31, 2017. Cases with the diagnosis of adenocarcinoma of the colon or rectum, aged between 18 and 70 years at the time of diagnosis and who had the report of IHC MLH1, MSH2, MSH6 and PMS2, or who had paraffin-embedded and formalin-fixed tissue available for such analysis, were included. Cases in which access to the clinical record was not obtained or that did not have an available and interpretable IHC were excluded.

A case was considered suspicious of LS if the tumor had: (a) isolated absence of wild BRAF MLH1 staining (no mutation), (b) absence of wild BRAF MLH1 and PMS2 staining, (c) isolated absence of MSH2 staining, (d) isolated absence of MSH6 staining, (e) absence of MSH2 and MSH6 staining, f) isolated absence of PMS2 staining. For this study, it was not possible to analyze MLH1 methylation in a complementary way, nor were molecular studies of IMS.

### Clinical Information

Demographic information was obtained from the clinical file, as well as the personal and family history of neoplasms. The characteristics of each participant's tumor were obtained from the pathology report. Information related to gender, age of diagnosis, tumor location, histological characteristics, history of CCR or extracolonic neoplasms, as well as all neoplasms documented in family members with the respective age at diagnosis were collected. The family pattern of neoplasm presentation was classified as: hereditary (when it met the Amsterdam criteria), familial (when there was a report of neoplasms in the family, but it did not meet the inheritance criteria), sporadic (when there was no aggregation of neoplasms in the participant's relatives) and unclassifiable (if the available information did not allow it to be grouped into any of the previous ones).


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The case was defined as meeting the Amsterdam I criteria if there were at least 3 relatives with CCR and all of the following criteria: a) one person must be a first-degree relative of the other two, b) at least 2 successive generations must be affected, c) at least one CCR must be diagnosed before the age of 50, d) familial adenomatous polyposis was ruled out, e) the tumors were confirmed by pathology.

The case was defined as meeting the Amsterdam II criteria if there were at least 3 relatives with a cancer associated with LS such as CCR, endometrium, small intestine, ureter or renal pelvis, stomach, ovary, hepatobiliary, brain, skin and meets the other criteria: a) one must be a first-degree relative of the other two, b) at least 2 successive generations must be affected, c) at least one case must be diagnosed before the age of 50, d) the familial adenomatous polyposis, e) the tumors were confirmed by pathology. For the analyses, the cases that complied with Amsterdam II were used, since they also covered all the cases that approved Amsterdam I.

The case was defined as supporting Bethesda's criteria if it presented at least one of the following:

1) A CCR diagnosed at an age younger than 50 years, 2) presence of synchronous or metachronous CCR or other tumor associated with LS, 3) a CCR with histology suggestive of MSI such as lymphocyte infiltration into the tumor, Crohn's-like lymphocytic reaction, mucinous differentiation or signet ring cells, bone marrow growth pattern in a patient under 60 years of age, 4) a CCR diagnosed in one or more first-degree relatives with an LS-related tumor with one of them diagnosed before the age of 50 years. 5) A CCR diagnosed in two or more first -or second-degree relatives with LS-related tumors regardless of age.

The PREMM5 score was calculated in each participant using the online calculator available from the Dana Farber Cancer Institute (http://premm.dfci.harvard.edu), a clinical prediction model that estimates the cumulative probability that an individual will carry a germline variant of the LS genes based on the cancer cases of the patient and his or her family; When the age of the cancer diagnosis of the relatives was not recorded, estimates were made based on the different notes in the file to complete the form.

**Immunohistochemistry and molecular diagnostics**

Hematoxylin-eosin-stained slides from endoscopic or surgical specimen biopsies were identified as representative areas of the tumor. IHC staining was carried out on paraffin-embedded and formalin-fixed tissue. Tissue slices with a thickness of 4 micrometers were made, which were dried in an oven at 63°C for a period of 45 to 60 minutes. Tissue recovery was performed using EnVision FLEX Retrieval Solution High pH TARGET with Dako's epitope recovery equipment. Subsequently, the IHC technique was applied using the Dako Autostainer Link platform48 and antibodies against MLH1 (ES05 clona); MSH2 (clona M363), MSH6 (clona EP51), and PMS2 (clona EP49).

