Clinicopathological and molecular characterization of Brazilian families at risk for Lynch syndrome

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Highlights

- Largest study of germline mutations in Lynch Syndrome-related genes in the Brazil
- New pathogenic variants were identified.
- Co-occurrence of germline variant and somatic MLH1 hypermethylation
- Recurrent and founder variants could lead to a cost-effective genetic testing
- *MLH1* somatic methylation can be a second hit in some Lynch Syndrome tumors.
- Predictive genetic testing enables more appropriate surveillance and prevention.

Clinicopathological and molecular characterization of Brazilian families at risk for Lynch syndrome

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Abstract

Lynch syndrome (LS), is the most common hereditary colorectal cancer syndrome. However, it´s poorly characterized in Brazil. Therefore, we aimed to determine the spectrum of Mismatch Repair (MMR) genes variants and investigate the *MLH1* promotor methylation role as a second hit in LS tumors. Tumor screening through microsatellite instability and immunohistochemistry for MMR proteins, were performed in 323 cases who met clinical criteria. *BRAF*-V600E and *MLH1* promoter methylation were analyzed for all mlh1-deficient tumors. Patients with MMR deficient tumor proceeded to germline genetic testing. MMR deficient tumors were detected in 41% of patients recruited. About half of patients carried a pathogenic germline variant. Two recurrent variants in *MLH1* and three novel pathogenic variants were identified. Furthermore, pathogenic germline variants with concomitant somatic *MLH1* hypermethylation were found in 6% of cases. Predictive genetic testing was offered to 387 relatives. Overall, 127 tumors were diagnosed in 100 LS patients, from 62 unrelated families. Our molecular data provide new information about the spectrum of MMR mutations, which contributes to a better characterization of LS in Brazil. Furthermore, we call attention to the possibility of failure in the diagnosis of germline *MLH1* mutation carriers when somatic *MLH1* hypermethylation is used to rule out LS.

Keywords: Lynch Syndrome; DNA Mismatch Repair; DNA methylation; Inherited cancer; Colorectal cancer.

1. Introduction

Lynch syndrome (LS) is the most common hereditary colorectal cancer (CRC) syndrome; it accounts for approximately 3% of all colorectal cancers [1]. Subjects with LS are at a much higher risk of developing CRC and endometrial cancer with a lifetime risk of 52–82% and 25–60% respectively[2]. Other cancers, including gastric, small bowel, ovarian, hepatobiliary, pancreatic, urinary, and brain cancers, are also associated with LS [1].

The major genetic factor associated with LS is the presence of germline mutations on DNA mismatch repair genes (MMR), namely *MLH1* (MutL Homolog 1), *MSH2* (MutS homolog 2), *MSH6* (MutS homolog 6) and *PMS2* (PMS1 Homolog 2) [3]. About 41% of LS cases are attributed to the *MLH1*, 39% to *MSH2*, 13% to *MSH6* and 6% to *PMS2* [2]. Up to 3% of LS are due to mutations in the *EPCAM* gene, which is directly upstream to *MSH2* loci[4].

From a clinical point-of-view, the identification of LS families depends on individuals and/or families meeting clinicopathological criteria, mainly Amsterdam I/II and revised Bethesda criteria [7,8]. Moreover, microsatellite instability (MSI) or immunohistochemical (IHC) testing of tumors coupled with molecular genetic testing of *BRAF* V600E and *MLH1* promoter hypermethylation are the molecular gold standard strategies to select patients for subsequent germline diagnostic testing [7,8]. Although *MLH1* promoter methylation analysis have been used, together with *BRAF* V600E mutation, to discriminate LS from sporadic CRC, there are, in the literature, reports showing co-occurrence of somatic *MLH1* methylation and germline *MLH1* mutation in LS patients [9,10].

In Brazil, the clinical and molecular characterization of families with LS is scarce. There are only two comprehensive molecular studies [11,12]. Thus, in the present study we aimed to fill the existing gap in the knowledge of the Brazilian LS population, by providing a detailed description of LS families from a reference cancer hospital in Brazil. In addition, the

occurrence of *MLH1* promotor methylation as a second hit in LS tumors will also be further evaluated.

2. Material and Methods

2.1. Recruitment of families

A total of 323 probands who met the Amsterdam and/or Bethesda Criteria were recruited over a period of seven years at Barretos Cancer Hospital (BCH). BCH is a reference cancer hospital located in the countryside of São Paulo state (Brazil). The patients come from 14 states, representing all the Brazilian geographical regions (80.8% from southeast, 13.9% central-western, 3.1% north, 1.2% northeast and 0.9% south). This study was approved by the local IRB (nº 745/2013).

2.2. Clinical and pathological data

Personal and familial cancer history data were obtained from the pedigree of each family. Confirmation of the cancer family history was attempted, by the medical geneticist, in all cases, through pathology and medical reports and/or death certificates. The site and type of cancer, histopathological information, tumor grade and age of each patient were obtained from the patient's clinical record.

2.3. Strategy for Lynch syndrome screening

The screening strategy consisted of tumor tissue analysis through MSI and IHC of the four MMR proteins (MLH1, MSH2, MSH6, PMS2). Cases with MLH1 loss of expression (by IHC) were referred to *BRAF*-V600E mutation and *MLH1* methylation analysis [1]. Figure 1 depicts the flowchart for LS identification adopted in this study. All patients with MMR deficient tumors were subjected to molecular genetic testing of the MMR genes according to altered gene(s) indicated by the IHC. Cascade genetic testing were performed to all interested relatives from families in which pathogenic/likely pathogenic variants were identified.

2.4. Microsatellite instability (MSI) analysis

The MSI evaluation was performed using a multiplex PCR comprising five quasi-monomorphic mononucleotide repeat markers (BAT-25, BAT-26, NR-21, NR-24, and NR-27) as reported by our group [13,14]. The results were analyzed using GeneMapper v4.1 software (Applied Biosystems). Samples were considered MSI-High (MSI-H) when two or more markers were altered, MSI-Low (MSI-L) when one marker was altered, and stable (MSS) if no altered marker was identified.

2.5. Immunohistochemical (IHC) analysis

The immunohistochemical reactions were performed at Dako Autostainer Link 48 equipment using a Dako EnVision™ FLEX detection system kit (Dako), according to the manufacturer's instructions. The anti-human primary antibodies used in the present study were: FLEX monoclonal mouse anti-MutL protein homolog 1 (clone ES05); FLEX monoclonal mouse anti-MutS protein homolog 2 (clone FE11); FLEX monoclonal rabbit anti-post meiotic segregation increased 2 (clone EP51); and FLEX monoclonal rabbit

anti-MutS protein homolog 6 (clone EP49). All cases were analyzed by an expert pathologist who classified each protein by its expression based on nuclear staining.

