UNIVERSITY OF SÃO PAULO
RIBEIRÃO PRETO MEDICAL SCHOOL

Reginaldo Cruz Alves Rosa

Genetic etiology of DNA-mismatch repair deficiency in cancer

Ribeirão Preto
2022

REGINALDO CRUZ ALVES ROSA

Genetic etiology of DNA-mismatch repair deficiency in cancer

Original version

Doctoral thesis presented to the Graduate Program in Biological Sciences (Genetics) at the Ribeirão Preto Medical School, University of São Paulo to obtain a doctoral degree (Ph. D.) in Science.

Concentration area: Genetics

Advisor: Prof. Dr. Victor Evangelista de Faria Ferraz
Co-advisor: Prof. Dr. Wilson Araújo da Silva Júnior

Ribeirão Preto

2022

I authorize the reproduction and total or partial dissemination of this study, through conventional or electronic means, for academic and research purposes, conditioned to the appropriate citation.

Autorizo a reprodução e divulgação total ou parcial deste trabalho, por qualquer meio convencional ou eletrônico, para fins de estudo e pesquisa, desde que citada a fonte.

Rosa, Reginaldo Cruz Alves

Etiologia genética da deficiência do sistema de reparo de pareamento incorreto de DNA em câncer

“Genetic etiology of DNA-mismatch repair deficiency in cancer”

Ribeirão Preto, 2022.
208 f;

Tese apresentada à Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, para obtenção do título de Doutor em Ciências. Área de concentração: Genética.

Orientador: Prof. Dr. Victor Evangelista de Faria Ferraz
Coorientador: Prof. Dr. Wilson Araújo da Silva Júnior

1. Câncer de endométrio. 2. CRISPR/Cas9. 3. Instabilidade de microssatélites. 4. Reparo de DNA. 5. Sequenciamento de nova geração.

ROSA, R. C. A. **Genetic etiology of DNA-mismatch repair deficiency in cancer**. Thesis (Ph.D. in Biological Sciences - Genetics) – Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, 2022.

Approved on: December 14, 2022

Thesis Committee

President: Prof. Dr. Victor Evangelista de Faria Ferraz

Institution: Ribeirão Preto Medical School – FMRP-USP

Signature: ______________________________

Prof. Dr: ______________________________

Institution: ______________________________

Judgment: ______________________________

Signature: ______________________________

Prof. Dr. ______________________________

Institution: ______________________________

Judgment: ______________________________

Signature: ______________________________

Prof. Dr. ______________________________

Institution: ______________________________

Judgment: ______________________________

Signature: ______________________________

FUNDING

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code: 001.

Reginaldo C. A. Rosa received a scholarship from the Capes/PrInt-USP Program (process number: 88887.574953/2020-00) to finance part of his internship at the Institut Gustave Roussy – France.

This thesis would not have been possible without the financial support from the Conselho Nacional de Desenvolvimento Científico e Tecnológico – Brasil (CNPq). Process number: 130281/2016-6 (scholarship); Process number: 2017/04283-8 (Universal grant).

This study was also financed in part by the Fundação de Amparo à Pesquisa do Estado de São Paulo – Brasil (FAPESP). Process number: 2017/04283-8 (Regular grant).

ACKNOWLEDGMENTS

First and utmost, I would like to thank my supervisors, Dr. Victor Ferraz and Dr. Patricia Kannouche, whose constant support was essential for developing this project. I don´t have enough words to express my gratitude for all the guidance, advice, and motivation I received from them during those years. Thank you for believing in my potential and stimulating me to become a creative, responsible, and independent scientist.

I want to express my gratitude to the UMR9019 members at IGR for having accepted me as one of yours and for sharing the resources, protocols, and knowledge needed for the conclusion of part of this project.

A huge thank you to the TLS team, especially Caro, Pierre, Jordane, Basile, and Vianney. I am grateful for all the good moments shared with you in France, from the discussions of countless western blot results to the excellent and strong drinks at the 48 Cocktail Club in Montparnasse.

I wish to express my gratitude to Prof. Dr. Wilson Araújo for welcoming me into his lab, which allowed me to meet incredible scientists who also became great friends, particularly Isabela, Simone, Ádamo, Danielle, Camila, and Greice.

A huge thank you to the fantastic friends I made in France, especially to the “mismatches” (Laeti and Louise), the “drama team” (Jordane, Pierre, and Maeva/Nana), Gabriela, and to Umit. I cannot thank you enough for making my experience in France way easier and very pleasing. Thank you for the unforgettable memories in France, including the soirées, raclettes, crêpes, happy hours, festivals, and all the other great moments I will never forget. I can´t wait to see you all in Brazil for my Ph.D. defense.

Thank you to the professors of the Department of Genetics at the University of São Paulo for contributing to my training as a geneticist.

I cannot forget to thank the great friends I met in Ribeirão Preto, particularly Romário, Hugo, Maritza, and Gabriela. Thank you for sharing with me all the challenging, frustrating, and happy moments reserved for those who are brave enough to live a Ph.D. experience.

I extend a huge thank you to all who contributed directly or indirectly to this Ph.D. project. I am deeply honored to have been surrounded by such kind and wise people during those five intense years.

Finally, I thank the thesis committee for kindly agreeing to read and review this thesis. I have no doubts that I will receive valuable contributions to the improvement of this study.

ABSTRACT

ROSA, R. C. A. **Genetic etiology of DNA-mismatch repair deficiency in cancer**. 2022. Thesis (Ph.D. in Biological Sciences - Genetics) – Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, 2022.

DNA mismatch repair (MMR) is a highly conserved pathway that maintains genomic integrity by repairing base-base mismatches and insertion-deletion loops generated during DNA replication. MMR deficiency is detected in a substantial fraction of tumors, especially endometrial cancer (EC), and is used as an indicator of cancer predisposition and a marker of resistance to certain chemotherapies, such as 6-thioguanine (6-TG). Germline and somatic inactivation of *MLH1*, *MSH2*, *MSH6*, and *PMS2* genes, which encode the main components of the MMR pathway, is the leading cause of MMR deficiency. However, some MMR-deficient tumors do not harbor any alteration in MMR genes, suggesting that other genes could also drive the MMR-deficient (MMR-D) phenotype in cancer. To investigate the genetic etiologies of MMR deficiency, we established a Brazilian cohort of 242 EC cases and assessed the MMR status on tumors by immunohistochemistry, microsatellite instability, and *MLH1*-methylation. MMR deficiency was detected in 38.4% of tumors, and germline mutation in the main MMR genes was investigated in 37 MMR-D cases. We found germline pathogenic variants in 10/37 (27%) patients. Next, we explored the etiology of the 27 unexplained MMR-D tumors by germline and somatic next-generation sequencing of 63 genes related to cancer-predisposition and DNA repair. Germline variants in *ATM*, *ATR*, *CHEK2*, *FAN1* and *MUTYH* genes were found in 26% of cases and were associated with a pronounced family history of cancer. Tumor sequencing revealed inactivating mutations in MMR genes, mainly in *MSH6,* as the leading cause of MMR deficiency in EC. Mutations in the exonuclease domain of *POLE* were found to be a frequent driver of MMR deficiency, probably by increasing mutation rates, resulting in the inactivation of MMR genes. Previous studies have identified WDHD1, an essential component of the eukaryotic replisome, as an MSH2 partner. Therefore, we constructed a *Wdhd1*-mutant cell line by CRISPR/Cas9 and interrogated the impact of the disrupted WDHD1-MSH2 interaction on the repair of replication errors and sensitivity to 6-TG. Disruption of WDHD1-MSH2 interaction did not increase the number of spontaneous mutations and did not lead to an MSI phenotype. On the other hand, *Wdhd1*-mutant cells acquired mild resistance to 6-TG. In conclusion, we have confirmed the inactivation of MMR genes as the main cause of MMR deficiency in EC. Additionally, germline mutations in other DNA repair genes are found in individuals with MMR-D tumors and may explain the high cancer incidence in their relatives. Finally, *WDHD1* is not an alternative driver of MMR deficiency but might participate in the MMR-mediated response to 6-TG.

Keywords: CRISPR/Cas9. DNA repair. Endometrial cancer. Microsatellite instability. Next-generation sequencing.

RESUMO

ROSA, R. C. A. **Etiologia genética da deficiência do sistema de reparo de pareamento incorreto de DNA em câncer**. 2022. Tese (Doutorado em Ciências Biológicas - Genética) – Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, 2022.

O reparo de pareamento incorreto de bases (MMR) é um mecanismo de reparo de DNA altamente conservado que mantém a integridade genômica através do reparo de bases malpareadas e de alças geradoras de inserções e deleções que ocorrem durante a replicação de DNA. Uma parcela considerável de tumores, sobretudo câncer de endométrio (CE), apresentam deficiência na via MMR (MMR-D), que é utilizada como um indicador de predisposição genética à câncer e como preditor de resistência à certos quimioterápicos, tais como 6-tioguanina (6-TG). A inativação germinativa ou somática dos genes *MLH1*, *MSH2*, *MSH6,* ou *PMS2*, que são os principais genes da via MMR, é a causa mais frequente da MMR-D. Contudo, alguns tumores com deficiência nessa via não apresentam nenhuma alteração nesses genes, o que sugere o envolvimento de outros genes na deficiência do sistema MMR em câncer. Com o objetivo de investigar as causas genéticas dessa deficiência, uma coorte brasileira composta por 242 casos de CE foi caracterizada por meio de imuno-histoquímica, instabilidade de microssatélites e metilação do gene *MLH1*. 38,4% dos tumores apresentaram deficiência na via MMR. A análise de variantes germinativa foi realizada em 37 destes casos. Foram encontradas variantes germinativas patogênicas em 10/37 (27%) pacientes. A etiologia dos 27 tumores MMR-D, sem uma causa determinada pela abordagem anterior, foi investigada por meio de sequenciamento de nova geração para análise de variantes germinativas e somáticas em 63 genes relacionados à predisposição à câncer e a vias de reparo de DNA. Variantes germinativas nos genes *ATM*, *ATR*, *CHEK2*, *FAN1* e *MUTYH* foram encontradas em 26% dos casos, muitos destes associados à uma história familiar de câncer. Por meio do sequenciamento de DNA tumoral, foi possível confirmar a inativação de genes da via MMR, principalmente do gene *MSH6*, como a principal causa do fenótipo de MMR-D em CE. Ainda, os dados de sequenciamento dos tumores mostraram que mutações no domínio com atividade de exonuclease codificado pelo gene *POLE* são uma causa frequente desse fenótipo, provavelmente advindo do aumento do número de mutações com consequente inativação de genes da via MMR. Estudos anteriores identificaram a proteína WDHD1, que é um componente essencial do replissoma em eucariotos, como um parceiro de interação da proteína MSH2. Deste modo, geramos um modelo celular contendo mutações no gene *Wdhd1* por CRISPR/Cas9 para avaliar o impacto da ruptura na interação entre WDHD1 e MSH2 no reparo de erros de replicação e na sensibilidade à 6-TG. A ausência de interação entre WDHD1 e MSH2 não gerou um aumento no número de mutações espontâneas e não desencadeou instabilidade de microssatélites no nosso modelo celular. Contudo, as células mutantes apresentaram resistência moderada à 6-TG. Este trabalho confirmou a inativação de genes MMR como a principal causa da deficiência da via MMR em CE. Mutações germinativas em genes de outras vias de reparo de DNA podem ser encontradas em indivíduos com tumores MMR-D, e podem explicar a alta incidência de câncer nas famílias desses pacientes. Por fim, o gene *WDHD1* não é uma causa alternativa para o fenótipo de deficiência da via MMR, mas pode estar envolvido na resposta celular à 6-TG mediada por essa via.

Palavras-chave: Câncer de endométrio. CRISPR/Cas9. Instabilidade de microssatélites. Reparo de DNA. Sequenciamento de nova geração.

LIST OF FIGURES

INTRODUCTION

Figure 1	Mismatch repair pathway in *E. coli*…………………………………….…..	18
Figure 2	Mismatch repair pathway in eukaryotes…………………………………….	20
Figure 3	Proposed models to explain the mechanisms of MMR-mediated cell cycle arrest and apoptosis under exposure to DNA-damaging agents. ……………	22
Figure 4	Immunohistochemistry staining of MMR proteins in a tissue sample of an endometrial carcinoma case……………………………………………...	25
Figure 5	Capillary electrophoresis result of an MSI-High endometrial carcinoma…...	27
Figure 6	Single-base substitution mutational signatures associated with Mismatch repair deficiency in human cancers………………………………………...	30

CHAPTER I

Figure 1	Flowchart summarizing the result of the strategy used for detection of Lynch syndrome………………………………………………………….	102
Figure 2	Molecular and clinical findings of the LS patient carrying a CNV in the *MLH1* gene…………………………………………………………….....	105

CHAPTER II

Figure 1	Oncoprint of germline variants identified in 27 cases of Lynch-like syndrome…………………………………………………………………	128
Figure 2	Oncoplot of somatic driver variants found in 20 Lynch-like tumors………	131
Figure 3	Relative contribution of COSMIC SBS mutational signatures to the mutational profile of somatic single-nucleotide substitutions observed in LLS tumors………………………………………………………………	136
Figure S1	Lollipop plot of somatic variants identified in four tumors with both POLE-exo* and *MSH6* mutations…………………………………………	135
Figure S2	96 trinucleotide mutational profile of 10 LLS tumors with a minimum of 20 single-nucleotide substitutions……………………………………......	137
Figure S3	Replicative strand bias………………………………………………...	138

CHAPTER III

Figure 1	Identification of *WDHD1* mutations in Unexplained MSI tumors from the TCGA dataset…………………………….…………………………....	159
Figure 2	Validation of WDHD1-MSH2 interaction……………………………….	161
Figure 3	Validation of the minimal MSH2-binding site in WDHD1 protein……..…..	163
Figure 4	Accumulation of spontaneous mutations and microsatellite instability in cells lacking WDHD1-MSH2 protein interaction…………………….….	165
Figure 5	Effect of 6-thioguanine on the surviving fraction of *Wdhd1*-mutant cells…..	166

Figure 6	Cell cycle analysis of cells treated with 6-thioguanine……………………	167
Figure S1	Microsatellite unstable tumors among 33 tumor types from the TCGA dataset………………………………………………………………	160
Figure S2	Validation of WDHD1-MSH2 interaction by immunoprecipitation of the GFP-MSH2 protein…………………………………………………………	162
Figure S3	Characterization of *Wdh1*-mutant clones…………………………………..	164

LIST OF TABLES

INTRODUCTION

Table 1 - Clinical criteria originally defined for LS diagnosis in CRC………………. 45

Table 2 - Clinical criteria elaborated by the American Society of Gynecologic Oncology for the detection of individuals at risk for hereditary endometrial, colorectal, and other LS-associated cancers…………..…………………… 46

Table 3 - Simplified SGO clinical criteria for detection of individuals diagnosed with endometrial cancer at risk of having LS……………………………….... 46

CHAPTER I

Table 1 - Clinical and pathological characteristics of 242 Brazilian cases of endometrial cancer…………………………………………………………….. 101

Table 2 - Germline variants identified among 37 patients diagnosed with unmethylated dMMR endometrial tumors………………………………….. 104

Table 3 - Comparison between clinicopathologic variables and tumor MMR status and germline findings………………………………………………………. 107

CHAPTER II

Table 1 - Clinicopathologic features of 27 Lynch-like patients according to the presence of pathogenic germline variants…………………………………... 127

Table 2 - Germline variants, including pathogenic, likely pathogenic, and variants of uncertain significance, identified in the cohort of LLS patients……………. 130

Table S1 Molecular findings of the endometrial cohort of Lynch-syndrome patients.. 133