The complete absence of nuclear staining in tumor cells in the presence of positive control (nuclear staining of non-neoplastic cells) was considered as loss of expression of the corresponding protein in the tumor.

In tumors with loss of MLH1 expression or combined loss of MLH1 with PMS2, the *BRAF* V600E mutation was analyzed in a complementary manner. In each case, the pathology professional selected and marked the paraffin-embedded block containing the largest number of viable tumor cells (identified in the corresponding sheet stained with hematoxylin-eosin) to obtain 4-micrometer sections. DNA was extracted using the QIA amp DNA Paraffin-Embedded Tissue Kit; the quantification and purity of the extraction product was measured with ultraviolet spectrophotometry (Nanodrop 2000c). The DNA obtained was amplified by polymerase chain reaction and the mutation in codon 600 of the *BRAF* gene was analyzed by pyrosequencing technique (PyroMark Quiagen with therascreen BRAF pyro kit) according to the manufacturer's specifications. Finally, the results were analyzed using the PyroMark Q24 software.

**Statistical analysis**

Qualitative and ordinal variables were analyzed as frequencies and percentages. Continuous variables were expressed as median ± standard deviation. Prevalence was calculated by dividing the number of people with dMMR tested with IHC who met the criteria for a suspected case of LS by the total number of participants. Categorical variables were compared using Fisher's exact test. Cohen's Kappa coefficient was used to analyze the agreement between the clinical-pathological criteria and the state of the dMMR tumor. A value of $p < 0.05$ was considered as statistical significance when comparing the probability of dMMR in tumors according to the following clinical and pathological variables: gender, age at diagnosis of CCR, anatomical location of the tumor, histology, history of other neoplasms, and the Amsterdam, Bethesda, and PREMM criteria$_5$. For statistics, the OpenEpi program was used.

## Results

The sample included a total of 249 patients, with a median age of 57.8 ± 9.4 years; 126 (50.6%) were women with a median age of 57.2 years and 123 men with a median age of 58.4 years. Forty-three (17.2%) of the participants were diagnosed at a young age, i.e. under 50

years of age. Most of the participants lived in the Greater Metropolitan Area, where 82% corresponded to cantons known for a high incidence of CCR (San José 21.6%, Goicoechea 20.0%, Montes de Oca 11.6%, Coronado 11.2%, Moravia 9.2% and Curridabat 8.4%). Figure 1 shows the patients included in the study period.