2.6. *BRAF* mutational analysis

The hotspot region of the *BRAF* oncogene (codon 600) was subjected to PCR, followed by direct sequencing, as reported [15].

2.7. Methylation analysis

2.7.1. Sodium bisulfite treatment

For methylation analysis, the DNA samples from tumor were treated with sodium bisulfite using EpiTect Bisulfite Kit (Qiagen), according to the manufacturer's instructions. The samples were stored at -80°C until use.

2.7.2. Pyrosequencing analysis

To evaluate *MLH1* somatic methylation in tumor tissues from cases lacking MLH1 expression, pyrosequencing was done using PyroMark Q24 CpG MLH1 kit (Qiagen). Each PCR reaction included 5 μL of bisulfite-treated DNA (10 – 50 ng/ μL), 0.8 μL of each primer (5 pmoles of each PCR primer), 12.5 μL of Master Mix Multiplex (Qiagen) in a final volume reaction of 20 μL. The biotinylated PCR products from 10μL of PCR reaction mix was captured on streptavidin-coated beads and underwent pyrosequencing using Pyro Gold Reagents - PyroMark Q24 (Qiagen). Pyrosequencing method considered each CpG site as a C/T polymorphism and generates quantitative data that provided a proportion of methylated

versus unmethylated allele. The data obtained were analyzed by PyroMark software v.2.0.7 (Qiagen). The Pyrosequencing assay analyzed the "C" region of the *MLH1* gene promoter. The 10% cut-off point was used for the characterization of methylated (> 10%) and unmethylated samples (≤10%), which was based on studies of the methylation status of the *MLH1* promoter region available in the literature and previously validated [16,17]. Besides, to evaluated the methylation threshold stablished, DNA from normal colon tissue (n=10) and colon tumor with MSH2/MSH6 loss of expression of (n=7) were analyzed and presented *MLH1* methylation mean of 0.13% ±0.06% and 0.22% ±0.47%, respectively. The DNA from RKO colorectal cancer cell line was used as a biallelic *MLH1* methylation positive control.

In cases with somatic methylation of *MLH1*, constitutional epimutations were sought in DNA from blood cells.

2.8. Germline genetic testing

Cases with MSI-High/Low and/or altered IHC and *BRAF* WT were referred for genetic testing, regardless of *MLH1* hypermethylation status. The genomic DNA was extracted from blood specimens using QIAamp DNA Blood Mini Kit (Qiagen). Genetic testing was performed by Sanger sequencing from 2011 to 2015, and by Next Generation Sequencing (Ion Torrent – PGM) from 2016 to 2018.

2.8.1. Sanger Sequencing

From 2011 to 2015, genetic tests were performed by Sanger sequencing. All exon-intron boundaries and the complete coding region of the *MLH1* (NM_000249.3), *MSH2* (NM_000251.2), *MSH6* (NM_000179.2), and *PMS2* (NM_00535.5) genes were amplified by PCR [18]. Due to the presence of highly homologous *PMS2* pseudogenes, for exons 11 and

13, long-range followed by nested PCRs were performed using LongRange PCR Kit (Qiagen) and HotStarTaq Master Mix Kit (Qiagen). All pathogenic variants in *PMS2* gene were confirmed in cDNA following protocols reported elsewhere [19]. After PCR amplification, the products were purified with ExoSap-IT (Thermo Fisher Scientific), bi-directionally sequenced (BigDye terminator v3.1; Applied Biosystems) and analyzed using SeqScape v2.7 software (Applied Biosystems). All pathogenics variants were confirmed by a second PCR/sequencing reaction in a new sample.

2.8.2.NGS Sequencing

2.8.2.1. Ion Torrent PGM library preparation

From 2016 to 2018, genetic tests were performed by Next Generation Sequencing. The entire coding regions of the *MLH1*, *MSH2*, *MSH6* and *PMS2* genes were amplified using the Ion AmpliSeq Custom Panel (Thermo Fisher Scientific) consisting of three primer pools, covering the target regions in 209 amplicons, including all exons and 15 bp of intronic flanking sequences. Ion Torrent adapter-barcode ligated libraries were generated using Ion AmpliSeq™ Library Kit 2.0 (Thermo Fisher Scientific) and Ion Xpress™ Barcode Adapter Kit (Thermo Fisher Scientific) according to the manufacturer's procedures.

2.8.2.2. Emulsion PCR and sequencing

The concentration of the libraries was determined using the Ion Library TaqMan Quantitation Kit (Thermo Fisher Scientific). After quantification, each amplicon library was diluted to 20 pM and pooled to perform the emulsion PCR reaction using an Ion Chef System (Thermo Fisher Scientific) according to the manufacturer's instructions.

Massive Parallel Sequencing was carried out with Ion Torrent PGM sequencer system (Thermo Fisher Scientific) using the Ion Torrent PGM Hi-Q Sequencing Kit and Ion 316v2 Chip (Thermo Fisher Scientific), according to manufacturer's instructions.

2.8.2.3. NGS Data Analyses

Primary analysis was performed by Torrent Server™ (v 5.0) and further by Ion Reporter™ Server hosting informatic tools (Ion Reporter™ Software v5.0) for variant analysis, filtering, and annotations. Custom workflow (Application: DNA, Sample group: Single, Reference: hg19, Variant type: Germline) with preconfigured parameter settings was utilized (minimal variant frequency of 0.1, minimum coverage of 5 ×, maximum strand bias of 0.98). The average depth of total coverage was set at minimum 50 ×.

2.9. Rearrangement analysis

The analysis of *MLH1*, *MSH2*, *MSH6*, and *PMS2* rearrangements was performed with the Multiplex Ligation-dependent Probe Amplification Kit (MLPA) (MRC Holland) according to the manufacturer's protocol. Fragment separation was conducted by capillary electrophoresis on an automated ABI 3500xL Genetic Analyzer (Applied Biosystems).

2.10. Variant classification

Interpretation of the variants was performed according to ACMG[20], InSiGHT [21], ClinVar [22], and HGMD [23] databases. The variants were then classified according to the five-tier IARC system recommended by the International Agency for Research on Cancer

(IARC) [24]. All variants were described according to HGVS (Human Genome Variation Society) guidelines.