LIST OF ABBREVIATIONS

5-FU	5-fluorouracil
6-TG	6-thioguanine
ATP	Adenosine triphosphate
CpG	Cytosine-guanine dinucleotide
CRC	Colorectal cancer
CMMRD	Constitutional mismatch repair deficiency syndrome
CNV	Copy-number variation
CTLA-4	Cytotoxic T lymphocyte Antigen 4
DDR	DNA damage response
DNA	Deoxyribonucleic acid
EC	Endometrial cancer
ExoI	Exonuclease I
FDA	Food and Drug Administration
FIGO	Federation of Gynecology and Obstetrics
FFPE	Formalin-fixed paraffin-embedded
FMRP	Ribeirão Preto Medical School
GPV	Germline pathogenic variant
HC	General Hospital
ICIs	Immune checkpoint inhibitors
IDL	Insertion-deletion loop
IHC	Immunohistochemistry
LOH	Loss of heterozygosity
LS	Lynch syndrome
LLS	Lynch-like syndrome
MGMT	O^6-Methylguanine-DNA Methyltransferase
MLPA	Multiplex ligation-dependent probe amplification
MMR	Mismatch repair
MMR-D	Mismatch repair-deficient
MMR-P	Mismatch repair-proficient
MNNG	N-methyl-N -nitro-N-nitrosoguanidine
MSI	Microsatellite instability
MSI-H	Microsatellite instability high
MSI-L	Microsatellite instability low
MSS	Microsatellite stability
MutLα	MutL homolog alpha
MutLγ	MutL homolog gamma
MutSα	MutS homolog alpha
MutSβ	MutS homolog beta
NGS	Next-generation sequencing
PARP	Poly (ADP)-ribose polymerase
PCNA	Proliferating cell nuclear antigen

PCR	Polymerase chain reaction
PD-1	Programmed Cell Death
PD-L1	PD-1 ligand
Pol III	DNA polymerase 3
Polα	DNA polymerase alpha
Polδ	DNA polymerase delta
Polε	DNA polymerase epsilon
POLE-exo*	Mutations in the exonuclease domain of Polε
PPAP	Polymerase proofreading-associated polyposis
RFC	Replication factor C
RNA	Ribonucleic acid
RPA	Replication protein A
SBS	Single-base substitutions
SSB	Single-strand DNA-binding proteins
ssDNA	Single-strand DNA
SNV	Single-nucleotide variation
TCGA	The Cancer Genome Atlas project
TMA	Tissue microarray
TMB	Tumor mutation burden
TMZ	Temozolomide
USP	University of São Paulo
UTR	Untranslated region
VAF	Variant allele frequency
VUS	Variant of uncertain significance
WDHD1	WD Repeat And HMG-Box DNA Binding Protein 1

SUMMARY

INTRODUCTION 17

1 Mismatch repair pathway 17
1.1 DNA damage signaling function 20
2 Mismatch repair deficiency 22
2.1 Detection 23
2.1.1 Immunohistochemistry 23
2.1.2 PCR-based MSI 25
2.1.3 NGS-based MSI 28
2.1.4 Mutational signatures 29
2.2 Frequency in human tumors 31
2.3 Clinical implications 32
2.3.1 Prognosis in cancer patients 32
2.3.2 Resistance to chemotherapy 33
2.3.3 Response to immunotherapy 34
2.3.4 Screening of cancer predisposition syndromes 35
3 Etiology of Mismatch repair deficiency in cancer 36
3.1 *MLH1* methylation 37
3.2 Constitutional Mismatch Repair Deficiency 38
3.3 Lynch Syndrome 39
3.3.1 History 40
3.3.2 Clinical phenotype 41
3.3.3 Etiology 42
3.3.4 Screening approaches 43
3.3.4.1 Universal screening of tumors 47
3.3.5 Molecular diagnosis 48
3.4 Lynch-like Syndrome 49
3.4.1 Clinical phenotype 49
3.4.2 Etiology 50
3.4.2.1 False-positive tumor screening 51
3.4.2.2 MicroRNAs 52
3.4.2.3 Somatic inactivation of MMR genes 52
3.4.2.4 Missed cases of Lynch syndrome 54
3.4.3.5 Non-MMR genes 55
4 Relevance of the study 58

REFERENCES 60

OBJECTIVES 91

CHAPTER I: Lynch syndrome identification in a Brazilian cohort of endometrial cancer screened by a universal approach 92

CHAPTER II: Mutational landscape of Lynch-like syndrome in endometrial cancer 120

CHAPTER III: Uncovering the role of WDHD1 in the DNA mismatch repair pathway 150

CONCLUSION 180

SCIENTIFIC PRODUCTIONS ACHIEVED DURING THE PH.D. 182

Articles published in scientific journals 182
Oral and poster presentation 183
Scholarships 184

APPENDIX..185

Case report of germline and somatic POLE-exo mutations in an EC case 185

ANNEX 208

Ethics committee approval 208

INTRODUCTION

DNA replication in eukaryotic cells requires three DNA polymerases: Polymerase alpha (Polα), delta (Polδ), and epsilon (Polε). Polα acts as a primase by synthesizing short RNA primers required to initiate replication at the replication origins. After synthesizing a few nucleotides, Polα is replaced by Polε in the leading strand and Pol δ in the lagging strand to synthesize DNA in a high-processivity and fidelity manner (SHCHERBAKOVA; BEBENEK; KUNKEL, 2003).

Replicative polymerases are highly accurate machines with proofreading capabilities. Pol α is the most error-prone of the three polymerases. However, it synthesizes the least amount of DNA, and most of the bases it incorporates are removed during the later replication steps. Pol δ has intermediate fidelity, and the leading strand polymerase, Pol ε, has the highest fidelity in replicating DNA (CORTEZ, 2019).

Despite the high fidelity, replicative polymerases insert the incorrect nucleotide approximately every 10^4-10^5 polymerization events (KUNKEL; ERIE, 2015). The distorted geometry of these misincorporations triggers the exonuclease activity inherent in the replicative polymerases to excise the incorrect base, lowering the error rate by 10 to 100-fold. Mismatches that escape this proofreading activity are substrates for the mismatch repair (MMR) system, which functions as a spellchecker to lower the error rate to as low as $2x10^{-10}$ substitutions per base per cell division (KUNKEL; ERIE, 2015).

1 Mismatch repair pathway

MMR is a highly conserved DNA repair mechanism that plays a critical role in DNA fidelity, mutation avoidance, and genome stability (LIU; KEIJZERS; RASMUSSEN, 2017). This essential repair pathway was first discovered in *Escherichia coli,* and its main steps were further reconstituted *in vitro* from purified proteins. The prokaryotic methyl-directed MMR pathway comprises three main stages: recognition, excision, and gap-filling by DNA resynthesis, which requires several components, including the homodimers MutS and MutL, as well as the restriction endonuclease MutH, the helicase II UvrD, Exo I, and other single-strand specific exonucleases, single-strand DNA-binding proteins (SSB), the DNA polymerase III (Pol III) holoenzyme, and the DNA ligase (LU, 2021) (**Figure 1**).

The single-nucleotide mismatches and short insertion-deletion loops (IDLs) generated during replication are recognized by the MutS homodimer, which further recruits MutL and

MutH, forming a complex that translocates through the DNA duplex in an ATP-dependent manner until it reaches a hemimethylated d(GATC) sequence (LIU; KEIJZERS; RASMUSSEN, 2017). The absence of methylation at this restriction site is used as a signal to direct the repair to the newly synthesized strand. MutH nicks the unmethylated strand at the 5' position of the guanine at the GATC site to introduce an entry point for the excision reaction mediated by UvrD and exonuclease enzymes (FUKUI, 2010). The single-strand DNA generated during the excision step is stabilized by SSBs. Finally, the gap produced during the excision of the daughter DNA strand is filled in by DNA polymerase III, and the nick is ligated by DNA ligase (GROOTHUIZEN; SIXMA, 2016).

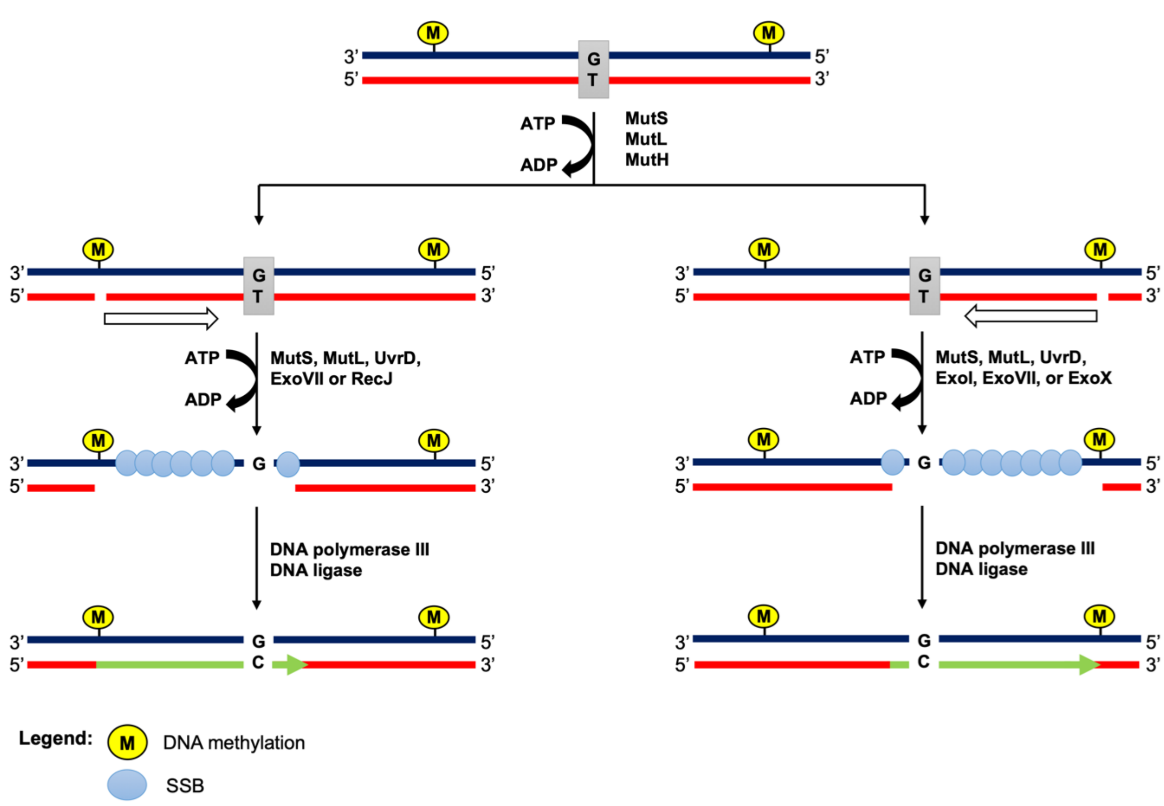


Figure 1 – Mismatch repair pathway in *E. coli*. The first step involves the recognition of the mismatch (represented by the G-T mismatch) by the MutS homodimer. Next, MutL and MutH are recruited to the mismatch site, forming a complex with MutS. The MutS-MutL-MutH complex translocates in an ATP-dependent manner to a hemimethylated GATC restriction site located either upstream or downstream of the mismatch site. MutH cleaves the newly synthesized strand (in red) at the 5' side of the guanine of the restriction site. MutS and MutL hydrolyze ATP to trigger downstream repair steps, comprising resection of the unmethylated DNA strand by helicases (UvrD) and exonucleases (ExoI, ExoVII, ExoX, or RecJ, depending on the position of the strand nick relative to the mismatch site). SSB proteins protect single-strand DNA resulting from this process. The last step comprises the resynthesis of DNA to fill the gap generated during the resection step by DNA polymerase III and the nick sealing by DNA ligase. Open arrows represent the direction of resection from the GATC sequence to the mismatch site, while thick green arrows show the direction of resynthesis. Adapted from LU, 2021.

The MMR pathway in eukaryotes is less well-understood than in prokaryotes. However, its three main steps are conserved across all species, with some differences (LIU; KEIJZERS; RASMUSSEN, 2017) (**Figure 2**). In eukaryotes, the MMR pathway is initiated by the recognition of mismatches by the heterodimer MutS homolog alpha (MutSα), composed of MSH2 and MSH6 proteins, or MutS homolog beta (MutSβ), formed by MSH2 and MSH3. MutS recognizes preferentially base-base mismatches and short IDLs, whereas MutSβ is responsible for recognizing mainly more prominent IDLS. A second heterodimer, the MutL homolog alpha (MutLα), formed by MLH1 and PMS2 proteins, is recruited to the mismatch site and, together with MutSα or MutSβ, mediates the excision of the newly synthesized strand to remove the replication error. Differently from bacteria, the strand discrimination in eukaryotes MMR does not rely on the hemimethylated state of parental and daughter strands during DNA replications. Instead, it has been proposed that strand discrimination in eukaryotes depends upon the occurrence of daughter-strand nicks generated during the DNA replication (PUTNAM, 2021).

Eukaryotic MMR faces a temporal problem of detecting DNA mismatches and initiating repair before chromatin is assembled. To overcome this challenge, it has been suggested that MutSα/β heterodimers interact directly with the DNA replication machinery by interacting with MSH6 or MSH2 proteins and the replisome processivity clamp, proliferating cell nuclear antigen (PCNA) (MANHART, 2021). Since eukaryotes lack a MutH homolog, the nick on the daughter strand required for the excision of the newly synthesized strand is introduced by the latent endonuclease activity of MutLα, in a reaction that also requires PCNA, the replication factor C (RFC), and ATP (FUKUI, 2010). Nicks generated by MutLα are used as initiation points for excision by the 5’-3’exonuclease I (ExoI), creating single-strand gaps as large as a thousand nucleotides, which are protected by replication protein A (RPA) (MANHART, 2021). Replicative DNA polymerases fulfill those gaps and DNA ligase I, repairing the mismatches previously identified in the daughter strand (GENG et al., 2011).

Other MMR proteins have been identified, including PMS1, which can interact with MLH1 to form the MutLβ heterodimer, and MLH3, which interacts with MLH1, forming the MutL homolog gamma (MutLγ) dimer. Both heterodimers have a minor role in mitotic MMR repair. However, MutLγ is required to promote crossing over in meiosis. In addition, the MMR dimer formed by MSH4 and MSH5 has been identified as vital for meiosis but not involved with the mitotic MMR repair (MANHART; ALANI, 2016).

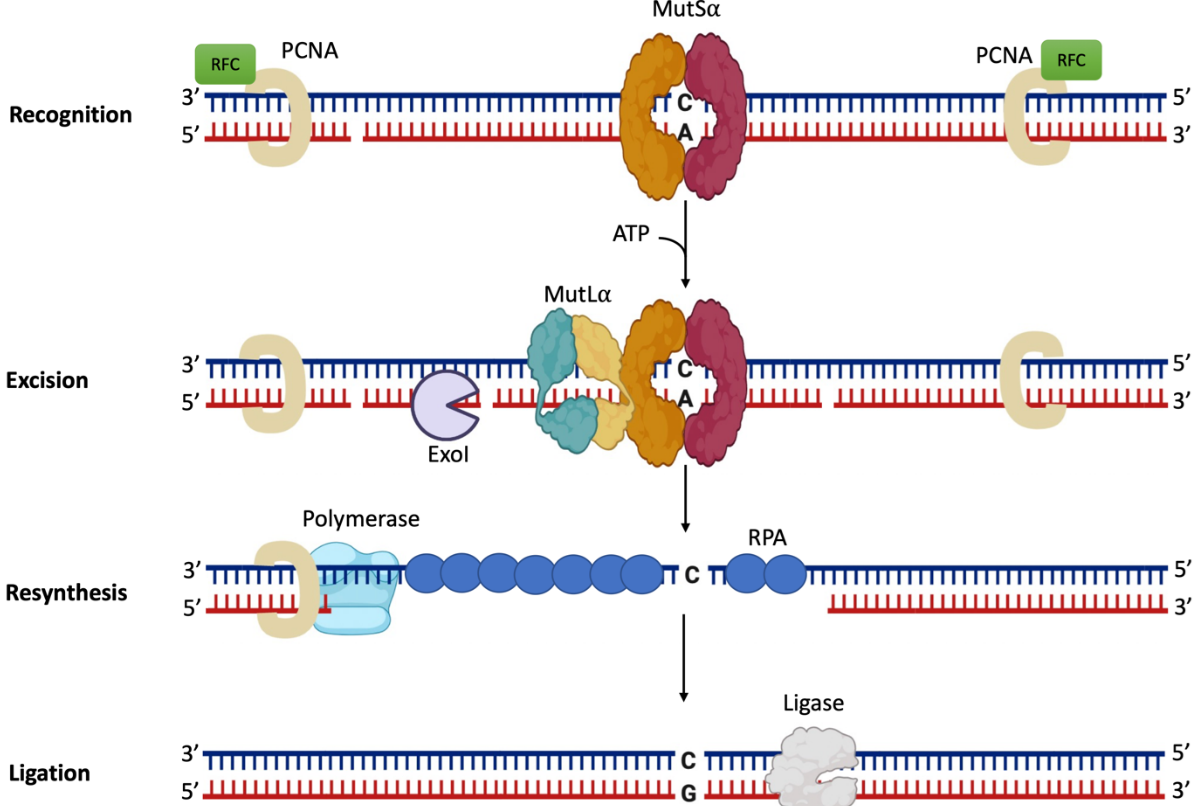


Figure 2 – Mismatch repair pathway in eukaryotes. The pathway starts with the recognition of a replication mismatch (represented by a C-A mispairing) by either the MutSα or MutSβ heterodimers. The newly synthesized strand (red) is distinguished from the parental strand (blue) based on the existence of nicks on the daughter strand generated during DNA replication. The MutS complex recruits MutLα and activates its latent endonuclease activity to introduce a nick in the daughter strand in an ATP-PCNA-RFC-dependent manner. ExoI uses the incision generated by MutL to excise the newly synthesized strand containing the mismatch. RPA proteins stabilize the resulting single-strand strand. The last step comprised the error-free resynthesis of DNA by replicative polymerases and the ligation of gaps by DNA ligase. Adapted from YANG; HSIEH, 2016.