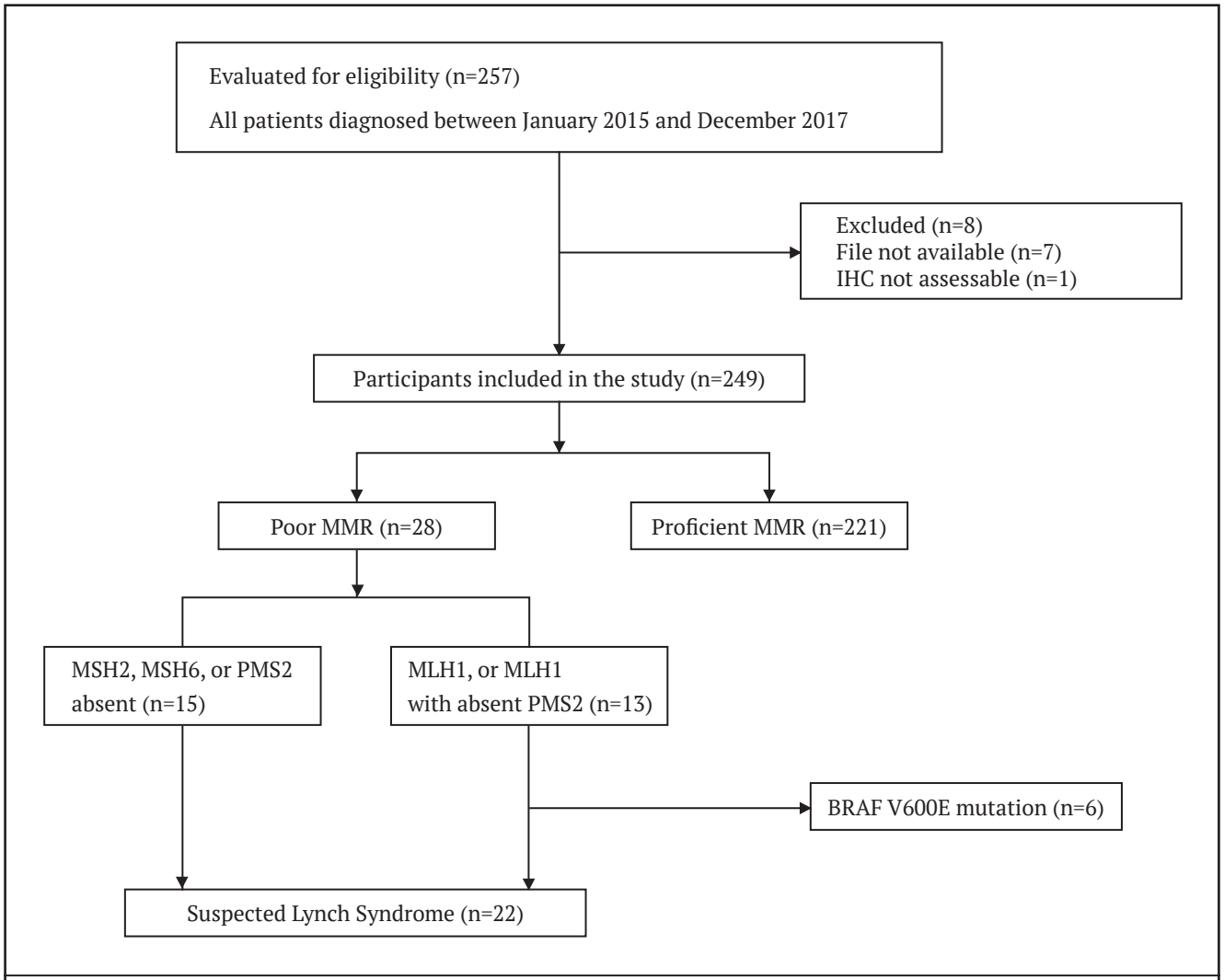


**Figure 1.** Flow chart of patients < 70 years of age with colorectal adenocarcinoma diagnosed between January 2015 and December 2017 at the Rafael Ángel Calderón Guardia Hospital.

Twenty-two (8.8%) of the participants had a finding in the IHC that classified them as a suspected case of LS and that would merit genetic counseling and, eventually, a germline diagnosis to confirm this syndrome. In total, an alteration in IHC compatible with dMMR was demonstrated in 28 individuals (11.2%), distributed as follows: 13 (46.4%) with joint loss of MLH1 and PMS2, 8 (28.6%) with isolated loss of PMS2, 5 (17.9%) with joint loss of MSH2 and MSH6, 2 (7.1%) with isolated loss of MSH6. The *BRAF* V600E mutation was identified in 6 of the 13 cases of tumors with the absence of MLH1.

The variables analyzed are summarized in Table 1. A large part of the neoplasms was located on the left side, only 17.7% had a characteristic histological pattern of tumors with dMMR. Among the 29 participants who had another diagnosed neoplasm, a total of 36 synchronous or metachronous cancers were registered, where 44.4% correspond to neoplasms classically associated with LS (8 CCR, 3 gastric, 2 renal, 1 endometrial, 1 small intestine, 1 ovary)

In cases where there was a family history of cancer, a total of 357 neoplasms were documented, in 297 (83.2%) of these antecedents, the age at the time of diagnosis was not recorded in the file. In no case was an elaborate genealogy found.