2.11 Statistical analysis

Statistical analysis was conducted using SPSS v.20 software (Chicago, IL, USA). The comparisons between clinical and molecular characteristics employed a simple analysis using a Chi-square (or Fisher's exact) test. Mean age at cancer diagnosis among the four MMR genes was compared using Analysis of Variance (ANOVA). Quantitative variables were analyzed using the Student t test. Differences of 5% were considered statistically significance in all tests. The sensitivity of MSI and IHC for identifying mutation carries were calculated using two-by-two contingency table. Only cases with results for MSI, IHC and genetic testing were used to calculate sensitivity.

3. Results

3.1. Features of Probands

A total of 323 probands with family history suggestive of LS were referred to screening. The main clinical and histopathological features of the probands are summarized in Table 1. All patients were cancer-affected, with a previous diagnosis of CRC and endometrial cancer in 66% and 34% of cases, respectively. The mean age at cancer diagnosis was 50 years (range 16–76 years). The male/female ratio was 30%/70%.

3.2. Tumor testing

Tumor analysis (IHC and MSI) was carried out on the 323 study probands. Figure 2 depicts the detailed results of the cascade tumor screening. MSI and IHC status were compared in 316 tumor cases. MSI and/or IHC tumor analysis produced inconclusive results in 7 cases and proceeded directly to genetic testing. Assuming that all cases with proficient MMR were negative for pathogenic germline mutations, the sensitivity of MSI and IHC was 96% and 100%, respectively.

In total, 134 tumors were MMR-deficient (Table 1 and Figure 2). Among all patients with MMR-deficient tumors, 11% fulfilled the Amsterdam criteria. *BRAF*-V600E mutation and *MLH1* hypermethylation analyses were performed as part of the screening in all tumors with loss of MLH1 expression. *BRAF*-V600E mutation was identified in 14 cases, all of them with concomitant *MLH1* hypermethylation. Moreover, 30 *BRAF*-WT tumors showed *MLH1* hypermethylation (these cases were referred for genetic testing, given that tumor methylation was not used, in this study, to exclude patients from genetic testing). The *MLH1* promoter methylation level was 53% (SD= 21%) in the *BRAF* wild-type group, and 57% (SD= 22%) in the *BRAF*-mutated group showing no difference between both groups (p=0,594).

3.3. Germline genetic testing

Genetic testing was performed on 127 probands, of whom 62 (49%) had pathogenic/likely pathogenic variants in one of the MMR genes. Table 2 describes all the pathogenic germline variants identified, as well as the cancers reported by the probands and their relatives. The frequency of mutated genes was 39% (24/62) in *MLH1*, 37% (23/62) in *MSH2*, 18% (11/62) in *MSH6*, e 6% (4/62) in *PMS2*. We did not detect any patients with an *EPCAM* mutation or constitutive *MLH1* methylation. Frameshift (37%, 23/62) and nonsense (27%, 17/62) mutations were the most common alterations, following by large rearrangement (13%), missense (11%), splicing (9,5%) and in-frame (2%) (Table 2).

Although the pathogenic variants identified were distributed all over the genes, one variant in *MLH1*, c.1276C>T, p.(Gln426Ter), was identified in 10% (6/62) of the families (Table 2), and one Portuguese founder mutations in *MLH1,* c.(1897-?_2271+?)del (also known as exon 17-19 deletion), were found in 5 families. Besides, 3 new pathogenic/likely pathogenic variants (two in *PMS2* and one in *MLH1*) were identified and are described in detail in Table 2.

Genetic counselling and cascade testing were offered to all interested relatives of the 62 pathogenic variants carriers probands. Among the 387 relatives who underwent predictive genetic testing, 45% (n=175) were positive for the pathogenic variant segregating in the family. Among the 175 mutated-relatives, 38 had a LS-related cancer and 127 were unaffected at the time of testing (Table 2).

Interestingly and contrary to what it would be expected, somatic *MLH1* hypermethylation was detected in four patients harboring pathogenic germline variant. Three patients with *MLH1* pathogenic variants (*MLH1*: c.1853delAinsTTCTT; c.677+1G>A; c.1897-?_2271+?del), and in one patients with a *PMS2* pathogenic variant (*PMS2*: c.2185_2186delCT), representing 6% (4/62) of the all mutated patients. All of them developed CRC at ages 62, 55, 32 and 68 respectively (Table 2). The somatic *MLH1* promotor methylation values in the germline mutation carriers were 22.5%, 19.2%, 70% and 57%. There was not statistically significant difference in the *MLH1* methylation values between the germline mutation carriers (42%) and non-carriers (55%) (p=0,231).

3.4. Cancer occurrence in Lynch syndrome patients

In total, 127 tumors were reported in the 100 pathogenic/likely pathogenic variants carriers (62 probands and 38 relatives) (Table 3). The majority had only one LS associated cancer (76%), while 17% of patients had two cancer diagnosis, and 7% had three or more

associated cancers. Overall, 66% (n = 84) colorectal tumors and 34% (n = 43) extra-colonic tumors were reported. Proximal colon (43%) was the most common location and the mean age of CRC diagnosis was 47 years. Of the female patients with pathogenic variant who developed cancer, 23% presented endometrium cancer with mean age at diagnosis of 48 years. Among females, 9 developed both CRC and endometrial cancer, and in 66% of the cases, endometrial cancer was the first tumor diagnosed. The other extracolonic tumors diagnosed are detailed in table 3. The mean age at cancer diagnosis was compared among the *MLH1, MSH2, MSH6* and *PMS2* mutation carriers. Although the mean age at cancer diagnosis was lower for *MLH1* mutation carriers, no statistically significant difference was identified (p=0.158) (Table 3). Besides, when we compare the stage at colorectal cancer diagnosis (localized versus metastatic), among the patients with germline pathogenic mutation in the four genes, no difference is found (p=0.280).

4. Discussion

This is a detailed study involving the screening and diagnosis of families at risk for LS in Brazil. Although detection of Lynch syndrome has evolved from a clinical criterion to tumor-based universal screening, some possible barriers, such as limited access to genetic testing and financial sources, may hinder the successful implementation of LS screening programs, especially in developing countries. Our study demonstrates that clinical criteria followed by tumor screening through MSI and IHC is a feasible and effective way to select individuals for molecular genetic testing. Of the 323 probands initially selected, only 39% were considered eligible for the molecular genetic test for MMR genes. MSI and IHC showed almost equally high sensitivities. Similar results have been obtained in previous studies [27].