1.1 DNA damage signaling function

In addition to its well-described role in the repair of mismatches generated during DNA replication, the MMR pathway is involved in the cellular response to DNA lesions produced by a variety of genotoxic agents, including cisplatin, carboplatin, 5-fluorouracil, 6-thioguanine (6-TG), N-methyl-N -nitro-N-nitrosoguanidine (MNNG), and iododeoxyuridine (MARTIN et al., 2010). Once the MMR proteins recognize the drug-induced DNA damage, this repair machinery can trigger different responses, including repair of the damage, cell cycle arrest, and apoptosis (LI; PEARLMAN; HSIEH, 2016; NARINE et al., 2010).

Although the mechanism by which MMR triggers cellular responses under exposure to DNA damage agents is not entirely understood, it is assumed to be initiated similarly to that of

replication-derived mismatches (GUPTA; HEINEN, 2019). It has been shown that MutSα and MutLα heterodimers interact with ATR and ATM, which are essential components of different DNA damage response (DDR) pathways, activating those proteins and resulting in phosphorylation of p53, p73, and other downstream DDR components (XU; LI, 2021).

Two models have been proposed to describe the role of MMR proteins in DDR, the futile cycling and the direct signaling models (**Figure 3**). The futile cycling model suggests that once the mismatch generated by drug-induced DNA adduct in the template DNA strand is recognized, the MMR pathway undergoes repetitive futile attempts of repair, causing secondary DNA damages, mainly single-strand DNA (ssDNA) gaps in the newly synthesized DNA strand and, ultimately, accumulating extensive double-strand breaks (DSBs), which provokes G2/M cell cycle arrest and subsequent apoptosis (GUPTA; HEINEN, 2019; MARTIN et al., 2010). On the other hand, The direct signaling model suggests that upon recognizing a mismatch generated by a drug-induced adduct, MutSα and MutLα complexes could directly trigger cell cycle arrest and apoptosis via the recruitment of DDR components (LI; PEARLMAN; HSIEH, 2016). In both models, the action of MMR components on DNA adducts activates a DDR signaling cascade mediated by ATM and/or ATR, resulting in cell death. However, the detailed mechanism of these models needs to be better understood (XU; LI, 2021).

Not surprisingly, the loss of functional MMR components, most frequently MLH1, PMS2, MSH2, and MSH6 proteins, results in cellular tolerance to certain DNA-damaging agents (LI; PEARLMAN; HSIEH, 2016). Since many of those agents are commonly used in chemotherapy for different tumors, the assessment of MMR functional status has direct clinical implications, mainly in chemo-resistance, as recurring drug-resistant tumors are frequently MMR-deficient (MMR-D) (GUPTA; HEINEN, 2019). On the other hand, restoring the MMR activity in MMR-D tumors might be explored to reverse resistance and turn those tumors sensitive to chemotherapy (MENG; DAI; GUO, 2008; PLUMB et al., 2000; STRATHDEE et al., 1999).

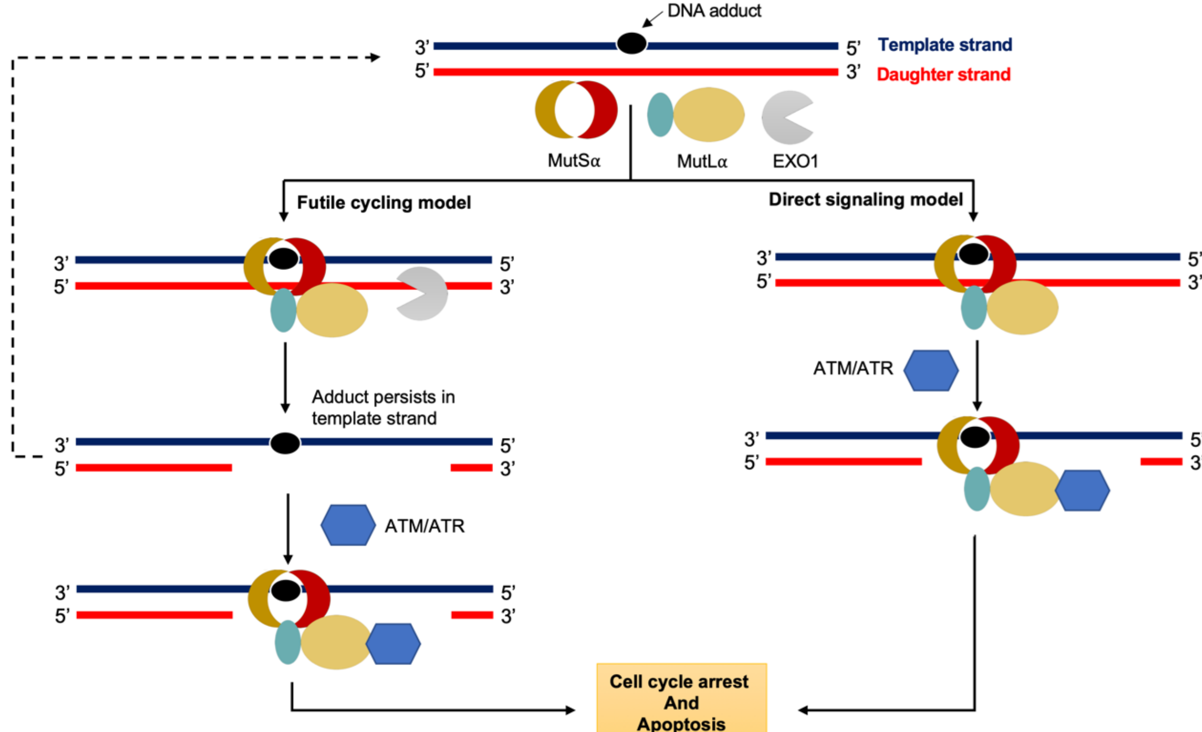


Figure 3 – Proposed models to explain the mechanisms of MMR-mediated cell cycle arrest and apoptosis under exposure to DNA-damaging agents. The futile cycling model (on the left) suggests that DNA adduct (solid black circle) on the template strand induces mismatches that MMR proteins can recognize. Since MMR only targets the daughter strand, the adduct cannot be repaired, provoking repetitive cycles of MMR and ultimately triggering cell cycle arrest and apoptosis via the ATM and/or ATR damage signaling. The direct signaling model (on the right) proposes that MMR proteins recruit ATM/ATR immediately after recognizing the DNA adduct, activating the downstream DNA damage response and resulting in cell cycle arrest and apoptosis. Adapted from HEWISH et al., 2010.

2 Mismatch repair deficiency

Loss of functions of one or more MMR proteins impairs the repair of mismatches and loops generated during DNA replication, leading to the accumulation of spontaneous mutations throughout the genome and affecting the function of several genes, including tumor suppressor and pro-oncogene genes, which increases the likelihood of initiating a malignant transformation (BATEMAN, 2021). Indeed, MMR deficiency is observed in various cancers, which exhibit high mutation rates, 100-1,000-fold greater than MMR-proficient tumors (BARETTI; LE, 2018).

The high mutation rates commonly observed in MMR-D tumors are mainly represented by mutations in microsatellite regions, which are short tandem repeated DNA sequences distributed throughout the genome (POULOGIANNIS; FRAYLING; ARENDS, 2010). Microsatellites are highly susceptible to replication errors caused by slippage of polymerases

during replication of those repetitive sequences, resulting in the accumulating insertions and deletions in several microsatellite loci, a molecular phenotype known as microsatellite instability (PAL; PERMUTH-WEY; SELLERS, 2008). Several proto-oncogenes and tumor suppressor genes, such as *TGFBR2*, *IGF2R*, *PTEN*, *BAX*, *MRE11*, *RAD50*, and various DNA repair genes, including *MSH6*, *MSH3*, and *MLH3*, contain microsatellites in their coding sequences and, consequently, are highly prone to be mutated in MMR-D cells, which could drive the tumorigenic process in those cells (GUILLOTIN; MARTIN, 2014; HEWISH et al., 2010).

The identification of tumors with MMR deficiency has several clinical implications, ranging from identifying individuals potentially at risk of having a cancer-predisposing syndrome to the prediction of outcome and response to anti-cancer therapies. Therefore, the different methodologies to identify MMR deficiency, as well as the prevalence of this phenotype in human tumors, and the details of its diverse clinical implications will be discussed in the following sections.

2.1 Detection

Improvements in molecular techniques have facilitated the routine assessment of MMR status in different tumor types as part of the medical care (HEWISH et al., 2010a). In clinical practice, MMR deficiency can be detected at a genetic, protein, or functional level. The presence of MMR proteins in the nuclei of tumor cells is commonly assessed by immunohistochemistry (IHC) (BATEMAN, 2021). In addition, the tumor mutator phenotype is traditionally measured through polymerase chain reaction amplification (PCR)-based microsatellite instability (MSI) analysis from tumor DNA (TOGNETTO et al., 2017).

Several centers worldwide take advantage of next-generation sequencing (NGS) technology to detect MMR deficiency and other clinically relevant biomarkers in different tumor types in single testing, saving time and tumor tissue (XIAO et al., 2021). Importantly, NGS-based approaches have high concordance rates with the traditional IHC and PCR-based MSI techniques (KANG et al., 2022; SHIMOZAKI et al., 2021).

The technical aspects, benefits, and limitations of MMR IHC, PCR-based MSI, and current NGS-based approaches to detect MMR deficiency in human tumors will be discussed in the following sections.

2.1.1 Immunohistochemistry

The analysis of MMR protein expression by IHC has become a widely used approach to detect MMR deficiency in solid tumors. The functionality of the MMR pathway can be inferred by using antibodies against MLH1, MSH2, MSH6, and PMS2 proteins. In addition to detecting MMR-D tumors, lack of expression of one or more of those proteins also indicates what MMR gene is most likely defective due to the protein heterodimerism between MLH1/PMS2 and MSH2/MSH6 proteins (BATEMAN, 2021; VILAR; GRUBER, 2010). For instance, the simultaneous loss of MLH1 and PMS2 protein expression suggests an alteration in the *MLH1* gene since the PMS2 protein is destabilized in the absence of MLH1. Similarly, loss of both MSH2 and MSH6 protein expression indicates the inactivation of *MSH2,* as MSH6 is unstable in the absence of the MSH2 protein. On the other hand, isolated loss of MSH6 or PMS2 protein expression suggests the existence of an alteration in *MSH6* or *PMS2* genes, respectively (LYNCH et al., 2015).

Some studies have argued that the analysis of PMS2 and MSH6 over the IHC panel comprising the four MMR proteins would be a more cost-effective approach to detecting MMR deficiency in tumors usually harboring this phenotype, including colorectal cancer (CRC), endometrial cancer (EC), and skin cancer (HALL et al., 2010; MOJTAHED et al., 2011). However, several centers use the panel composed of the four MMR proteins to assess MMR deficiency in solid tumors since the overall cost of this later approach is still relatively low. Additionally, the use of four IHC markers can be helpful in cases where the expression of one or more proteins cannot be assessed due to technical issues (BATEMAN, 2021).

Most antibodies used for IHC analysis of MMR proteins usually result in stable and consistent nuclear staining patterns with retained or lost staining (JOOST et al., 2014; RAFFONE et al., 2020). However, some difficulties can be encountered in the interpretation of MMR IHC analysis, which may include weak protein staining throughout the tumor and in stromal and inflammatory cells (commonly used as internal controls); absent MMR protein staining in both tumor and internal control cells; patchy loss of MMR protein staining in the tumor, and cytoplasmic MMR protein expression within tumor cells (BATEMAN, 2021). Additionally, a small portion of tumors may exhibit heterogeneous loss of MMR protein expression, which renders the interpretation of results even more challenging (MCCARTHY et al., 2019). Even though some of those issues might have an underlying biological reason, most cases are related to technical artifacts related to preanalytical variables, such as the fixation and storage of samples, and problems occurring during the IHC protocol (ENGEL; MOORE, 2011). Thus, internal controls are mandatory to accurately assess MMR deficiency in tumor tissue (**Figure 4**).

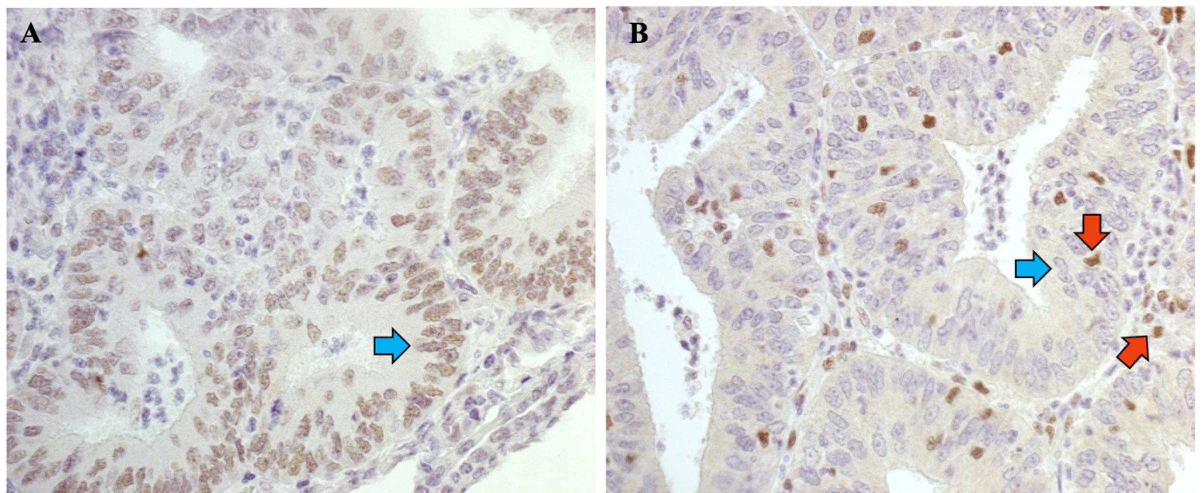


Figure 4 - Immunohistochemistry staining of MMR proteins in a tissue sample of an endometrial carcinoma case. The positive staining of MMR proteins is confirmed by the presence of brown staining in the nuclei of tumoral cells, while blue nuclei indicate loss of MMR protein expression. (A) Positive nuclear staining of MLH1 protein in tumoral cells. (B) Negative staining of MSH6 protein in tumoral cells with retained MSH6 expression in infiltrating inflammatory cells (internal control). The Blue arrow shows the tumoral nuclei, while the red arrows indicate inflammatory cells. Original magnification 200x. Source: Author's repository.