**Table 1. Clinical and pathological characteristics of 249 cases with colorectal adenocarcinoma diagnosed at an age ≤ 70 years between January 2015 and December 2017 at the Hospital Rafael Ángel Calderón Guardia**

| Variable | n (%) |
|---|---|
| **Location of the cancer** | |
| Right colon | 47 (18.9) |
| Transverse colon | 12 (4.8) |
| Left and straight colon | 190 (76.3) |
| **Histology** | |
| None | 187 (75.1) |
| Mucinous | 39 (15.7) |
| Signet Ring | 2 (0.8) |
| Mucinous and signet ring | 2 (0.8) |
| Lymphocyte infiltration | 1 (0.4) |
| Core pattern | 0 (0) |
| Not reported | 18 (7.2) |
| **Personal history of another colorectal cancer or other neoplasm** | |
| No | 220 (88.4) |
| Yes | 29 (11.6) |
| **Family history of cancer** | 61 (24.5) |
| Yes | 174 (69.8) |
| Unknown | 14 (5.6) |
| **Familial pattern according to history of neoplasms** | |
| Sporadic | 126 (50.6) |
| Familiar | 100 (40.2) |
| Hereditary | 9 (3.6) |
| Not classifiable | 14 (5.6) |
| **Amsterdam I (meeting all criteria)** | |
| No | 230 (92.4) |
| Yes | 4 (1.6) |
| Not classifiable | 15 (6.0) |
| **Amsterdam II (meeting all criteria)** | |
| No | 225 (90.4) |
| Yes | 9 (3.6) |
| Not classifiable | 15 (6.0) |
| **Bethesda Revised (any number of criteria)** | |
| No | 96 (38.5) |
| Yes | 141 (56.7) |
| Not classifiable | 12 (4.8) |
| **PREMM5 Score** | |
| < 2.5% | 113 (45.4) |
| ≥ 2.5% | 136 (54.6) |

Right-sided tumors (proximal to the splenic angle of the colon), as well as Bethesda criteria 3 and 4, were found to be the only ones with statistical significance in the probability of being associated with a tumor with dMMR, with odds ratios of 5.46 (95% CI 2.24-13.70), 4.15 (95% CI 1.29-12.28) and 8.43 (95% CI 1.86-36.61), respectively. No significant difference was found in the analysis by gender, age at diagnosis, tumor histology,

and personal history of other neoplasms, even when they belonged to the SL criteria, Amsterdam criteria, Bethesda criteria 1, 2 and 5, also according to the PREMM score$_5$. Table 2 presents the analyses for the study variables that demonstrated significant probability.

The concordance between the clinical-pathological criteria and the dMMR tumor status was negligible (Cohen's Kappa coefficient <0.2): Amsterdam (0.095), Bethesda (0.060), PREMM$_5$ (0.010), Histology (0.085).

**Table 2. Clinical-pathological variables with a statistically significant probability of presenting failed DNA incompatibility repair in people diagnosed with colorectal adenocarcinoma at an age ≤70 years between January 2015 and December 2017 at the Rafael Ángel Calderón Guardia Hospital**

| | Normal MMR n (%) | Poor MMR n (%) | P value |
|---|---|---|---|
| Location of colorectal cancer (n=249) | | | |
| Right | 43 (72.9) | 16 (27.1) | **0.0001** |
| Left | 178 (93.7) | 12 (6.3) | |
| Bethesda Criterion 3 (n=237) | | | |
| Yes | 18 (72.0) | 7 (28.0) | **0.016** |
| No | 194 (91.5) | 18 (8.5) | |
| Bethesda Criterion 4 (n=237) | | | |
| Yes | 6 (54.5) | 5 (45.5) | **0.005** |
| No | 206 (91.2) | 20 (8.8) | |

In the 22 cases listed as suspected of LS by dMMR, none of the clinical and pathological criteria were met in 100% of cases, as shown in Figure 2. In the people who did verify some criterion, it was shown that most did not present the alteration of suspicion in the IHC: 7 out of 9 who met the Amsterdam criterion did not acquire dMMR, as well as 122 out of 141 for any Bethesda criterion, 120 out of 136 for PREMM5, 35 out of 44 for the histology standard.