Although deficiency of the MMR system can be used as a predictor of LS, one should take into consideration that MMR deficiency can also result from somatic inactivation [28]. Analysis of somatic mutations in *BRAF* gene is currently the most widely used negative predictor of germline mutations in MMR genes [15]. Likewise, *MLH1* promoter methylation analysis has also been proposed as an effective strategy to discriminate LS from the sporadic form of MSI-CRC. However, some reports from the literature already called attention to the possibility that *MLH1* somatic methylation can be a second hit in some LS tumors [9,10,29]. Our data showed that pathogenic variants were found in four patients with concomitant *MLH1* hypermethylation. Thus, we report that 6% of the MMR mutated patients with LS also exhibit somatic *MLH1* methylation, probably as a second hit. So, our findings reinforce the need to revisit the worldwide flowchart for the identification of LS patients, to avoid misdiagnose a significant proportion of LS families.

MMR pathogenic/likely pathogenic variants were identified in 49% of our families. This result is similar to that reported by Rossi BM et al in LS patients from Latin America (47%), in which part of our cohort was also included[30]. In the present study, variants shared among families (recurrent mutations) were not common. Although, we identified one recurrent variant in *MLH1* (c.1276C>T), present in 13 individuals from 6 unrelated families. This variant was first described in Danish families, and previously described in one Brazilian family from Sao Paulo, Brazil [31]. We also identified a Portuguese founder mutation in *MLH1* gene, c.(1897-?_2271+?)del (also known as exon 17-19 deletion), in five unrelated Brazilian families in the present study. Additional research on a larger number of Brazilian families is necessary to determine the prevalence of such alterations, because the presence of founder mutations in specific populations could lead to a cost-effective alternative for genetic testing, mainly in underdeveloped and developing countries.

Two new frameshift variants in the *PMS2* gene and a large deletion in the *MLH1* gene were found. In the *PMS2* gene, the variant c.2188_2192del variant was identified in a patient

with colon cancer at the age of 29 years. The c.2185_2186del variant was identified in 2 individuals from the same family, one with colon cancer at the age of 69 years and the other with endometrial cancer at the age of 48. In the *MLH1* gene, the deletion of exons 16-17 (c.(1732-?_1989+?)del) was identified in a man with rectum cancer, diagnosed at 41 years of age.

Concerning the clinicopathological characteristics, we described 127 tumors in 100 individuals, from 62 unrelated families. In total, 84 colorectal tumors were observed among the mutated patients (75% in the probands and 55% in the relatives), 47% of them were *MLH1* mutation carriers. Approximately half of all CRC tumors were located in the proximal colon and moderately differentiated, similarly as reported by Haraldsdottir and collaborators in LS families [32].

Extracolonic tumors were associated with *MSH2* and *MSH6* in 63% of cases. Furthermore, only one *MSH6* mutation carrier family met the Amsterdam II criteria, corroborating the hypothesis that *MSH6* has a lower penetrance than the other MMR genes [2,12]. Among family members, endometrial cancer was the most prevalent extra-colonic tumor (82%). This high frequency is supported by the fact that Lynch syndrome accounts for approximately 2% of all endometrial cancers [33]. Prostate cancer was detected in 3 *MSH2* mutation carriers, two of them with Gleason score > 7 and diagnosed at 53 and 64 years of age respectively. Several studies have demonstrated an increased risk for prostate cancer among MMR mutation carriers, mainly associated with *MSH2* [2,34]. For breast cancer, the risk associated with mutations in the MMR genes is still controversial [2,35,36]. In the present study, only one individual with a pathogenic variant in *MLH1* developed breast cancer.

Regarding clinically suspected LS patients with MMR-proficient tumors, we identified 13 families that fulfils Amsterdam criteria, called as FCCTX families (Familial Colorectal Cancer Type X). Although FCCTX families also fulfils Amsterdam criteria, it normally presents a later age of onset, a distal location of tumors in the colon, and a lower risk of

developing extracolonic tumors [37]. Even though the genetic basis for FCCTX has been a topic of intensive research, the cause of the increased risk of cancer in these families remains unclear [38].

The main limitation of this study was that MSI/IHC accuracy, specificity, negative and positive predictive value estimates were not possible to obtain, because genetic testing was not performed on the proficient MMR cases.

5. Conclusion

The present report is a detailed study involving the screening and diagnosis of families suspected of LS in a public cancer hospital from Brazil. In addition to the broad spectrum of alterations throughout the MMR genes, we add new information about *MLH1* and *PMS2* gene variants, and identified two recurrent variant in *MLH1* gene, contributing to a better characterization of LS in Brazil. Furthermore, we call attention to the possibility of failure in the diagnosis of germline *MLH1* mutation carriers when somatic *MLH1* hypermethylation is used to rule out LS.

Author Statement

André Escremim de Paula: Conceptualization, Methodology, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Visualization. **Henrique de Campos Reis Galvão**: Conceptualization, Data Curation, Investigation, Writing - Original Draft. **Murilo Bonatelli**: Methodology, Investigation, Writing - Original Draft. **Gabriela Carvalho Fernandes**: Methodology, Investigation. **Gustavo Noriz Berardinelli**: Methodology, Investigation. **Cristina Sabato**: Methodology, Investigation. **Carlos Eduardo Mattos Andrade**: Investigation. **Maximiliano Cadamuro Neto**: Investigation. **Luis Gustavo Capochim Romagnolo**: Investigation. **Natalia Campacci**: Investigation. **Cristovam Scapulatempo-Neto**: Methodology, Investigation. **Rui Manuel Reis:** Writing - Review & Editing, Funding acquisition. **Edenir Inêz Palmero**: Conceptualization, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

Author contributions: André Escremim de Paula: Conceptualization, Methodology, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Visualization. Henrique de Campos Reis Galvão: Conceptualization, Data Curation, Investigation, Writing - Original Draft. Murilo Bonatelli: Methodology, Investigation, Writing - Original Draft. Gabriela Carvalho Fernandes: Methodology, Investigation. Gustavo Noriz Berardinelli: Methodology, Investigation. Cristina Sabato: Methodology, Investigation. Carlos Eduardo Mattos Andrade: Investigation. Maximiliano Cadamuro Neto: Investigation. Luis Gustavo Capochim Romagnolo: Investigation. Natalia Campacci: Investigation. Cristovam Scapulatempo-Neto: Methodology, Investigation. Rui Manuel Reis: Writing - Review & Editing, Funding acquisition. Edenir Inêz Palmero: Conceptualization, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Table 1: **Clinical and Histopathological features of the probands.**