The detection of MMR deficiency by IHC is highly concordant with the PCR-based MSI analysis, achieving more than 90% of concordance in both endometrial and colorectal tumors (LOUGHREY et al., 2021; MCCONECHY et al., 2015). Therefore, those methods are considered to be complementary, with tumors harboring loss of at least one MMR protein by IHC being referred to as MMR-D and being commonly found also to harbor an MSI phenotype, and tumors with a retained expression of the four MMR proteins are classified as MMR proficient (MMR-P) and are commonly found to be microsatellite stable (MSS) (KAWAKAMI; ZAANAN; SINICROPE, 2015). The combination of PCR-based MSI and IHC increases the specificity and sensitivity of MMR deficiency detection. However, the costs of this combined approach are also higher (COHEN; PRITCHARD; JARVIK, 2019). For this reason, most centers choose between one of these two techniques.

2.1.2 PCR-based MSI

MSI analysis is an alternative technique for diagnosing MMR deficiency in solid tumors and consists of comparing the length of a panel of microsatellite loci by PCR amplification from both normal and tumor DNA to detect somatic changes (MCCONECHY et al., 2015). In contrast to IHC, the analysis of MMR deficiency by MSI is not able to indicate which MMR gene is most likely inactivated. However, the MSI technique can detect MMR-D tumors

harboring mutations in MMR genes that affect their function but do not compromise the expression of their proteins and, rarely, in tumors whose MMR deficiency might be driven by alterations in non-MMR genes (BATEMAN, 2021).

The first well-established test of MSI in solid tumors was proposed in 1998 during an international meeting organized by the American Institute of Cancer Research, comprising a panel of five microsatellite markers composed of three dinucleotide (D2S123, D17S250, and D5S346) and two mononucleotide (BAT25 and BAT26) markers, to detect MSI in CRC. This set of microsatellites is known as the Bethesda panel (MCCONECHY et al., 2015). A few years later, an alternative MSI assay composed of a multiplexed fluorescent PCR analysis in a panel of five mononucleotide microsatellites was described as a rapid, accurate, and high-throughput screening method for MSI detection in different tumor types (SURAWEERA et al., 2002) (**Figure 5**). Panels composed of mononucleotide markers were found to be more sensitive to detecting MSI than those with dinucleotide microsatellite loci, particularly in EC and other extracolonic tumors, where a considerable fraction of MMR-D cases are related to defects in the *MSH6* gene (BUHARD et al., 2004; WONG et al., 2006). In addition, due to the quasimonomorphic nature of this mononucleotide panel, the analysis of MSI would be performed in tumors without the need for matching normal DNA (BUHARD et al., 2006). Currently, a commercial MSI testing based on a panel of five mononucleotide markers is widely used to detect MSI in different tumor samples (BARETTI; LE, 2018).

The instability is usually determined by comparing the length of nucleotide repeats of a microsatellite locus between tumor and normal DNA, which can be obtained from adjacent normal tissue, blood, or mucosa. The PCR-amplified microsatellites were initially analyzed in denaturing acrylamide gels and radiolabeled primers. Currently, most centers use fluorescent primers and capillary electrophoresis to perform the MSI testing (VILAR; GRUBER, 2010). In the absence of matching normal DNA, a quasi-monomorphic variation range calculated from non-tumoral DNA in a population-representative sample of healthy individuals is used as a reference for detecting somatic variation in microsatellite length (CAMPANELLA et al., 2014).

Tumors are classified as MSI-High (MSI-H) when length alterations are observed in at least two of the five markers or more than 30% of loci when a larger panel is used, whereas tumors with alteration in a single marker or 10 to 30% of markers for a larger panel of microsatellites are classified as MSH-Low (MSI-L). Tumors with no alterations in microsatellite markers are considered microsatellite stable (MSS) (MCCONECHY et al., 2015; VILAR; GRUBER, 2010). Some authors group MSI-L and MSS tumors together based on studies showing no difference in the prognosis of patients with MSS and MSI-L tumors

(HEWISH et al., 2010a). However, this classification is controversial since the biological defect underlying the MSI-L phenotype is not well understood, and some studies have associated the MSI-L phenotype with a poor prognosis in comparison with MSS tumors (KOHONEN-CORISH et al., 2005; LEE et al., 2015; NAZEMALHOSSEINI MOJARAD et al., 2016).

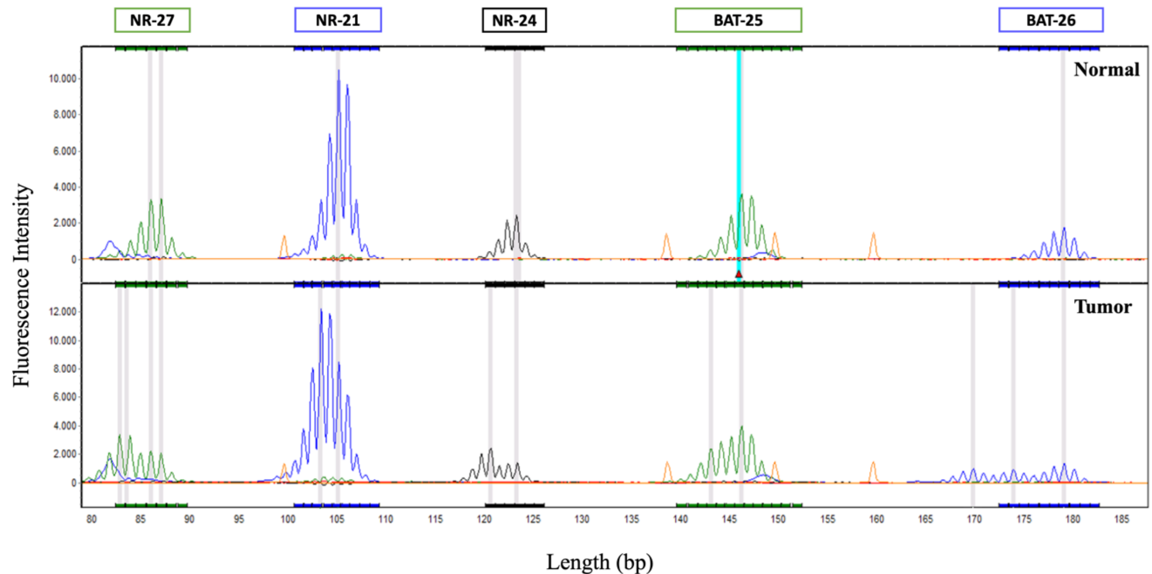


Figure 5 – Capillary electrophoresis result of an MSI-High endometrial carcinoma. Electropherograms of the fluorescent amplification of five mononucleotide microsatellites (NR-27, NR-21, NR-24, BAT-25, and BAT-26) from normal and tumor DNA from an endometrioid adenocarcinoma. Normal DNA was obtained from adjacent normal tissue. All five markers have somatic instability, as observed by the appearance of new alleles in tumor DNA compared to the normal counterpart (alleles for each microsatellite loci are shown as horizontal gray bars). Standard fragments used for sizing fragment lengths are labeled in orange. Source: Author's repository.

PCR-based MSI is considered a non-expensive and highly accurate approach to detecting MMR-D tumors, but it contains some limitations. First, it requires skilled analysts to accurately interpret the variation in fragment length observed in different tumors. This interpretation is especially challenging in endometrial tumors where the shifts in microsatellite lengths are more discrete, usually comprising changes in a single nucleotide, compared with CRC, which may generate high rates of false-negative results (WANG et al., 2017). Additionally, the sensibility to detect MMR deficiency depends on the panel used for MSI analysis and the tumor type under investigation. A large proportion of MSH6-deficient cases, overrepresented in endometrial tumors, are classified as MSS when the MSI analysis is assessed by the Bethesda panel since the MutSα dimer (composed of MSH2 and MSH6 proteins) is involved, mainly with the repair of base-base mismatches and single-nucleotide insertion-deletion loops, therefore, not repairing more extensive alterations, such as those that may

accumulate in dinucleotide microsatellite sequences in MSI tumors (BARETTI; LE, 2018; HEWISH et al., 2010a).

2.1.3 NGS-based MSI

With the crescent use of NGS technologies in oncology, detecting MSI from various tumor sequencing data is becoming a standard clinical practice. NGS-based techniques can detect MSI tumors with high sensibility and specificity rates, similar to those achieved by combining traditional IHC and PCR-based methods, but with the advantage of evaluating MSI from hundreds of microsatellite loci in addition to several other genomic variations in a single assay, and requiring minimal amounts of tumor samples (DEDEURWAERDERE et al., 2021; LI et al., 2020a; ZHENG et al., 2020).

Different NGS panels are commercially available for the detection of MSI, along with hotspot mutations and structural variations in a pre-defined set of genes with therapeutic and prognostic value, including ColoSeq (PRITCHARD et al., 2012), MSIPlus (HEMPELMANN et al., 2015), and the MSK-IMPACT (CHENG et al., 2015). Alternatively, the genomic landscape of MSI can be detected from whole-exome sequencing (WES) and whole-genome sequencing (WGS) data (CORTES-CIRIANO et al., 2017).

Several computational methods for MSI detection from NGS data have been developed, with MSIsensor and MANTIS being some of the most broadly used in a variety of tumor types. The MSIsensor is a C++ software with the potential to detect microsatellite changes from WES data by comparing the length distribution of microsatellites between paired normal and tumor samples. Later, this software was improved to support the detection of MSI in tumor samples without the need for a normal DNA (JIA et al., 2020). MSIsensor is incorporated in the pipeline of MSI detection using the MSK-IMPACT, the NGS-based tumor profiling panel approved by the American Food and Drug Administration (FDA) to select cancer cases most likely to benefit from immune checkpoint inhibitors (RATNER, 2018). More recently, the detection of MSI from circulating cell-free DNA was included in the MSIsensor family (HAN et al., 2021). Alternatively, MANTIS (Microsatellite Analysis for Normal Tumor InStability) can also detect MSI from normal-tumor paired WES and WGS in different cancer types, with high sensibility and specificity in comparison with other methods, including MSIsensor (KAUTTO et al., 2016).

In addition to detecting MSI from DNA, some authors have also described the identification of MSI gene expression signatures extracted from RNA sequencing (RNAseq)

data (DANAHER et al., 2019; LI; FENG; WANG, 2020; PAČÍNKOVÁ; POPOVICI, 2019). Recently, those expression signatures were experimentally validated in a study comprising more than a thousand colorectal, endometrial, gastric, uterine carcinosarcoma, and esophageal cancer samples (SOROKIN et al., 2021). However, the clinical application of those MSI signatures remains to be explored.

2.1.4 Mutational signatures

Different mutational processes generate mutations with a specific preference for sequence contexts, resulting in characteristic patterns of somatic mutations termed as mutational signatures (STEELE; PILLAY; ALEXANDROV, 2022). Those mutational signatures are defined by the relative frequency of six possible base substitutions (C > A, C > G, C > T, T > A, T > C, and T > G) and their sequence context, defined as the immediate 5' and 3'bases (MEIER et al., 2018). In addition to single-base substitutions (SBS), mutational signatures can also be defined from dinucleotide substitutions, small indels, and structural variations (BRADY; GOUT; ZHANG, 2022).

Mutational signatures are extracted from a catalog of somatic mutations, usually found in cancer samples, by mathematical algorithms that were first introduced in 2013 by Alexandrov *et al*. By the time, the authors extracted 21 single-base substitutions (SBS) mutational signatures from 30 cancer types, and most of them were addressed to an etiology (ALEXANDROV et al., 2013). This repertoire was recently updated, and a total of 67 SBS signatures are available in the Catalog of Somatic Mutations in Cancer (COSMIC), 49 of them with a potential biological origin (ALEXANDROV et al., 2020; TATE et al., 2019).

Defects in DNA repair pathways have been associated with the etiology of different mutational signatures (VOLKOVA et al., 2020). Seven distinct SBS mutational signatures have been associated with deficiency of the MMR pathway so far (**Figure 6**). Some MMR-D signatures are characterized by an enrichment of C>T substitutions (SBS6 and SBS15), while others have a high frequency of T>C mutations (SBS21 and SBS26). Additionally, the mutational signature SBS44 is characterized by high amounts of C>A, C>T, and T>C substitutions (FANG et al., 2021). The signatures SBS14 and SBS20 are linked to combined MMR deficiency and mutations in the exonuclease domain of *POLE* and *POLD1* exonuclease mutations, respectively, which encode the proofreading and polymerase domains of the eukaryotic replicative polymerases (HARADHVALA et al., 2018).

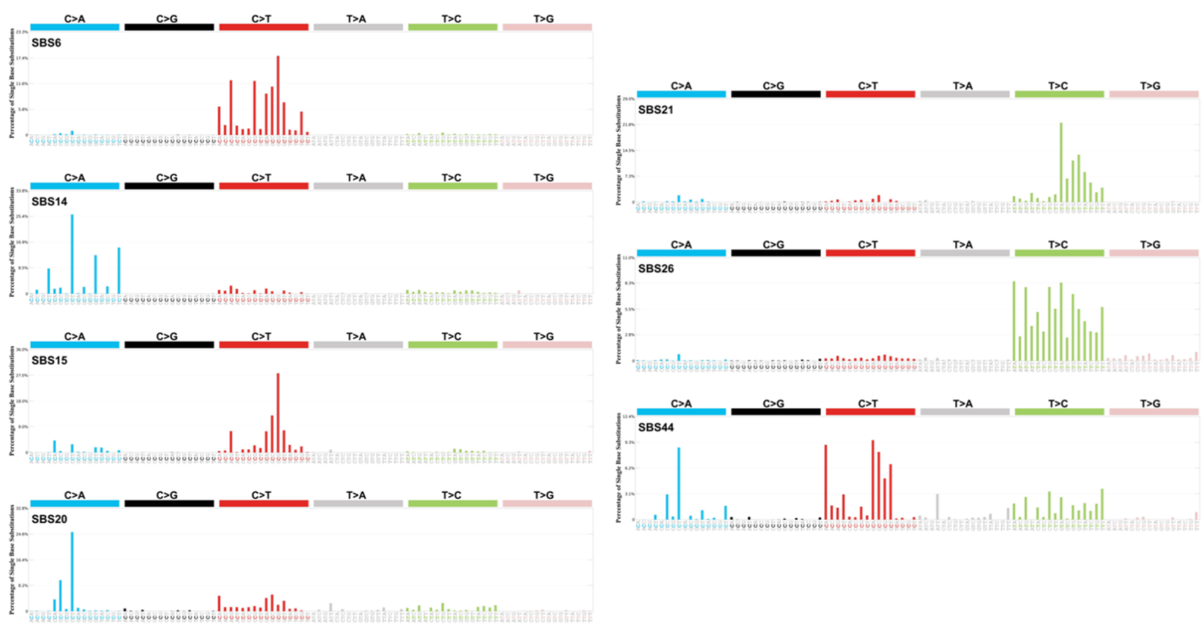


Figure 6 – Single-base substitution mutational signatures associated with Mismatch repair deficiency in human cancers. Source: COSMIC and ALEXANDROV et al., 2020.

The fact that defects in the MMR pathway, a highly conserved process in different species, may result in seven different mutational signatures remains to be elucidated. The various methods for mutational signature extraction and the complexity of datasets used to extract them may partially explain this curious fact (NÉMETH et al., 2020). However, some biological mechanisms have already been described as potential drivers of some of those mutational signatures, as described for signatures SBS14 and SBS20 (HARADHVALA et al., 2018). Additionally, a recent study conducted by Fang *et al*. demonstrated that most C>T mutations in CpG sites present in some MMR-D SBS signatures are significantly enriched in MSH2 and MSH6-deficient tumors and are linked to replication-independent repair of 5-methyl cytosine (5mC) deamination-induced mismatches mediated by those MMR proteins (FANG et al., 2021).