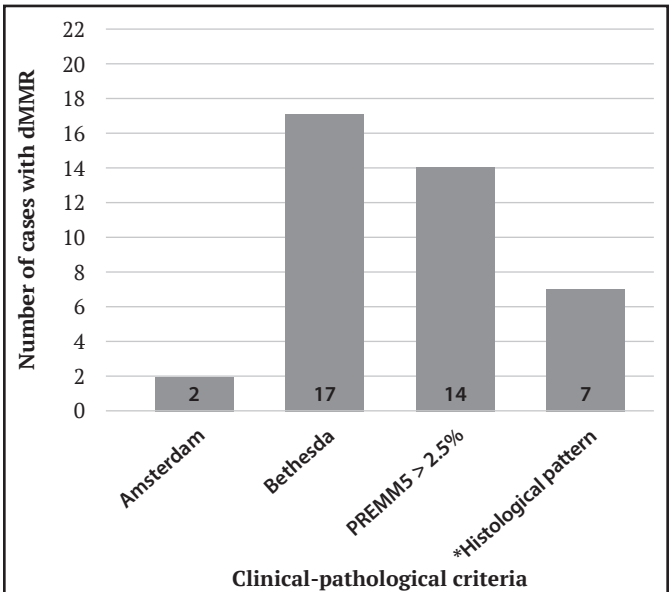


**Figure 2.** Number of cases with colorectal adenocarcinoma suspected of Lynch syndrome due to failed DNA incompatibility repair (dMMR) that also meet clinical-pathological criteria for suspicion of the syndrome.
*Histological pattern: mucinous, signet ring, lymphocyte infiltration.

## Discussion

The results of this study suggest that analyzing the MLH1, MSH2, MSH6, PMS2 proteins in the tumor of people diagnosed with colorectal adenocarcinoma at the age of ≤ 70 years could catalog more suspected cases of LS that merit referral for genetic diagnosis to confirm the syndrome, regardless of the existence or not of clinical criteria such as those of Amsterdam. Bethesda, standards in histology or calculators such as PREMM$_5$. If these criteria were used as a requirement to request the IHC test in the CCR, there would be a potential loss of suspected cases for IHC at this frequency: 90% for Amsterdam, 23% for Bethesda, 36% for PREMM$_5$, 68% for the histological pattern.

The prevalence of dMMR tumors found in this study is within the range of 8 to 20% based on previous reports in different regions of the world.[17–23] The MLH1-related pathway (absence of PMS2-inducing MLH1) is frequently related to the mechanism of hypermethylation of the *MLH1* gene promoter in the genesis of CCR, which is largely due to a somatic event, which can be inferred in 46% of the cases analyzed in which a V600E mutation in the BRAF gene was concomitantly demonstrated. This combination of protein loss in IHC has been reported by other centers as the most frequent tendency when the use of IHC has been implemented as a strategy to select cases of sampling.[24] A rare finding was the high percentage of cases with the PMS2 pathway as the main alteration. In a study applied in China, this phenomenon was found in which it was the main altered pathway and represents

32.8% of the cases of tumors with dMMR.[25] The isolated loss of PMS2 in the presence of all other proteins is an uncommon phenotype of tumors with dMMR that could also be due to other factors: the small sample size, for example four of the cases were endoscopic biopsies, of which the analysis was performed on a 2-3 mm sample that could alter the validity, as well as management in the preanalytical phase of biopsy where suboptimal fixation could affect expression. In addition, there are data in cases of endometrial cancer and LS that suggest that MLH1 methylation could also be a cause of isolated PMS2 loss.[26] For this study, the diagnosis of methylation was not available to corroborate this possible explanation.