	Total (n=323)	**MMR Tumor Proficient (n=182)**	**MMR Tumor Deficient †**	**p-value**
Clinical criteria‡				
Revised Bethesda	323	182 (56.3%)	141 (43.7%)	NA*
Amsterdam II	29	13 (44.8%)	16 (55.2%)	0.190
Age at diagnosis				
< 30 y	24	13 (54.2%)	11 (45.8%)	
30-39 y	52	36 (69.2%)	16 (30.8%)	0.239
40-49 y	86	47 (54.7%)	39 (45.3%)	
>= 50	161	86 (53.4%)	75 (46.6%)	
Sex				
Male	98	41 (41.8%)	57 (58.2%)	0.001
Female	225	141 (62.7%)	84 (37.3%)	
Primary Tumor§				
Colorectal	214	106 (49.5%)	108 (50.5%)	0.001
Endometrial	109	76 (69.7%)	33 (30.3%)	
CRC localization				
Proximal colon	70	10 (14.3%)	60 (85.7%)	
Distal colon	46	30 (65.3%)	16 (34.8%)	<0.001
Rectum	78	57 (73.1%)	21 (26.9%)	

Colon total or unknown	20	9 (45%)	11 (55%)	
CRC Differentiation Grade				
Well (grade I)	21	12 (57.1%)	9 (42.9%)	0.098
Moderated (grade II)	149	76 (51%)	73 (49%)	
Poor (grade III)	34	11 (32.4%)	23 (67.6%)	
Unknown	10	7 (70%)	3 (30%)	

† Microsatellite instability (MSI) and immunohistochemical (IHC) discordant cases (n=8), and MSI and/or IHC inconclusive cases (n=7) were considered in the MMR-deficient group; ‡ Overlap between Bethesda and Amsterdam criteria data; * No statistics computed because the variable was a constant; § For patients with more than one tumor, only the tumors used for MSI and IHC analysis were considered. CRC: colorectal cancer.

Table 2: Pathogenic and likely pathogenic variants (class 4 and 5) identified

GENE	PROBAND ID	Nº OF TESTED (MUTATED) RELTIVES	BT	AMII	BRAZILIAN REGION	VARIANT	ACMG	INSIGHT	CLINVAR	HGMD	PRIMARY TUMOR IN PROBAND/RELATIVES (Sex, Dx)
MLH1	141	19 (7)	Yes	No	Southeast	c.83C>T (p.Pro28Leu)	Class 4	Class 5	Class 5	DM	Colon (♂, 28) / Nonmelanoma skin (♂,51)
MLH1	839	13 (6)	Yes	No	Southeast	c.117-1G>T	Class 5	Class 4	Class 4	DM	Colon, Endometrium (♀, 44, 47)/ Colon (♂, 43), breast (♀, 45)
MLH1	968	11 (5)	Yes	Yes	Southeast	c.122A>G (p.Asp41Gly)†	Class 4	Class 5	Class 5	DM	Colon, rectum (♂,45, 65)/ Colon (♂,40)
MLH1	814	13 (12)	Yes	No	Southeast	c.306+5G>A	Class 4	Class 5	Class 5	DM	Endometrium, colon (♀, 56, 76)/ Colon (♂, 56), colon (♀, 54)
MLH1	934	3 (2)	Yes	No	Southeast	c.332C>T (p.Ala111Val)	Class 4	Class 4	Class 4	DM	Rectum (♂, 42)/
MLH1	153	5 (2)	Yes	No	Southeast	c.503delA (p.Asn168Ilefs*34)	Class 5	Class 5	ND	DM	Colon (♂, 23)/ Rectosigmoid (♀,25)
MLH1	1324 ‡	2 (1)	Yes	No	Central-west	c.677+1G>A	Class 5	Class 4	Class 4	DM	Colon (♂, 55)/

MLH1	26	5 (3)	Yes	Yes	Southeast	c.1276C>T (p.Gln426*)	Class 5	Class 5	Class 5	DM	Colon transversum, colon sigmoideum (♂, 44, 44)/ Colon (♂, 28), sebaceous (♂,59)
MLH1	110	4 (1)	Yes	No	Central-west	c.1276C>T (p.Gln426*)	Class 5	Class 5	Class 5	DM	Colon (♂, 51)/
MLH1	227	12 (7)	Yes	No	Southeast	c.1276C>T (p.Gln426*)	Class 5	Class 5	Class 5	DM	Colon (♂, 22)/ Endometrium, colon (♀, 41, 52), colon, prostate, (♂, 74, 76)
MLH1	925	15 (6)	Yes	Yes	Central-west	c.1276C>T (p.Gln426*)	Class 5	Class 5	Class 5	DM	Colon, rectum (♀, 43,43)/ Rectum (♀, 29), colon (♂, 50)
MLH1	1131	4 (0)	Yes	Yes	Southeast	c.1276C>T (p.Gln426*)	Class 5	Class 5	Class 5	DM	Colon (♂, 63)/
MLH1	1486	10 (5)	Yes	Yes	Southeast	c.1276C>T (p.Gln426*)	Class 5	Class 5	Class 5	DM	Rectosigmoid (♂, 34)/ Rectum (♀, 39)
MLH1	509	6 (0)	Yes	No	Southeast	c.1333C>T (p.Gln445*)	Class 5	Class 5	ND	DM	Rectum, colon, small bowel (♀, 32, 45, 47)/
MLH1	124	6 (0)	Yes	No	Southeast	c.1690_1693delCTCA (p.Leu564Phefs*26)	Class 5	Class 5	Class 5	DM	Colon (♂, 42)/
MLH1	135 ‡	16 (7)	Yes	No	Southeast	c.1853delAinsTTCTT (p.Lys618Ilefs*4)	Class 5	Class 5	Class 5	DM	Colon (♂, 62)/ Colon (♀, 44), colon (♂, 48), colon (♂, 48), colon (♂, 48, 48)
MLH1	553	2 (2)	Ye	No	Central-west	c.2059C>T (p.Arg687Trp)	Class 5	Class 5	Class 5	DM	Colon, renal (♂, 51,51)/