In addition to identifying cancer etiologies, analysis of mutational signatures can also bring some therapeutic and prognostic insights (BRADY; GOUT; ZHANG, 2022). Since mutational signatures provide genomic evidence of DNA repair deficiency, regardless of the nature of the causative event, this approach may be more advantageous than the analysis of candidate genes since some driver events, such as epigenetic alterations, could be missed by genotyping approaches (MEIER et al., 2018). Some examples of the therapeutic value of mutational signatures in cancer are the association between mutational signatures related to homologous deficiency and sensitivity to poly(ADP)-ribose polymerase (PARP) inhibitors, as

well as the association between APOBEC-related mutational signatures and favorable response to ATR inhibitors (BRADY; GOUT; ZHANG, 2022). In the same way, mutational signatures associated with the MMR-D phenotype could be explored as markers to predict response to therapeutic approaches targeting MSI/MMR-D cancers.

2.2 Frequency in human tumors

The MMR-D phenotype has been detected in different cancer types, including colorectal, endometrial, gastric, and ovarian tumors, with the prevalence ranging from 8 to 30% of cases, depending on the tumor type and methodology used to detect this phenotype (VILAR; GRUBER, 2010).

A meta-analysis conducted in 12,633 cases of EC detected an MMR-D phenotype in 27% of tumors based on PCR-based MSI, similar to the prevalence reported in studies that used IHC to screen tumors for MMR deficiency (RYAN et al., 2019). The proportion of MMR deficiency was also estimated in CRC by a recent meta-analysis comprising 28,580 cases of colorectal carcinomas, and approximately 10% of CRC tumors were found to be MMR-D based on IHC analysis (EIKENBOOM et al., 2022).

Bailey *et al.* accessed the MSI status in 9,423 tumors comprising the 33 cancer types present in The Cancer Genome Atlas project (TCGA) using the MSIsensor tool and found an MSI-high phenotype in 3.6% of the cohort in 19 tumor types. Endometrial carcinomas were those with the most significant prevalence of MSI-high phenotype, observed in 28% of cases. Additionally, 19% of stomach carcinomas and 15% of colorectal tumors were found to be MSI-high (BAILEY et al., 2018). Similar results have been reported in a large study comprising 11,139 tumors from 39 different types, where the prevalence of MSI was accessed from tumor-normal pairs of whole-exome sequencing data using MANTIS. The authors detected MSI in 3.8% of cases, distributed along 27 of the 39 tumor types in their analysis. Endometrial, colon, and stomach carcinomas were the tumor types with the greatest frequencies of MSI, with this phenotype being observed in 31.7%, 19.72%, and 19.09% of cases, respectively (BONNEVILLE et al., 2017).

Those results indicate that EC is the tumor type harboring the greatest proportion of MSI cases, followed by CRC and stomach cancer. The clinical implications of MSI detection on those tumors will be discussed in the next section.

2.3 Clinical implications

The MMR-D phenotype is associated with three main implications: prediction of prognosis for CRC patients, prediction of response and resistance to anti-cancer therapies, and screening for identification of individuals at risk of having a cancer-predisposing syndrome (BATEMAN, 2021; VILAR; GRUBER, 2010). Those clinical implications will be further explored in this section.

2.3.1 Prognosis in cancer patients

The potential of MSI as a predictive marker of prognosis has been extensively studied in CRC and, to a lesser extent, in EC. Many authors have reported an association between a good prognosis and the MSI phenotype in patients diagnosed with stage I and stage II CRC regarding a 5-year survival rate, disease recurrence, and deterioration rate. However, this association was not observed in patients with stage III and IV MSI CRC (LI et al., 2020a; WANG et al., 2019). A reduced occurrence of metastasis was also observed in MSI-high CRC compared with its MSS/MSI-Low counterpart (TORSHIZI ESFAHANI et al., 2019; VILAR; GRUBER, 2010). Additionally, MSI-high CRC patients with recurrence disease were associated with more local recurrent metastasis and fewer lung and liver metastatic tumors than patients diagnosed with MSI-L/MSS CRC tumors (KIM et al., 2016a). The association between a good prognosis in MSI CRC is maintained after adjustment for clinicopathological factors that influence prognoses, such as high lymph node harvest and poor tumor differentiation (KANG et al., 2018).

Unlike CRC, the MSI phenotype is not associated with a good prognosis in EC. Several studies have reported a greater prevalence of high tumor grade, deep myometrial invasion, presence of angiolymphatic invasion, and higher clinical stage in sporadic endometrial carcinomas with the MSI phenotype than in MSS EC (AN et al., 2007; KANOPIENE et al., 2014; MACKAY et al., 2010; MCMEEKIN et al., 2016). Despite being associated with worse prognostic markers, many studies have not found a significant difference in survival in patients diagnosed with MSI endometrial carcinomas in comparison with those with MSS EC (ALEXANDROV et al., 2013; ARABI et al., 2009; EVRARD; ALEXANDRE, 2021; KANOPIENĖ et al., 2014; ZIGHELBOIM et al., 2007). The MSI phenotype might counteract the negative effect of poor prognosis factors in MMR-D EC, resulting in no difference in survival compared with MMR-proficient EC (MCMEEKIN et al., 2016).

2.3.2 Resistance to chemotherapy

MMR-D tumors are resistant to various agents used in anti-cancer chemotherapy, including methylating agents, platinum-based drugs, fluoropyrimidine compounds, and purine analogs (GUILLOTIN; MARTIN, 2014).

Resistance to temozolomide (TMZ), an alkylant agent, is observed in approximately 50% of brain tumors, mainly glioblastoma, with *MGMT* (O^6-Methylguanine-DNA Methyltransferase) promoter methylation and is associated with acquired defects in the MMR pathway, mainly due to mutations in the *MSH6* gene (CRISAFULLI et al., 2022; LI et al., 2022; YIP et al., 2009). MMR-proficient tumor cells are sensitive to TMZ treatment due to the recognition of O^6-methylguanine DNA adducts derived from TMZ treatment by MMR proteins and consequent MMR-mediate cell cycle arrest and apoptosis. On the other hand, MMR-D tumors cannot recognize O^6-methylguanine adducts and, therefore, do not undergo apoptosis, becoming resistant to TMZ treatment (CAPORALI et al., 2004; GANESA et al., 2022). An additional negative consequence of acquired MMR deficiency in TMZ-treated brain tumors is the increase in mutation rates with potential implications for cancer progression (CAHILL et al., 2007; HUNTER et al., 2006).

DNA adducts generated by platinum agents, such as cisplatin, can be detected by MMR proteins (CHANEY et al., 2005). Recognizing cisplatin-induced DNA lesions by MMR components is vital to maintain cellular sensibility to those agents by MMR-mediated activation of DDR pathways and consequent cell death (SAWANT et al., 2015). Reduced expression of *MLH1*, *MSH2*, and *MSH6* genes is associated with platinum resistance in some cancer types (HUANG et al., 2021). In ovarian cancer, for example, proficient MMR tumors are associated with a better response to cisplatin-based chemotherapy (ZHAO et al., 2018), whereas loss of MMR protein expression is associated, at least partially, with resistance to carboplatin and cisplatin (DAMIA; BROGGINI, 2019).

Chemotherapy based on 5-fluorouracil (5-FU), a nucleoside analog of uracil and thymine, is frequently used to treat a variety of cancers, including CRC (IWAIZUMI; TSENG-ROGENSKI; CARETHERS, 2011). Several studies have reported resistance to 5-FU in MSI/MMR-D CRC. However, some authors have reported conflicting results showing a better response to 5-FU in MSI CRC than MSS tumors (JOVER et al., 2009; VILAR; GRUBER, 2010). Those discordant findings might be explained by differences in study design, sample size, adjuvant chemotherapies, and mutations in different MMR genes (GUILLOTIN; MARTIN, 2014). Despite those conflicting findings, most studies agree that MMR deficiency

confers resistance to 5-FU (HEWISH et al., 2010a). The resistance to 5-FU observed in MMR-D cells is caused by incorporating its metabolites into DNA during the S-phase in opposition to guanine nucleotides. The 5-FU:G pairing is recognized by MMR proteins and results in cell cycle arrest and apoptosis, most likely by the futile cycling model (HEWISH et al., 2010b; WYATT; WILSON, 2009).

6-Thioguanine (6-TG) is a purine analog commonly used as an anti-cancer drug for the treatment of different tumors, mainly of childhood leukemia (BAYOUMY et al., 2020; CHEN et al., 2020). Cytotoxicity of 6-TG is mediated by incorporating its metabolite, thioguanine, into DNA in competition with other purine bases (LARSEN et al., 2021; TOKSVANG et al., 2022). Once methylated, thioguanine forms mismatches with thymine during DNA replication, which are recognized by the post-replicative MMR pathway, leading to cell cycle arrest via ATR/Chk1 and posterior cell death (STOJIC et al., 2004; YAMANE; TAYLOR; KINSELLA, 2004). Not surprisingly, MMR-D cells are resistant to 6-TG treatment, with implications for the prognosis of cancer patients treated with this chemotherapeutic agent (OFFMAN et al., 2004; YAN et al., 2003).

2.3.3 Response to immunotherapy

Although significant improvements in cancer treatment through the use of classical anti-cancer therapies have been obtained in the second half of the 20th century, those therapeutic approaches have achieved their potential, and additional improvements in outcomes will require the use of alternative and more effective therapeutics (HOTEIT et al., 2021). In recent years, immunotherapy-based treatments have emerged as an alternative and promising anti-cancer therapeutic approach. Cancer immunotherapy is a group of treatments that uses components of a cancer patient's immune system to fight against cancer cells (YANG et al., 2022). Immunotherapies comprise a variety of strategies, including the adoptive transfer of genetically-engineered immune cells, cytokines therapy, vaccines, oncolytic viruses, and immune checkpoint inhibitors (ICIs) (DHAR et al., 2021).

Immunotherapies based on ICIs use antibodies to block the activity of checkpoint molecules, such as Programmed Cell Death 1 (PD-1) and the Cytotoxic T lymphocyte Antigen 4 (CTLA-4), that act as negative regulators of the adaptive immune response against cancer cells (PÉREZ-RUIZ et al., 2020). ICIs can restore the effector function of immune cells, mainly $CD8^+$ T cells, unleashing them to recognize and eliminate malignant cells (YANG et al., 2022). Monoclonal antibodies against PD-1, PD-L1 (the PD-1 ligand), and CTLA-4 have profoundly

impacted the scenario of cancer therapy and are currently being used as first and second-line treatments for a variety of cancers (CERCEK et al., 2022; WALDMAN; FRITZ; LENARDO, 2020; WANG; XIE; LIU, 2021).

Despite the significant improvements in cancer treatment achieved by ICI therapies, only a fraction of cancer patients will respond to this treatment, highlighting the need for the use of markers with the potential to predict which patients are more likely to benefit from ICI therapies (WANG; XIE; LIU, 2021). Inactivation in the MMR pathway and subsequent microsatellite instability leads to the accumulation of mutations, mainly frameshifts in the repetitive coding sequence of genes, that may lead to the expression of aberrant proteins in tumor cells, termed neoantigens, that can be recognized by immune cells and stimulate an anti-cancer immune-response (BARETTI; LE, 2018; YARCHOAN et al., 2017). Several studies have shown that MMR-D/MSI-H tumors are sensitive to ICI treatment, particularly for therapies based on anti-PD-1 and anti-PD-L1 antibodies, regardless of tumor location and histology (DUDLEY et al., 2016; LE et al., 2015, 2017; ZHAO et al., 2019). Those findings guided the first FDA site-agnostic approval of an anti-cancer drug (pembrolizumab, an anti-PD-1 antibody) in 2017. On this occasion, pembrolizumab was recommended for adult and pediatric patients diagnosed with MMR-D/MSI-high metastatic solid tumors, refractory to other cancer treatments, regardless of the histologic type (BOYIADZIS et al., 2018).

Promising results have supported the neoadjuvant use of ICI therapies beyond its administration in a refractory and adjuvant setting, as initially approved by the FDA. Recent data from a phase 2 clinical trial reported a complete clinical response in 100% of patients diagnosed with locally advanced rectal cancer, treated with neoadjuvant dostarlimab, an anti-PD-1 monoclonal antibody, with no surgery and chemotherapy being performed (CERCEK et al., 2022). Those findings highlight the MMR-D/MSI phenotype as a robust biomarker for identifying patients more likely to benefit from ICI immunotherapy.

2.3.4 Screening of cancer predisposition syndromes

Cancer predisposition syndromes (CPS) are caused by constitutional defects in genes involved with molecular pathways that, once altered, may lead to cancer development (CARTA et al., 2020). So far, dozens of CPS have been described, each conferring an increased risk for developing a more or less well-defined spectrum of tumors (GARBER; OFFIT, 2005).

Most CPS are caused by germline inactivating mutations in tumor suppressor genes involved with cell cycle regulation, apoptosis, senescence, and cell differentiation.

Additionally, some of those genes are involved with DNA damage and repair pathways (MCGEE; NICHOLS, 2016). In the context of MMR, there are two CPS caused by constitutional defects in MMR genes: Lynch syndrome (LS) and Constitutional mismatch repair deficiency syndrome (CMMRD). LS is an autosomal dominant disease that predisposes individuals to a broad spectrum of tumors, mainly colorectal, endometrial, and gastric tumors (LYNCH et al., 2015). On the other hand, CMMRD is an autosomal recessive disease caused by homozygous or compound heterozygous germline mutations that predispose to various childhood cancers, including hematologic and brain malignancies and tumors from the LS spectrum (ABEDALTHAGAFI, 2018).

Tumors arising in LS and CMMRD patients exhibit MMR deficiency, which can be detected by the methods described previously in this chapter. The identification of MMR-D tumors is routinely used as a screening method to identify individuals at risk of having LS of CMMRD. Once identified, genetic testing is recommended on those individuals and their respective family members, and specific clinical management of cancer surveillance protocols are offered to the positive cases (ARONSON et al., 2022; CURTIUS; GUPTA; BOLAND, 2022).

The clinical and molecular characteristics of LS and CMMRD will be discussed in the next section as hereditary causes of MMR deficiency.

3 Etiology of Mismatch repair deficiency in cancer

The MMR-D phenotype observed in a portion of human cancers is driven mainly through the inactivation of MMR genes, mostly in *MLH1*, *MSH2*, *MSH6*, or *PMS2*, by different mechanisms, including epigenetic silencing mediated by promoter methylation, point mutations, indels, CNVs, structural rearrangements, and (OLDFIELD et al., 2021; POULOGIANNIS; FRAYLING; ARENDS, 2010). Those mechanisms may occur constitutively, leading to an inherited predisposition to cancer, or be restricted to somatic cells, giving rise to sporadic tumors (RICHMAN, 2015). MMR genes generally act as classical tumor suppressor genes, where biallelic loss-of-function alterations must occur for the inactivation of their tumor suppressive activity, according to the Knudson two-hit hypothesis (HEWISH et al., 2010a).

The known etiology of MMR deficiency and its associated clinical phenotypes will be discussed in the following sections.

3.1 *MLH1* methylation

DNA methylation is an epigenetic mechanism with essential functions in several cellular processes, playing a pivotal role in cancer by regulating the expression of genes related to the formation and progression of several tumor types (LOCKE et al., 2019; MOORE; LE; FAN, 2013). Methylation occurs most frequently on the fifth carbon of cytosines within a cytosine-guanine dinucleotide (CpG) context through an enzymatic reaction catalyzed by DNA methyltransferases (NISHIYAMA; NAKANISHI, 2021).

A large CpG island, a cluster of CpG dinucleotides in a CG-rich DNA sequence (HAN; ZHAO, 2009), has been identified in the promoter region of *MLH1*. Deng et al. initially divided this CpG island into four regions, and the methylation status of each was correlated with the expression of *MLH1* in 24 cancer cell lines. Methylation in region C, located between the nucleotides -248 and -178 upstream from the transcription start site and containing 8 CpG sites, was strongly associated with transcriptional silencing of the *MLH1* gene, which was not always the case for the other three regions (DENG et al., 1999).