The three variables that were associated with a higher probability of dMMR could be useful to design a diagnostic flow to detect cases of suspected LS, in case the resource is not available to implement immunohistochemistry in all cases diagnosed with colorectal cancer. The anatomical location in the right colon is compatible with the molecular consensus classification of colon cancer, in which subtype 1 is characterized by a higher frequency of IMS and a higher right location;[27] In addition to the implications for the possibility of detecting cases of LS, this condition may have prognostic value for those who suffer from the disease.[28,29] Although the criteria of the Revised Bethesda Guideline were validated to improve the identification of people at risk of LS and indication for the search for MSI in the tumor,30,31 only criterion 3 (CCR with an MSI phenotype diagnosed before the age of 60 years) and 4 (diagnosis of CCR in the person with a first-degree relative with a tumor associated with LS) were shown in this examination at an age < 50) had a significant relationship. In different considerations of this finding, it was not possible to find a possible explanation for the non-relationship of the other criteria beyond considering that the sample of cases with dMMR is small. It should be taken into account that even in previous research where all Bethesda criteria are taken into account, it has not been possible to demonstrate a detection of all cases of LS when compared with tools such as the IMS, so caution should be exercised if only the Bethesda criteria were to continue to be used for the detection of LS.[32] In the present study, it was identified that 3.6% of the participants met the Amsterdam criteria, that is, they remained within the expected range of the hereditary pattern of colorectal cancer in the population.[33] Although this was not associated with the detection of dMMR tumors in the analysis, it should be taken into account that it is still one of the criteria that determine the risk not only of LS, but also of the risk of CCR in family members.[34] To meet these criteria, a detailed collection of the history of neoplasms is required, which does not reflect the reality of the medical records and shows the high percentage (83%) in which the age of diagnosis was not defined. This lack of data could also be the explanation for the high percentage of a family pattern shown in Table 1, since these details allow a better classification of the presentation of neoplasms in a family. Genealogy continues to be one of the most valuable tools when looking for a hereditary pattern of a disease, however, it is not routinely used in medical records, which could be because it requires more time in the anamnesis, even the lack of training in the symbology and elaboration process.

This study is considered to include a representative sample since it was possible to include 97% of all people diagnosed with CCR in the study period and most of them came from cantons previously identified as frequent places of residence of women and men with CCR in Costa Rica (Statistical bulletin of incidence of the most frequent malignant tumors in Costa Rica for the year 2013: Ministry of Health; 2015. [revised on 10-11-2022]. Available at: https://www.ministeriodesalud.go.cr/index.php/biblioteca-de-archivos-left/ documents-ministry-of-health/informational-material/ published-material/statistics-and-databases/statistics-and-databases-health-surveillance/statistics-health-surveillance/statistics-health-surveillance/cancer-statistics-national-tumor-registry/cancer-incidence-bulletins/1725-cancer-incidence-bulletin-2013 /file). In addition, the pathological characteristics of the included cases are like previous reports in other countries, both for the location of CCR, which is more frequent on the left side, between 70-80% of cases,20,24,35, and for some histological characteristics such as the mucinous component around 15%.[36]

This project has several limitations, including the potential extrapolation in terms of being an analysis focused on a single center, which may not represent the reality and quality of the tumor diagnosis protocols of other hospitals. In addition, there was a suboptimal record of family history in the participants' records that could modify the findings and criteria found in this sample, which is relevant to correct since in other Hispanic populations an increase in the capture of cases of LS has been demonstrated associated with an improvement in the quality of family history collection.[37] At the time of development of this research, the Institution did not yet have the resource of a genetic counseling consultation (available as of 2019), so it was not possible to confirm the LS by analyzing various germline pathogenic agents in cases where a GS could be suspected. Therefore, immunohistochemistry is a test that, by itself does not allow confirming this syndrome. Another limitation to be noted would be that there was no complementary molecular study for the analysis of IMS or methylation of the promoter of the *MLH1 gene*.

The results of this research could be useful for decision-making in the organization of detection and referral for a confirmatory diagnosis of hereditary CCR syndromes, whose attention is inconsistent in Latin American health systems.[38] To our knowledge, this is the first analysis at the national level that investigates the potential role of IHC in detecting cases of suspected SL.

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