			s								
MLH1	354	5 (2)	Yes	Yes	Southeast	c.(1897-?_2271+?)del	Class 5	ND	ND	DM	Colon (♂, 40)/
MLH1	1508.1 ‡	1 (1)	Yes	No	South	c.(1897-?_2271+?)del	Class 5	ND	ND	DM	Colon (♀, 32)/ Liposarcoma (♀,59)
MLH1	623	2 (2)	Yes	No	Southeast	c.(1897-?_2271+?)del	Class 5	ND	ND	DM	Endometrium, ovarian, colon (♀,30, 31, 44)/
MLH1	906	3 (2)	Yes	No	Southeast	c.(1897-?_2271+?)del	Class 5	ND	ND	DM	Colon (♂, 67)/ Rectum (♂, 45)
MLH1	1160.5	5 (2)	Yes	Yes	Southeast	c.(1897-?_2271+?)del	Class 5	ND	ND	DM	Colon (♂, 46)/ Rectum (♀,67)
MLH1	524	19 (9)	Yes	No	Southeast	c.(117-?_207+?)del	Class 5	Class 5	Class 5	DM	Endometrium (♀,43)/ Colon (♀, 60), occult primary tumour (♀, 59)
MLH1	692	6 (2)	Yes	No	Southeast	§c(1732-?_1989+?)del	Class 5	ND	ND	ND	Rectum (♂, 41)/
MSH2	378	0 (0)	Yes	No	Southeast	c.71delA (p.Gln24Argfs*40)	Class 5	Class 5	ND	DM	Rectum (♀, 56)/
MSH2	989	2 (1)	Yes	No	North	c.96dupC (p.Thr33Hisfs*49)	Class 5	Class 5	ND	DM	Endometrium (♀, 38)/ Endometrium (♀, 40)
MSH2	18	18 (10)	Yes	Yes	Southeast	c.174dupC (p.Lys59Glnfs*23)	Class 5	Class 5	ND	DM	Colon (♀, 47)/ Endometrium (♀, 39), colon (♀, 41), bladder, colon (♀, 69,

											72)
MSH2	246	13 (7)	Yes	Yes	Southeast	c.388_389delCA (p.Gln130Valfs*2)	Class 5	Class 5	Class 5	DM	Endometrium (♀, 53)/ Colon (♀, 50)
MSH2	1253	12 (4)	Yes	No	Southeast	c.645+1_645+10delins	Class 4	Class 5	ND	DM	Rectosigmoid (♀,44)/ Renal, endometrium (♀,53,54)
MSH2	1262	8 (2)	Yes	No	Southeast	c.711_727del17 (p.Ile237Metfs*13)	Class 5	Class 5	ND	DM	Rectosigmoid (♀, 52)/
MSH2	16	0 (0)	Yes	Yes	Southeast	c.862C>T (p.Gln288*)	Class 5	Class 5	Class 5	DM	Colon (♂, 27)/
MSH2	1248	1 (0)	Yes	No	North	c.942+3A>T	Class 4	Class 5	Class 5	DM	Colon (♀, 16)/
MSH2	836.1	7 (3)	Yes	No	Central-west	c.1076+1_1076+2delGT	Class 4	Class 5	ND	DM	Colon (♀, 28)/ Prostate (♂,53)
MSH2	215	2 (0)	Yes	No	Southeast	c.1147C>T (p.Arg382*)	Class 5	Class 5	Class 5	DM	Colon, colon, endometrium (♀,23, 52, 52)/
MSH2	370	1 (1)	Yes	No	Central-west	c.1255C>T (p.Gln419*)	Class 5	Class 5	Class 5	DM	Endometrium (♀,52)/
MSH2	1654	0 (0)	Yes	No	Southeast	c.1444A>T (p.Arg482*)	Class 5	Class 5	Class 5	DM	Colon (♀,72)/
MSH2	229	1 (1)	Yes	Yes	Central-west	c.1705_1706delGA (p.Glu569Ilefs*2)	Class 5	Class 5	Class 5	DM	Colon (♂, 41)/

MSH2	473	3 (1)	Yes	No	Central-west	c.1738G>T (p.Glu580*)	Class 5	Class 5	Class 5	DM	Colon, rectum (♀, 54, 54)/
MSH2	323	6 (2)	Yes	No	Central-west	c.1786_1788delAAT (p.Asn596del)	Class 5	Class 5	Class 5	DM	Colon, Gastric, endometrium (♀, 55, 62, 62)/ Colon (♀, 32)
MSH2	339	21 (9)	Yes	No	Southeast	c.1861C>T (p.Arg621*)	Class 5	Class 5	Class 5	DM	Endometrium, rectum (♀, 55, 62)/ Endometrium (♀, 38)
MSH2	1721	0 (0)	Yes	No	Southeast	c.1935delA (p.Asp646Metfs*39)	Class 5	Class 5	ND	DM	Endometrium, rectum (♀, 35, 36)/
MSH2	1411	16 (7)	Yes	No	Southeast	c.2089T>C (p.Cys697Arg)	Class 5	Class 5	Class 5	DM	Colon (♀, 38)/
MSH2	310	2 (2)	Yes	No	Central-west	c.2131C>T (p.Arg711*)	Class 5	Class 5	Class 5	DM	Rectum (♀, 21)/ Colon (♂, 47)
MSH2	1959	0 (0)	Yes	No	Southeast	c.2152C>T (p.Gln718*)	Class 5	Class 5	Class 5	DM	Endometrium (♀,46)/
MSH2	40	0 (0)	Yes	Yes	Southeast	c.2525_2526delAG (p.Glu842Valfs*3)	Class 5	Class 5	Class 5	DM	Colon (♂, 24)/
MSH2	243	15 (6)	Yes	No	Southeast	c.(646-?_1076+?)del	Class 5	Class 5	Class 5	DM	Rectum (♂, 51)/
MSH2	871	0 (0)	Yes	No	Southeast	c.(1077-?_1276+?)del	Class 5	Class 5	Class 5	DM	Colon, prostate (♂, 64, 64)/
MSH6	688	12 (4)	Yes	No	Southeast	c.23_26delACAG (p.Tyr8Serfs*8)	Class 4	Class 5	ND	DM	Colon (♀, 63)/