There is a strong correlation between sporadic MMR-D tumors, including CRC, EC, and other cancer types, and hypermethylation of the *MLH1* promoter region, usually derived from the CpG island methylator phenotype (CIMP), which is characterized by the regional hypermethylation of CpG islands (BATEMAN, 2021; HEWISH et al., 2010a; KIM et al., 2016b). Methylation-mediated silencing of *MHL1* has been described as the primary cause of MMR deficiency in endometrial and colorectal cancers (HERMAN et al., 1998; SIMPKINS et al., 1999). Several studies have estimated that approximately 10% to 15% of sporadic uterine and colorectal carcinomas have hypermethylation in the promoter region of the *MLH1* (BENHAMIDA et al., 2020; LOUKOVAARA; PASANEN; BÜTZOW, 2021; MIYAKURA et al., 2003; MOREIRA et al., 2015; SCARPA et al., 2021). The majority of CRC tumors with MMR deficiency, detected mainly by loss of *MLH1* protein expression in tumor tissue, are related to somatic *MLH1* methylation, with frequencies ranging from 53.5% to 86.5% of tumors (ADAR et al., 2017; LI et al., 2013b; MALOBERTI et al., 2022). Similarly, the majority of MMR-D endometrial carcinomas are caused by methylation in the promoter region of *MLH1*, with 71% to 94.5% of MMR-D tumors harboring *MLH1* methylation (BRUEGL et al., 2014; BUCHANAN et al., 2014a; GURIN et al., 1999; HAMPEL et al., 2006).

Various methodologies for DNA methylation identification have been used to assess the methylation status of *MLH1* in different tumor types. Those methodologies are based on methylation-sensitive restriction enzymes, bisulfite modification of DNA, or a combination of

both methods (BONORA et al., 2019; NELL et al., 2020). Many downstream techniques are used to detect DNA methylation, including direct DNA sequencing, methylation-specific PCR, single-strand conformational polymorphism, melting curve analysis, denaturing gel electrophoresis, and real-time PCR (BETTSTETTER et al., 2007; OLDFIELD et al., 2021). High-throughput methodologies, including pyrosequencing and array-based platforms, can also detect methylation in *MLH1* (BENHAMIDA et al., 2020; NEWTON et al., 2014).

Due to the high frequency of MMR-D tumors harboring somatic inactivation of *MLH1* by promoter methylation, the testing for *MLH1* methylation is strongly recommended for EC and CRC cases exhibiting aberrant MLH1 protein expression or high levels of MSI to distinguish between sporadic and potential hereditary MMR-D cancers (BENHAMIDA et al., 2020; DESHPANDE et al., 2020; GAUSACHS et al., 2012; PEĆINA-ŠLAUS et al., 2020).

3.2 Constitutional Mismatch Repair Deficiency

CMMRD, also known as Biallelic mismatch repair deficiency, is a highly penetrant autosomal recessive childhood cancer predisposition syndrome caused by biallelic germline mutations in MMR genes (SHLIEN et al., 2015). CMMRD was first described in 1999 in the offspring from a consanguineous marriage between LS individuals in two different families (RICCIARDONE et al., 1999; WANG et al., 1999). Since then, more than a hundred CMMRD patients diagnosed with pediatric and young adult cancers have been reported (WIMMER et al., 2014).

In opposite to LS, where patients develop tumors at adult age, CMMRD is characterized by a broad spectrum of early-onset tumors during childhood, including brain tumors and hematological malignancies, and a variety of pre-malignant and malignant lesions of the gastrointestinal tract, which are typically found in LS patients at a later age (WIMMER; ETZLER, 2008; WIMMER; KRATZ, 2010). High-grade gliomas are the most common central nervous system tumors diagnosed in CMMRD and are the primary cause of death in these children (DURNO et al., 2021). Hematologic malignancies are diagnosed in CMMRD patients at a mean age of 6 years, while brain tumors and LS-associated tumors are diagnosed later, at mean ages of 9 and 17 years, respectively (WIMMER et al., 2014).

Even though biallelic mutations in the four main MMR genes can cause CMMRD, *PMS2* and *MSH6* are the genes most commonly mutated in those patients, contrary to the scenario of LS, where *MSH2* and *MLH1* are more frequently mutated (WIMMER et al., 2014). Interestingly, the clinical features of CMMRD vary according to the affected genes, with

patients with mutations in *MLH1* and *MSH2* being associated with a more severe phenotype than *MSH6/PMS2* carriers (WIMMER et al., 2014). CMMRD patients with mutations in *MLH1* and *MSH2* are more frequently affected by hematologic malignancies than *MSH6* and *PMS2* mutated patients. On the other hand, CMMRD patients with mutations in *MSH6* and *PMS2* genes have a higher prevalence of brain tumors than *MLH1/MSH2* mutated patients. Of note, tumors in *MLH1/MSH2* patients tend to occur at an earlier age in comparison with *MSH6/PMS2* CMMRD patients, but individuals from the latter group have a higher likelihood of surviving their first tumor and developing a second malignancy in comparison with individuals from the first group (WIMMER; KRATZ, 2010; WIMMER et al., 2014).

MSH2 and *MLH1* mutations, most commonly found in LS, are less common in CMMRD, while *PMS2* and *MSH6* are more widely observed in the latter syndrome. Some authors have hypothesized that highly penetrant mutations in *MSH2* and *MLH1* may be embryonically lethal when homozygous, resulting in a decreased frequency of mutations in those genes in CMMRD patients (BAKRY et al., 2014). Additionally, the higher survival observed in *MSH6/PMS2* mutated children may partially explain the overrepresentation of mutations in those genes compared to *MLH1/MSH2* carriers (WIMMER; KRATZ, 2010).

The differential diagnosis of CMMRD involves an extensive range of other cancer predisposition syndromes, including neurofibromatosis type 1 (NF-1), attenuated familial polyposis (FAP), MUTYH-associated polyposis, and Li-Fraumeni syndrome (ARONSON et al., 2022). Of note, CMMRD patients usually present with “café-au-lait” skin spots and other findings that mimic NF-1, a cancer predisposition disease that can manifest as tumors of the nervous system, in addition to other clinical characteristics (BAKRY et al., 2014; LEGIUS et al., 2021). A subset of CMMR patients also presents with a phenotype overlapping with FAP, with colorectal polyps being found in up to 32% of the patients, many of them being diagnosed with multiple synchronous adenomas, ranging from few to up to 100 polyps (HERKERT et al., 2011; JASPERSON; SAMOWITZ; BURT, 2011; WIMMER et al., 2014). This broad phenotypic spectrum supports the use of high-throughput sequencing technologies to obtain the diagnosis of CMMRD more accurately and in a shorter period than traditional methods, guided uniquely by clinical evaluations.

3.3 Lynch Syndrome

LS is one of the most common cancer predisposition syndromes, with an estimated population prevalence of approximately 1 in 250-1,000 individuals (TANAKAYA, 2019). This

syndrome is responsible for around 5% of CRC cases and 3% of EC cases, depending on the population of study and the approaches adopted for its screening and diagnosis (ABU-GHAZALEH et al., 2022; RYAN et al., 2019).

LS is an autosomal dominant cancer predisposition disease caused by germline heterozygous inactivating mutations in one of the four central MMR genes (EDWARDS; MONAHAN, 2022). Tumorigenesis in LS patients is initiated by the somatic inactivation of the wild-type MMR allele, disrupting the repair activity of the MMR pathway and resulting in the accumulation of mutations in genes involved in the cancer development (LYNCH et al., 2015).

The following sections will discuss the history, clinical features, genetic etiology, and diagnosis of this widespread MMR-D disease.

3.3.1 History

The history of LS starts with the description of one of the first comprehensive family clusters of cancer in 1895 by Dr. Warthin, a pathologist from the University of Michigan (LYNCH et al., 2015). Dr. Warthin noticed that the cancer phenotype was transmitted in this family according to the mendelian autosomal dominant model and published his findings in 1913, naming this pedigree as Family G (WARTHIN, 1913).

Almost five decades later, Dr. Henry Lynch, who was a medicine residency intern at the University of Michigan, described two other families (Families M and N) with a history of cancer similar to the one previously observed in Family G (LYNCH et al., 1966, 2009). Dr. Lynch also revised Family G and recorded the cancer prevalence among more than 650 of its members (LYNCH; KRUSH, 1971). Dr. Lynch observed that, despite the high frequency of CRC and other malignancies among different generations, individuals of those families were not affected by colonic polyps, differentiating those cases from the Familial Adenomatous Polyposis (FAP), the most favored diagnosis of familial CRC at the time. Therefore, Dr. Lynch hypothesized that this could be a different and undescribed hereditary cancer-predisposing syndrome characterized by an autosomal dominant transmission of CRC and extracolonic tumors (LYNCH et al., 2015). He referred to this new condition using the term "Cancer Family Syndrome" (BOLAND; LYNCH, 2013).

Later on, further reports of families sharing similar findings to those described by Lynch were published, with some families exhibiting only CRC cases while others were also represented by extracolonic tumors, mainly endometrial and gastric cancer, giving rise to the

terms Lynch Syndrome I and II, to distinguish between families without polyposis and with a predisposition to CRC-only from those with CRC and extracolonic cancers, respectively (BOLAND; LYNCH, 2013). In 1985, the term "hereditary nonpolyposis colorectal cancer" (HNPCC) was first used by Henry Lynch to encompass both manifestations of LS, which was widely used for many years (LYNCH et al., 1985). Nowadays, the term HNPCC is strongly discouraged for being considered a poor descriptor of a syndrome that does not manifest as CRC only (JASS, 2006). With the discovery of its genetic basis (discussed in a dedicated section of this chapter), the term Lynch syndrome is the most indicated, referring to individuals with hereditary heterozygous mutations in MMR genes, with a notable predisposition to a broad spectrum of cancers (JASS, 2006).

3.3.2 Clinical phenotype

LS confers an increased lifetime risk for developing non-polypoid colorectal malignancies and endometrial carcinomas. In addition, LS patients may also present with stomach, small bowel, hepatobiliary system, upper urologic tract, and ovary cancer (WATSON; RILEY, 2005). Those tumors occur at an earlier age compared to sporadic cancers, and the development of multiple primary malignancies, synchronous or metachronous, are also observed (TANAKAYA, 2019). More recently, breast, prostate, pancreatic, and adrenocortical malignancies have also been associated with LS (LYNCH et al., 2015). However, the occurrence of those tumors in LS patients is much smaller than the ones from the classical spectrum of the syndrome.

The lifetime risk for CRC in LS patients ranges from 20 to 70%, with age at diagnosis between 44 and 61 years, on average. The risk for EC ranges from 15-70%, with an average age at diagnosis of 48 to 62 years. Women with LS have a 4-12% risk for developing ovarian cancer, with a diagnosis at 42.5 years on average. In addition, the lifetime risk for gastric cancer is between 6% and 13% at an average age of 56. LS carriers also have a smaller but still significant risk for developing other extracolonic tumors, such as cancers affecting the small intestine, brain, skin, hepatobiliary, and urinary tract (DURATURO et al., 2019). Of note, the lifetime risk for EC in women with LS is superior to their risk for CRC and other malignancies, and, frequently, EC is the first malignancy to be identified in those patients, being considered the sentinel tumor (MEYER; BROADDUS; LU, 2009; RYAN et al., 2019).

A subset of LS individuals presents with sebaceous gland tumors (sebaceous adenomas and carcinomas and basal cell carcinomas) and keratoacanthomas of the skin associated with

visceral tumors (PONTI et al., 2016). Those patients are considered to have Muir-Torre syndrome, a variation of LS that is present in about 9% of LS patients (SOUTH et al., 2008). Skin tumors found in patients with Muir-Torre syndrome have MMR deficiency, with remarkable microsatellite instability and loss of MMR protein expression, suggesting that those tumors share the same etiology as the visceral malignancies observed in LS patients (MACHIN et al., 2002; MORALES-BURGOS et al., 2008). In addition to the early age at cancer diagnosis and the occurrence of synchronous and metachronous cancers, LS may also present with other remarkable clinical features, including preferential tumor localization in the right-sided colon, poor differentiation with mucinous features, Crohn's-like lymphoid reaction, and tumor-infiltrating lymphocytes (DURATURO et al., 2019).

3.3.3 Etiology

Most LS cases (70-90%) are attributed to pathogenic germline variants in *MLH1* and *MSH2*, whereas the remaining 10-30% are associated with mutations in *MSH6* and *PMS2* (COHEN; LEININGER, 2014). Those frequencies may vary according to the cancer type and the study population. By curating the catalog of MMR germline variants registered on the InSiGHT Colon Cancer Gene Variant Database, Thompson *et al*. revealed that 43% of germline pathogenic (class 5) and likely pathogenic variants (class 4) are found in *MLH1*, 39.2% in *MSH2*, 12.85 in *MSH6*, and 5% in *PMS*2 (THOMPSON et al., 2014). On the other hand, studies of germline variants in MMR genes from cohorts of EC show a prevalence of pathogenic variants in *MSH2* and *MSH6* genes, with an important but less frequent contribution of mutations in *MLH1,* followed by a small frequency of variants in *PMS2* (EGOAVIL et al., 2013a; RING et al., 2016; YANG et al., 2021).

The different frequencies of mutations in MMR genes observed between CRC and EC in LS patients may be explained partially by the cancer risk conferred by each of those genes. In a prospective study comprising 6,350 carriers of pathogenic variants in LS genes, Dominguez-Valentin *et al.* found that the cumulative incidence of cancer in any organ at the age of 75 years is higher in LS patients with pathogenic variants in *MSH2* (84.3% in women and 75.2% in men); followed by individuals with mutations in *MLH1* (81% in women and 71.4% in men); *MSH6* (61.8% in women and 41.7% in men), and *PMS2* (34.1% in both genders). This general observation is also confirmed when the cumulative risk is stratified by tumor type for most of the tumors from the spectrum of LS. For instance, women with pathogenic variants in *MSH2* and *MSH6* have a higher incidence of EC at the age of 75 than

those with variants in *MLH1* and *PMS2* (48.9% and 41.1% vs. 37% and 12.8%, respectively). On the other hand, the cumulative incidence of CRC at the age of 75 years is higher in carriers of pathogenic variants in *MLH1* (57.1% in men and 48.3% in women) than individuals with variants in the other MMR genes (DOMINGUEZ-VALENTIN et al., 2020).

In addition to mutations in *MLH1*, *MSH2*, *MSH6*, and *PMS2*, germline deletions comprising the last exon of the Epithelial cell adhesion molecule (*EPCAM)* gene are the genetic cause of up to 3% of LS cases and can be found in 20-25% of suspected LS cases whose tumors are negative for the MSH2 protein and without mutations in *MSH2* (RUMILLA et al., 2011; TUTLEWSKA; LUBINSKI; KURZAWSKI, 2013). Those deletions remove the polyadenylation site of *EPCAM*, abolishing its transcription termination and leading to the transcriptional read-through into the *MSH2* gene, which is located downstream of *EPCAM*, and resulting in *MSH2* inactivation in cells expressing *EPCAM* by hypermethylation of *MSH2* promoter (KUIPER et al., 2011; LIGTENBERG et al., 2009). Individuals with germline 3'end deletions in *EPCAM* have a 75% cumulative risk of developing CRC before 70 years and up to 55% risk of developing EC when those deletions are extended closer to *MSH2* (KEMPERS et al., 2011).

Rare cases of LS have also been attributed to constitutional monoallelic hypermethylation in the promoter region of the *MLH1* gene (HITCHINS, 2015). Those patients have tumors exhibiting loss of MLH1 protein without a germline mutation in the *MLH1* gene. Around 75 cases of constitutional *MLH1* hypermethylation have been reported so far, accounting for 2-3% of suspected LS cases (DÁMASO et al., 2018). Most cases associated with *MLH1* epimutations were described in LS patients diagnosed with CRC and rarely with EC (EGOAVIL et al., 2013b; HITCHINS; WARD, 2009).

3.3.4 Screening approaches

Initially, genetic testing was restricted to patients who met clinical criteria based mainly on the number and age of family members with LS-associated cancers (CURTIUS; GUPTA; BOLAND, 2022). In 1991, the International Collaborative Group on HNPCC devised standardized clinical criteria to identify potential LS cases for further studies aiming to decipher the etiology and pathogenesis of this genetic disease (VASEN et al., 1991). Those criteria were named Amsterdam I Criteria and focused on a strong family history of CRC at a young age of onset (LYNCH et al., 2015). Those criteria were modified in 1999 to include extracolonic malignancies belonging to the LS tumor spectrum, referred to as Amsterdam II Criteria

(VASEN et al., 1999). Amsterdam Criteria were highly specific for LS but had a low sensitivity, missing many LS cases (COHEN; PRITCHARD; JARVIK, 2019). With the discovery of MSI as a hallmark of LS tumors, the American National Cancer Institute developed criteria less restrictive than Amsterdam Criteria aiming to select CRC cases for MSI testing on tumor samples (LYNCH et al., 2015). Those criteria were published in 1997 and are currently known as Bethesda Guidelines (RODRIGUEZ-BIGAS et al., 1997). Bethesda Guidelines were revised in 2004 to include recommendations for identifying LS patients and a panel of five microsatellite loci to investigate MSI in tumors of patients suspected to have LS (DURATURO et al., 2019; UMAR et al., 2004). The Amsterdam I and II Criteria and Original and Revised Bethesda Guidelines are described in **Table 1**.

Even though extracolonic tumors, mainly endometrial cancer, have been observed in LS families since the description of this syndrome, the Amsterdam and Bethesda Criteria were elaborated to detect LS from CRC cases, highlighting the need for criteria of LS detection adapted for extracolonic cancer. In 2007, the Society of Gynecologic Oncologists Education Committee published guidelines for the detection of patients with gynecologic tumors at increased risk of having LS, whose genetic testing is recommended (LANCASTER et al., 2007). Those clinical criteria were partially based on Amsterdam II Criteria and Revised Bethesda Guidelines and the accumulated evidence of MMR deficiency in endometrial, colorectal, and other LS-tumors (MEYER; BROADDUS; LU, 2009). The SGO criteria are presented in **Table 2.**

Amsterdam I Criteria:

At least three relatives with histologically verified CRC:

1. One is a first-degree relative of the other two;
2. At least two successive generations affected;
3. At least one of the relatives with CRC is diagnosed at < 50 years of age;
4. Familial adenomatous polyposis (FAP) has been excluded.

Amsterdam II Criteria:

At least three relatives with an LS-associated cancer, including CRC, endometrial, stomach, ovary, ureter or renal pelvis, brain, small bowel, hepatobiliary tract, and skin (sebaceous tumors):

1. One is a first-degree relative of the other two;
2. At least two successive generations are affected;
3. At least one CRC should be diagnosed before age 50;
4. FAP should be excluded;
5. Tumors should be verified by pathology examination.

Bethesda Guidelines:

1. Individuals with cancer in families that meet the Amsterdam Criteria.
2. Individuals with two HNPCC-related cancers, including synchronous and metachronous CRC or associated extracolonic cancers[1].
3. Individuals with CRC and a first-degree relative with CRC and/or HNPCC-related extracolonic cancer and/or a colorectal adenoma; one of the cancers diagnosed at age < 40 years
4. Individuals with CRC or endometrial cancer diagnosed at age < 45 years, and the adenoma diagnosed at age < 40 years.
5. Individuals with right-sided CRC with an undifferentiated pattern (solid/cribriform) on histopathology diagnosed at age < 45 years[2].
6. Individuals with signet-ring-cell-type CRC diagnosed at age < 45 years[3].
7. Individuals with adenomas diagnosed at age < 40 years.

Revised Bethesda Guidelines:

To justify MSI testing, at least one of the following criteria must be fulfilled:

1. CRC diagnosed in a patient who is less than 50 years of age;
2. Presence of synchronous or metachronous CRC, or other LS-associated tumors[4], regardless of age;
3. CRC with MSI-high[5] histology[6] diagnosed in a patient who is less 60 years of age;
4. CRC or LS-associated tumor diagnosed in one or more first-degree relatives with an HNPCC-related tumor, with one of the cancers being diagnosed under the age of 50 years[7];
5. CRC or LS-associated tumor diagnosed in two or more first or second-degree relatives with HNPCC-related tumors, regardless of age[7].

Table 1 – Clinical criteria originally defined for LS diagnosis in CRC. [1] endometrial, ovarian, gastric, hepatobiliary, or small-bowel cancer or transitional cell carcinoma of the renal pelvis or ureter. [2] solid/cribriform defined as poorly differentiated or undifferentiated carcinoma composed of irregular, solid sheets of large eosinophilic cells and containing small gland-like spaces. [3] Composed of >50% signet ring cells. [4] LS-associated tumors were defined as colorectal, endometrial, stomach, ovarian, pancreas, ureter and renal pelvis, biliary tract, and brain (usually glioblastoma as seen in Turcot syndrome) tumors, sebaceous gland adenomas, and keratoacanthomas in Muir–Torre syndrome, and carcinoma of the small bowel. [5] MSI-high in tumors refers to changes in two or more of the five National Cancer Institute-recommended panels of microsatellite markers. [6] Presence of tumor-infiltrating lymphocytes, Crohn's-like lymphocytic reaction, mucinous/signet-ring differentiation, or medullary growth pattern. CRC: colorectal cancer. [7] Those criteria have been reworded to clarify the Revised Bethesda Guidelines. HNPCC: Hereditary nonpolyposis colorectal cancer. Adapted from LYNCH et al., 2015.

Patients with greater than approximately 20–25% chance of having an inherited predisposition to endometrial, colorectal, and related cancers and for whom genetic risk assessment is recommended (at least one of the following criteria):
1. Patients with endometrial or colorectal cancer who meet the Amsterdam II criteria. 2. Patients with synchronous or metachronous endometrial and colorectal cancer with the first cancer diagnosed prior to age 50. 3. Patients with synchronous or metachronous ovarian and colorectal cancer with the first cancer diagnosed prior to age 50. 4. Patients with colorectal or endometrial cancer with evidence of a mismatch repair defect (*i.e.* microsatellite instability (MSI) or immunohistochemical loss of expression of MLH1, MSH2, MSH6, or PMS2). 5. Patients with a first or second-degree relative with a known mismatch repair gene mutation.
Patients with greater than approximately 5–10% chance of having an inherited predisposition to endometrial, colorectal, and related cancers and for whom genetic risk assessment may be helpful (at least one of the following criteria):
1. Patients with endometrial or colorectal cancer diagnosed prior to age 50. 2. Patient with endometrial or ovarian cancer with a synchronous or metachronous colon or other Lynch/HNPCC-associated tumor[1] at any age. 3. Patients with endometrial or colorectal cancer and a first-degree relative with a Lynch/HNPCC-associated tumor[1] diagnosed prior to age 50. 4. Patients with colorectal or endometrial cancer diagnosed at any age with two or more first or second-degree relatives[2] with Lynch/HNPCC-associated tumors[1], regardless of age. 5. Patients with a first or second degree relative† that meets the above criteria.

Table 2 – Clinical criteria elaborated by the American Society of Gynecologic Oncology for the detection of individuals at risk for hereditary endometrial, colorectal, and other LS-associated cancers. [1] colorectal, endometrial, stomach, ovarian, pancreas, ureter and renal pelvis, biliary tract, and brain (usually glioblastoma as seen in Turcot syndrome) tumors, sebaceous gland adenomas and keratoacanthomas in Muir–Torre syndrome, and carcinoma of the small bowel. [2] First and second-degree relatives are parents, siblings, aunts, uncles, nieces, nephews, grandparents, and grandchildren. Adapted from LANCASTER et al., 2007.

A simplified version of the SGO criteria was published in 2015 without stratifying patients for their risk of having an inherited gynecologic cancer (LANCASTER et al., 2015), as shown in **Table 3**.

Patients with an increased likelihood of Lynch syndrome and for whom the genetic assessment is recommended (at least one of the following criteria):
1. Patients with endometrial or colorectal cancer with evidence of microsatellite instability or loss of a DNA mismatch repair protein (MLH1, MSH2, MSH6, PMS2) on immunohistochemistry. 2. Patients with a first-degree relative affected with endometrial or colorectal cancer who was either diagnosed before age 60 years or who is identified to be at risk for Lynch syndrome by a systematic clinical screen that incorporates a focused personal and medical history, as defined elsewhere[1]. 3. Patients with a first or second-degree relative[2] with a known mutation in a mismatch repair gene.

Table 3 – Simplified SGO clinical criteria for detection of individuals diagnosed with endometrial cancer at risk of having LS. [1] details of screening are described in ACOG PRACTICE BULLETIN NO. 147, 2014. [2] First-degree relatives are defined as parents, siblings, and Children. Second-degree relatives comprise aunts, uncles, nieces, nephews, grandparents, and grandchildren. Adapted from LANCASTER et al., 2015.

3.3.4.1 Universal screening of tumors

Screening individuals for LS genetic testing based on clinical criteria was important as an inexpensive approach to detect patients at risk on a population scale. However, this screening method relies on accurate information about cancer prevalence in pedigrees to estimate their risk of being families with LS (LAWRENCE et al., 2021). Moreover, screening approaches based on clinical criteria were found to have low sensitivity, with half of CRC-associated LS cases being missed, which required new methods to identify suspected LS in the genetic testing (COHEN; PRITCHARD; JARVIK, 2019).

In this context, several centers started to adopt the universal tumor screening approach, which comprises testing all individuals newly diagnosed with LS-associated tumors, mainly CRC and EC, regardless of family history or age at cancer diagnosis. (CRAIN et al., 2022). By this approach, patients whose tumors test positive for microsatellite instability or have MMR-D detected by the loss of expression of at least one MMR protein are suspected of having LS; therefore, genetic testing is recommended (EIKENBOOM et al., 2022).

In 2009, the American working group named Evaluation of Genomic Applications in Practice and Prevention (EGAPP) recommended screening all CRC for detecting LS (EGAPP, 2009). Currently, the universal use of tumor screening by either MSI or IHC for all newly diagnosed CRC and EC is highly recommended by several international institutions (DI MARCO et al., 2018; GIARDIELLO et al., 2014; JU et al., 2018; LAWRENCE et al., 2021; LEE et al., 2020; SYNGAL et al., 2015). In addition to CRC and EC, some authors also support universal screening in other LS-associated tumors, including ovarian, urinary, and sebaceous tumors (KUNNACKAL JOHN et al., 2022).

In addition to increasing the sensitivity to detect LS families that would be missed by clinical criteria, universal tumor screening also contributes to a better understanding of the epidemiology of MMR deficiency in different tumor types and more accurate estimates of LS prevalence among those tumors. The meta-analysis conducted by Ryan *et al*. reported an LS prevalence of 3% among 12,633 EC cases included in their study, representing 29% of endometrial tumors found to be MMR-D based on universal screening of cases by IHC and PCR-based MSI. Interestingly, the pre-selection of cases for clinical criteria, such as age at diagnosis, did not increase the proportion of cases with germline variants in the LS-associated genes (RYAN et al., 2019). The prevalence of LS in CRC was also assessed by a recent meta-analysis conducted by Eikenboom *et al*., who reported an LS prevalence of 2% among 58,580 CRC colorectal tumors included in their study, representing 33% of tumors with an MMR-D

phenotype (EIKENBOOM et al., 2022). Those robust meta-analyses highlight one of the main challenges in adopting universal tumor screening, which is the low frequency of LS in patients with an MMR-D tumor, corresponding to no more than 33% of cases (BENUSIGLIO et al., 2020).

3.3.5 Molecular diagnosis

A definitive diagnosis of LS is obtained by identifying a pathogenic germline variant in one of the main MMR genes (LYNCH et al., 2015). The traditional methods for the molecular diagnosis of LS include the direct sequencing of *MLH1, MSH2, MSH6*, and *PMS2* for the detection of single-nucleotide variants (SNVs) in their coding regions and splicing sites, as well as the multiplex ligation-dependent probe amplification (MLPA) technique for the investigation of copy-number alterations (CNVs) in both MMR genes and the 3' end of *EPCAM* (DURATURO et al., 2019).

With the advantage of next-sequencing technologies, the costs of sequencing-based genetic testing have decreased considerably, leading to revolutionary changes in the rationale for investigating a patient suspected of cancer predisposition syndrome. Instead of examining candidate genes individually, using NGS-based multigene panels is becoming the standard practice in the molecular diagnosis of hereditary cancer syndromes, including LS (COHEN; PRITCHARD; JARVIK, 2019). In addition to investigating a large number of genes simultaneously, NGS-based sequencing can detect both SNVs and CNVs at a single test, reducing the costs and time of molecular diagnosis of cancer-predisposing syndromes (SINGH et al., 2021).

The implementation of NGS technologies has revolutionized the molecular diagnosis of LS and supported important discoveries about the molecular pathology, epidemiology, and mechanistic of this syndrome. Several studies have explored the high-throughput potential of NGS platforms to investigate the molecular etiologies of MMR-D tumors without a detected variant in LS genes by expanding their analysis to the multi-gene, exonic, or genomic levels (DOS SANTOS et al., 2022; POPE et al., 2020; TALSETH-PALMER et al., 2016). Additionally, some authors have used NGS-based techniques to extend the detection of MSI to thousands of microsatellite loci in parallel in different cancer types (JIA et al., 2020; KAUTTO et al., 2016). Those advancements were critical to expanding the investigation of LS on a pan-cancer scale, with cases being identified in tumors not commonly associated with the spectrum

of LS, including breast (NIKITIN et al., 2020), lung (SUN et al., 2019), and several other tumor types (LATHAM et al., 2019).

3.4 Lynch-like Syndrome

Even though the presence of MMR deficiency is the hallmark of LS tumors, this molecular phenotype is not restricted to LS and can occur in other contexts, such as the somatic methylation of the *MLH1* promoter (LECLERC; VERMAUT; BUISINE, 2021). Therefore, this screening approach identifies many MMR-D tumors with no evidence of germline variants in the MMR genes (HARALDSDOTTIR et al., 2014). Patients with MMR-D tumors without germline variants in LS genes and not related to somatic hypermethylation in *MLH1* are termed as having Lynch-like syndrome (LLS) (POPE et al., 2020).

LLS accounts for 44% to 70% of CRC and EC tumors with MMR deficiency (BUCHANAN et al., 2014b; CARETHERS; STOFFEL, 2015; GOLUBICKI et al., 2021; LECLERC; VERMAUT; BUISINE, 2021; POPE et al., 2020). Tumors identified in LLS patients resemble those of LS concerning the presence of MMR deficiency, as observed by MMR IHC and MSI methods (CARETHERS; STOFFEL, 2015). However, in contrast to LS, the etiology of LLS is not entirely elucidated, which renders the clinical management of LLS patients and their relatives challenging (GOLUBICKI et al., 2021). The following sections will discuss the clinical features and potential etiology of LLS.

3.4.1 Clinical phenotype

Unlike patients with sporadic MMR-D tumors, LLS patients cannot be easily differentiated from individuals with LS, with the absence of germline variants in MMR genes being the central feature differentiating LLS patients from those with LS (CARETHERS; STOFFEL, 2015).