			s								
MSH6	149	6 (4)	Yes	No	Southeast	c.1133_1134delGA (p.Arg378Lysfs*3)	Class 4	Class 5	ND	DM	Rectum (♀, 46)/ Small bowel (♀, 44), colon, lung, bladder (♀, 73, 73 74)
MSH6	897	11 (8)	Yes	No	Southeast	c.1519dupA (p.Arg507Lysfs*9)	Class 5	Class 5	Class 5	DM	Endometrium (♀, 48)/ Endometrium (♀, 45)
MSH6	1339	1 (0)	Yes	No	South	c.1519dupA (p.Arg507Lysfs*9)	Class 5	Class 5	Class 5	DM	Colon (♀, 49)/
MSH6	1793	1 (0)	Yes	No	Southeast	c.1610_1613delAGTA (p.Lys537Ilefs*33)	Class 5	Class 5	ND	DM	Endometrium (♀,49)/
MSH6	302	6 (2)	Yes	No	Southeast	c.1913delinsAGA (p.Leu638Glnfs*11)	Class 5	Class 5	ND	DM	Endometrium (♀,56)/ Endometrium (♀, 52)
MSH6	1094	0 (0)	Yes	No	Southeast	c.2194C>T (p.Arg732*)	Class 5	Class 5	Class 5	DM	Colon (♀, 63)/
MSH6	196	8 (5)	Yes	No	Southeast	c.2332_2335dupCTTT (p.Cys779Serfs*7)	Class 4	Class 5	ND	DM	Endometrium, colon, (♀, 47, 48)/
MSH6	1032	2 (0)	Yes	No	Southeast	c.2659delC (p.Lys888Serfs*18)	Class 5	Class 5	ND	DM	Endometrium, colon (♀, 48, 53)/
MSH6	1049	1 (0)	Yes	Yes	Southeast	c.2983G>T (p.Glu995*)	Class 5	Class 5	Class 5	DM	Colon (♂, 34)/
MSH6	163	6 (2)	Yes	No	Southeast	c.3632T>C (p.Leu1211Pro)	Class 4	Class 5	Class 5	DM	Rectum (♀, 65)/ Rectosigmoid (♀, 70)

PMS2	607	0 (0)	Yes	No	Southeast	c.1A>G (p.Met1?)	Class 5	Class 4	Class 4	DM	Endometrium (♀, 57)/
PMS2	891	10 (2)	Yes	No	Southeast	[$]c.2185_2186delCT (p.Leu729Gnlfs*6)	Class 5	ND	ND	ND	Colon (♀, 32)/
PMS2	590 ‡	8 (6)	Yes	No	Southeast	[$]c.2185_2186delCT (p.Leu729Gnlfs*6)	Class 5	ND	ND	ND	Colon (♂, 68)/ Endometrium (♀, 48)
PMS2	875	0 (0)	Yes	No	Southeast	[$]c.2188_2192delAACTT (p.Leu731Cysfs*3)	Class 5	ND	ND	ND	Colon (♀, 29)/

BT= Bethesda criteria; AM II= Amsterdam II criteria; DX= diagnosis age; ACMG= American College of Medical Genetics and Genomics; InSIGHT= International Society for Gastrointestinal Hereditary Tumours; HGMD= Human Gene Mutation Database; ND= not described, DM= Disease causing mutation. † Aberrant splicing resulting in deletion of exon 2 (p.Cys39*) [25,26]; ‡ Patients with concurrent somatic *MLH1* methylation and pathogenic germline variant; [$] Novel predisposition disease variant identified.

Table 3: Colorectal cancer (CRC) and extra-colonic tumors diagnosed in MMR mutated probands and relatives

	Mean Age (range)	Tumors (n=127)	*MLH1* (n=59)	*MSH2* (n=43)	*MSH6* (n=20)	*PMS2* (n=5)	p value
Mean Age (range)	48 (16-76)	127	45 (16-76)	49 (21-74)	52.70 (25-73)	52.80 (32-68)	0.158
CRC	47 (16-76)	84	40 (47.2%)	28 (33.3%)	14 (16.7%)	2 (2.4%)	
Location							
Proximal colon	47 (16-76)	36	18 (50%)	11 (30.6%)	5 (13.9%)	2 (5.6%)	0.781
Distal colon	48 (24-74)	14	8 (57.1%)	4 (28.6%)	2 (14.3%)	0 (0%)	
Rectum	47 (21-70)	21	10 (47.6%)	8 (38.1%)	3 (14.3%)	0 (0%)	
Unknown	44 (28-60)	13	9 (69.2%)	3 (23.1%)	0 (0%)	1 (7.7%)	
Differentiation							
Well (grade I)	30 (25-36)	5	3 (60%)	1 (20%)	0 (0)	1 (20%)	0.300
Moderate (grade II)	47 (16-76)	42	19 (45.2%)	17 (40.5%)	4 (9.5%)	2 (4.8%)	
Poor (grade III)	44 (22-64)	9	5 (55.6%)	2 (22.2%)	2 (22.2%)	0 (0%)	
Unknown	50 (21-74)	28	18 (64.3%)	6 (21.4%)	4 (14.3%)	0 (0%)	
EXTRA-COLONIC TUMOR	51 (30-76)	43	14 (32.6%)	17 (39.5%)	10 (23.3%)	2 (4.7%)	
Endometrium	48 (30-62)	26	5 (19.2%)	12 (46.2%)	7 (26.9%)	2 (7.7%)	0.917
Bladder	72 (69-74)	2	0 (0)	1 (50%)	1 (50%)	0 (0)	
Breast	45	1	1 (100%)	0 (0)	0 (0)	0 (0)	
Gastric	62	1	0 (0)	1 (100%)	0 (0)	0 (0)	
Liposarcoma	59	1	1 (100%)	0 (0)	0 (0)	0 (0)	

Lung	73	1	0 (0)	0 (0)	1 (100%)	0 (0)
Occult primary tumor	59	1	1 (100%)	0 (0)	0 (0)	0 (0)
Ovarian	31	1	1 (100%)	0 (0)	0 (0)	0 (0)
Prostate	64 (53-76)	3	1 (33.3%)	2 (66.6%)	0 (0)	0 (0)
Renal	52 (51-53)	2	1 (50%)	1 (50%)	0 (0)	0 (0)
Sebaceous	59	1	1 (50%)	0 (0)	0 (0)	0 (0)
Nonmelanoma skin	51	1	1 (50%)	0 (0)	0 (0)	0 (0)
Small bowel	46 (44-47)	2	1 (50%)	0 (0)	1 (50%)	0 (0)

Two or more tumors were presented in 22 patients and are reported on this table. Tumors not classically related to Lynch Syndrome were reported by family members harboring pathogenic/likely pathogenic variant.

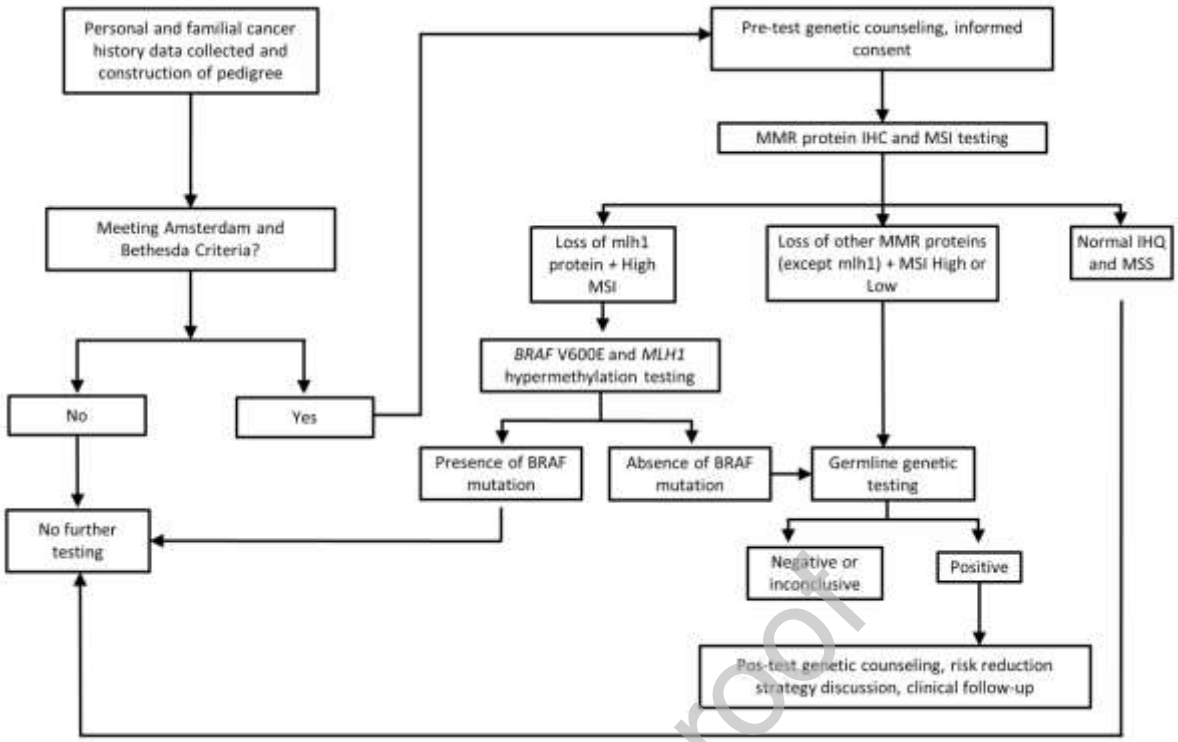

Figure 1: Algorithm illustrating the strategy used for the identification and genetic testing of patients at risk for Lynch syndrome (LS).

MMR= mismatch repair; IHC= immunohistochemical; MSI= microsatellite instability; MSS= stable microsatellite.

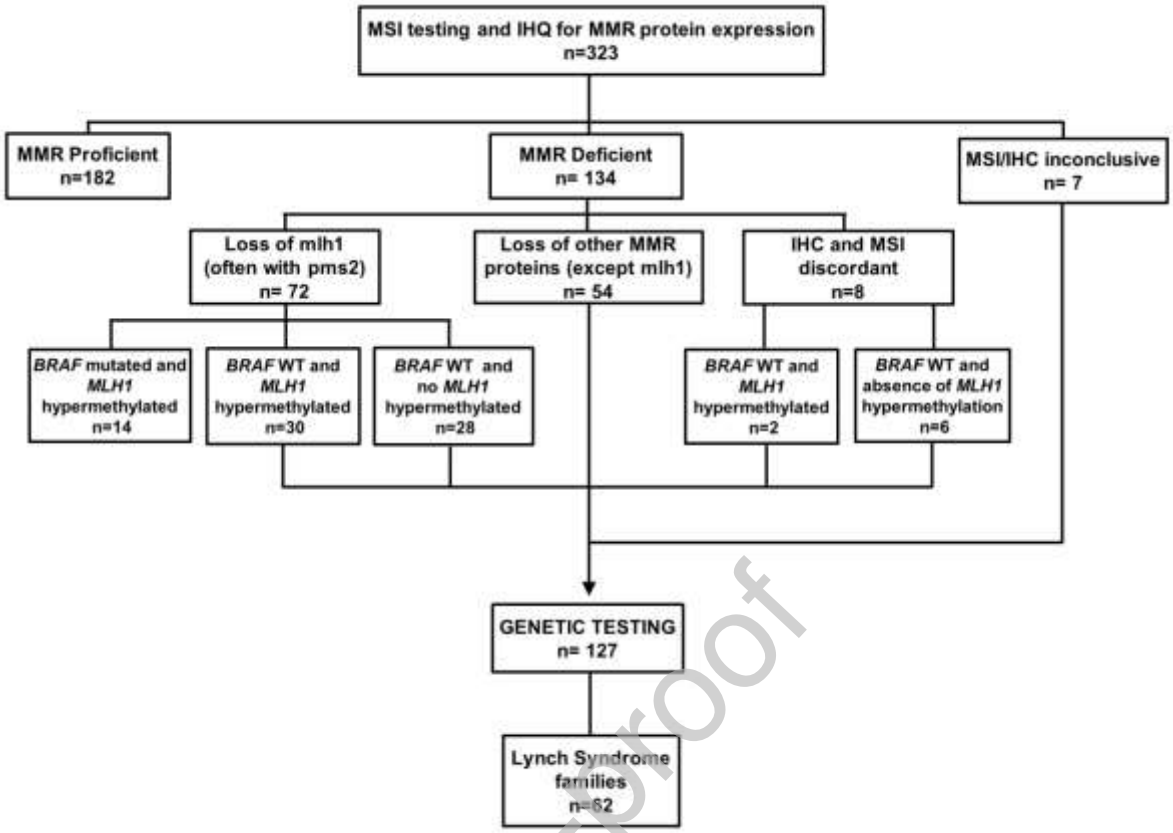

Figure 2: Summary of LS diagnostic strategy.