Patients with LLS and their first-degree relatives are considered at risk for cancer development, requiring specific screening and surveillance protocols (CASTILLEJO et al., 2014). Several studies have shown that patients with LLS develop cancer at younger ages, similar to LS patients (CARETHERS, 2014; CARETHERS; STOFFEL, 2015; RODRÍGUEZ-SOLER et al., 2013). The similarity of the age of onset at cancer diagnosis between LS and LLS patients was observed even when LLS cases with a sporadic origin of their MMR-D tumors were corroborated by the identification of somatic inactivating mutations in MMR

genes (HARALDSDOTTIR et al., 2014; MENSENKAMP et al., 2014). Additionally, LLS patients were found to develop cancer at earlier ages compared to sporadic MMR-D tumors due to *MLH1* methylation (MENSENKAMP et al., 2014). Those observations favor the hypothetic presence of germline variants in cancer-predisposing genes in a subset of LLS patients (CARETHERS; STOFFEL, 2015). On the other hand, several authors have reported that LLS patients have a lower lifetime risk of developing colorectal and extra-colonic cancers than patients with LS but with a higher risk than individuals diagnosed with *MLH1*-methylated MMR-D tumors (LECLERC; VERMAUT; BUISINE, 2021; MENSENKAMP et al., 2014; RODRÍGUEZ-SOLER et al., 2013; XU et al., 2020a).

Some other clinical differences between LLS and LS patients have also been reported, including a higher incidence of rectal cancer in LLS individuals in comparison with LS patients, a higher prevalence of CRC in LS families, and more cases of extra-colonic cancer in LLS pedigrees, and a higher prevalence of male patients in LLS families in comparison with LS (XU et al., 2020b). Additionally, opposite to CRC manifesting in LS patients, a lower proportion of CRC with mucinous differentiation was observed in individuals with LLS (XU et al., 2020a).

Therefore, LLS may be a heterogeneous disease, clinically represented by two categories. The first group may comprise patients with clinical characteristics, such as age at diagnosis and family history of cancer, similar to those of LS patients, whereas the second one might include LLS cases with features similar to those found in sporadic MMR-D tumors related to *MLH1* methylation (GOLUBICKI et al., 2021). The etiology of LLS will be discussed in the next section.

3.4.2 Etiology

The genetic etiology of LLS is not entirely understood, representing a complex disease composed of a mixture of heredity and sporadic cases (XU et al., 2020a). Several authors have elaborated different hypotheses to explain LLS tumors. Firstly, some LLS cases might be explained by false-positive tumor testing generated during the analysis of MMR deficiency by IHC or MSI (HARALDSDOTTIR et al., 2014; POPE et al., 2020). Secondly, LLS tumors may also be sporadic, being caused by the somatic inactivation of MMR genes by different mechanisms, including inactivating mutations, either biallelic or coupled with LOH events, and gene silencing by epigenetic factors (CARETHERS, 2014; HARALDSDOTTIR et al., 2014; MARTÍNEZ-ROCA et al., 2022; POPE et al., 2020). Lastly, considering a hereditary origin, a portion of LLS might be LS cases missed during the genetic testing, either due to variants in

genomic regions not commonly investigated, such as deep intronic and regulatory regions, or by missense variants in LS genes with an uncertain clinical significance. Additionally, a subset of LLS, particularly those with an early-age cancer diagnosis and with a family history of cancer, might be due to germline variants or other genetic mechanisms in other genes than those associated with LS etiology that could mimic the LS phenotype (CARETHERS; STOFFEL, 2015; MARTÍNEZ-ROCA et al., 2022; POPE et al., 2020).

All of these possibilities may be involved with the etiology of LLS since they are not exclusive mechanisms from each other, which could explain the intermediate phenotype observed in LLS patients compared with LS and sporadic tumors (CARETHERS, 2014). Determining the genetic etiology of LLS tumors is crucial to define clinical management and surveillance protocols and providing genetic testing to first-degree relatives of those cases with an increased risk of having a cancer predisposition syndrome (OLDFIELD et al., 2021).

The improvement of molecular diagnosis tools, mainly those based on NGS technologies, has led to the discovery of novel genetic events in LLS and has contributed to a better understanding of its etiology (XU et al., 2020a). Importantly, once the genetic etiology of a given LLS case is elucidated, it will no longer be considered LLS, and the clinical management of this case will be defined based on its newly defined etiology (MARTÍNEZ-ROCA et al., 2022).

3.4.2.1 False-positive tumor screening

Approximately 19% of tumors classified as MMR-D by either MMR IHC or MSI analysis are false-positive results (HARALDSDOTTIR et al., 2014). Most false-negative MMR-D tumors have discordant findings in IHC and MSI testing and are more frequent in extra-colonic cancers (HARALDSDOTTIR et al., 2014; JAFFRELOT et al., 2022). Part of those discordances can be explained by heterogeneous loss of MMR protein expression in tumor tissues which may be due to technical artifacts or biological reasons, rendering the interpretation of IHC challenging to (MCCARTHY et al., 2019). Additionally, the interpretation of PCR-based MSI can be tricking, particularly in EC, where the shifts in unstable microsatellite loci are smaller and more subtle than those observed in CRC, thus increasing the rate of false-positive results (WANG et al., 2017).

Thus, the review of MSI and IHC results should be considered before proceeding with a genetic investigation of MMR-D etiology, particularly in cases with discordant results between those two methodologies.

3.4.2.2 MicroRNAs

MicroRNAs are a class of endogenous small non-coding RNAs, with an average of 22 nucleotides in length, that play important roles in regulating gene expression in different organisms (O'BRIEN et al., 2018). MicroRNAs act as post-transcriptional repressors of several genes by guiding effector proteins to selected mRNAs via anti-sense complementarity to short sequences usually present in the 3' of untranslated regions (UTR) of their target mRNAs (DEXHEIMER; COCHELLA, 2020). The expression of different microRNAs is dysregulated in various human cancers, with implications for the tumorigenesis process by affecting the expression of both oncogenes and tumor suppressor genes (PENG; CROCE, 2016). Valeri *et al*. showed that the MMR pathway is modulated by a microRNA named miR-155 by demonstrating that overexpression of this microRNA downregulated the expression of *MLH1, MSH2*, and *MSH6*, leading to an MMR-D phenotype in CRC cell line (VALERI et al., 2010a). Importantly, those authors also found that miR-155 was overexpressed in CRC tumors with MMR phenotype without mutations and methylation in MMR genes, suggesting that overexpression of this microRNA could be a sporadic etiology for some LLS tumors.

The microRNA miR-22 was also found to target and downregulate MMR genes in another study conducted by Valeri *et al*. Those authors described that miR-22 could target *MSH2* and *MSH6* mRNAs by in silico and in vitro analyses and that the overexpression of this microRNA could reduce S/G2 cell cycle arrest in cells treated with 5-FU, leading to resistance to this drug in CRC cell lines and a xenograft CRC model (VALERI et al., 2010b). Even though resistance to 5-FU is evidence of MMR deficiency, the authors did not investigate whether the overexpression of miR-22 could lead to MSI phenotype and did not test its overexpression on unexplained MMR-D tumors. Therefore, the role of this microRNA in the etiology of LLS still needs to be determined.

The microRNA miR-137 was also described as a potential modulator of the MMR pathway by targeting the mRNA expressed by *MSH2*, both *in vitro* and *in vivo* experiments (LICCARDO et al., 2021). However, no evidence confirms that overexpression of miR-137 could result in an MMR-D phenotype in tumors. Thus, the involvement of miR-137 in the etiology of some LLS cases remains to be investigated.

3.4.2.3 Somatic inactivation of MMR genes

The somatic inactivation of MMR genes by biallelic mutations or monoallelic variants coupled with LOH events has been described by several authors as the significant cause of LLS tumors, especially colorectal and endometrial carcinomas (LECLERC; VERMAUT; BUISINE, 2021). Those cases are assumed to have a sporadic origin and are associated with older age at cancer diagnosis and a low lifetime risk of developing other cancers in both index cases and family members (GOLUBICKI et al., 2021).

The frequency of LLS tumors related to somatic inactivation of MMR genes varies according to the methods used to detect variants in tumor samples. Mensenkamp *et al.* combined Sanger and semiconductor sequencing to investigate somatic mutations in *MLH1* and *MSH2* genes in tumors of 25 LLS cases and found biallelic inactivating mutations in 52% of tumors (MENSENKAMP et al., 2014). Geurts-Giele *et al.* used an NGS-based sequencing approach to detect somatic mutations, together with multiplex PCR for LOH detection and in-situ fluorescent probes to investigate somatic CNVs in MMR genes in 40 LLS individuals and found two inactivating events, either biallelic mutations or a monoallelic variant with a LOH event, in 53% of tumors (GEURTS-GIELE et al., 2014). Lefol *et al*., by using two different NGS-based gene panels, identified double hit mutations in tumors from 63.7% of LLS patients, comprising CRC, EC, and other tumors from the LS spectrum (LEFOL et al., 2021). Xicola *et al*. used WES to investigate the occurrence of somatic events in LLS patients and found MMR biallelic inactivating mutations in 67% of patients (XICOLA et al., 2019). By using the ColoSeq assay, an NGS-based panel comprising 31 genes related to the hereditary cancer (PRITCHARD et al., 2012), Haraldsdottir *et al*. identified biallelic inactivating variants in MMR genes in 69% of their cohort of LLS patients diagnosed with CRC and EC (HARALDSDOTTIR et al., 2014). Porkka *et al.* detected two somatic events in MMR genes in 79% of LLS cases identified in a cohort of CRC (PORKKA et al., 2019). Pearlman *et al*. also used the ColoSeq assay in a cohort of early-onset CRC patients and found double somatic MMR mutations in 100% of LLS patients identified in their study (PEARLMAN et al., 2017).

Those studies corroborate that most LLS cases can be explained by double somatic mutations in MMR genes, indicating a sporadic etiology. However, the somatic inactivation of MMR genes can also be a result of germline events; therefore, a hereditary cancer predisposition cannot be automatically excluded, and clinical features, including the personal and family history of cancer in those cases, and a long-term follow-up of these families, should be considered (MARTÍNEZ-ROCA et al., 2022).

3.4.2.4 Missed cases of Lynch syndrome

About 30% of cases suspected to have LS are diagnosed with germline variants of uncertain significance (VUS) in the MMR genes (THOMPSON et al., 2014). Most VUS are missense mutations whose effect on the gene function cannot be determined due to the lack of clinical, epidemiological, molecular, or functional evidence (FEDERICI; SODDU, 2020). Some of those variants could be pathogenic; therefore, those LLS cases would be misdiagnosed in LS cases (XU et al., 2020a). Individuals carrying VUS and their relatives are advised to undergo clinical management according to their family history of cancer until the VUS can be reclassified as pathogenic or benign variants (SIJMONS; GREENBLATT; GENUARDI, 2013).

In addition to VUS in MMR genes, a substantial portion of LLS, mainly those with clinical features suggestive of LS, may be caused by germline variants not detected or difficult to detect by conventional techniques. Germline deletions at the 3' region of the *EPCAM* (RUMILLA et al., 2011) and epimutation in *MLH1* (HITCHINS; WARD, 2009*)*, alternative genetic etiologies of LS already discussed in this introduction are good examples of genetic events not detected by conventional sequencing methodologies. Structural variants in MMR genes have also been identified in suspected LS cases, including a recurrent inversion comprising exons 1-7 of the *MSH2* gene (MORK et al., 2017; POPE et al., 2020; RHEES; ARNOLD; BOLAND, 2014; WAGNER et al., 2002) and another inversion involving exons 2-6 of the same gene (LIU et al., 2016). Those inversions might be explained by the recombination events involving repetitive Alu sequences in *MSH2* (LIU et al., 2016; PÉREZ-CABORNERO et al., 2011). Inversions in *MLH1*, *PMS2*, and *MSH6* have also been reported in some LLS cases (KASPER et al., 2022; MEYER et al., 2009; MORAK et al., 2011).

Intronic regions are not routinely investigated in the context of the LS diagnosis (FULK et al., 2022; KIYOZUMI et al., 2018; PETERSEN et al., 2013; VAN DER KLIFT et al., 2015). Some authors have described the involvement of deep intronic variants, which are not included in most LS diagnosis approaches, as rare causes in LLS (CASADEI et al., 2019; CLENDENNING et al., 2011). Those variants might disrupt MMR gene functions by creating aberrant splicing and transcripts, generating mRNAs with pseudo-exons containing premature stop codons that would result in the expression of truncated proteins (KEEGAN; WILTON; FLETCHER, 2022). In addition to deep intronic variants, the insertion of an Alu-like sequence in the intron 7 of *MLH1* with consequent disruption of RNA splicing was reported in a suspected LS case (LI et al., 2020b).

Variants in regulatory regions, such as promoters and UTRs, of the MMR genes are not commonly analyzed in routine genetic testing for LS but might also be involved with the etiology of some LLS cases (LECLERC; VERMAUT; BUISINE, 2021). Rare variants in the promoter and 5'-UTR regions of *MLH1* and *MSH2* genes have been reported so far (GREEN et al., 2003; HESSON et al., 2015; SHIN et al., 2002). Those variants are thought to be pathogenic due to decreased expression levels of the affected MMR genes and could be associated with intermediate penetrance of the LS phenotype (HESSON et al., 2015). A duplication comprising 20 nucleotides in the *MSH6* polyadenylation site, another regulatory region, was identified in a family suspected to have LS (DECORSIÈRE et al., 2012). This duplication was found to reduce the expression of *MSH6* by lowering the efficiency of *MSH6* mRNA polyadenylation and, thus, could be the causal mutation in this family.

Rare cases of somatic mosaicism, *i.e.*, the presence of two or more cell populations with distinct genotypes in a particular individual (DE, 2011), have also been reported in suspected cases of LS. Two unrelated cases of somatic mosaicism in MSH2 have been reported so far (GUILLERM et al., 2020; SOURROUILLE et al., 2013). Additionally, two independent studies identified somatic mosaicism in *MLH1* in two unrelated suspected LS cases (GEURTS-GIELE et al., 2019; PASTRELLO et al., 2009). Somatic mosaicism is not easily identified by conventional sequencing methods, especially for mutations with a low allele fraction in healthy tissue, and the use of more sensitive techniques, such as deep-coverage NGS sequencing, is required to identify those rare cases of LS (MARTÍNEZ-ROCA et al., 2022).

3.4.3.5 Non-MMR genes

In addition to the somatic inactivation of MMR genes by biallelic mutations or epigenetic silencing mediated by microRNAs, and the presence of difficult-to-identify germline mutations in LS genes, the presence of germline or somatic variants in genes not related to the MMR pathway, could mimic LS by causing MMR-D and conferring predisposition to cancers belonging to the LS spectrum (GOLUBICKI et al., 2021; LECLERC; VERMAUT; BUISINE, 2021). Therefore, some LLS cases could be due to the pleiotropism of specific genes that, once mutated, could lead to a phenotype overlapping with other hereditary cancer syndromes, including LS (XU et al., 2020a). The intermediate-age diagnosis of cancer supports this hypothesis in LLS patients in comparison with LS and sporadic cases of cancer (MARTÍNEZ-ROCA et al., 2022).

Base excision repair (BER) is one of the most important DNA repair pathways, which recognizes and repairs a variety of endogenous and exogenous DNA damages, including damages induced by alkylation, oxidation, and deamination of nucleotides, in addition to repairing small, non-voluminous lesions that may be generated by several carcinogens (ROLDÁN-ARJONA; ARIZA; CÓRDOBA-CAÑERO, 2019; STRATIGOPOULOU; VAN DAM; GUIKEMA, 2020). The BER-mediated repair of DNA lesions involves a variety of proteins, including DNA glycosylases, which initiate the pathway by recognizing and excising the damaged bases (JACOBS; SCHÄR, 2012). In humans, eleven DNA glycosylases have been identified; one of them, an A/G-specific adenine DNA glycosylase, is coded by the *MUTYH* gene (KAIRUPAN; SCOTT, 2007). Homozygous or compound heterozygous germline mutations in *MUTYH* cause an autosomal recessive cancer predisposition disease associated with an increased risk for the development of colonic polyps and CRC, which can overlap with the clinical manifestation of some LS cases (VENESIO et al., 2012). Biallelic germline variants in *MUTYH* are found in approximately 1-3% of LLS cases (CASTILLEJO et al., 2014; MORAK et al., 2014). Interestingly, tumors of LLS patients harboring germline variants in *MUTYH* were found to have somatic inactivating mutations in MMR genes which are characterized by G>T transversion, typically found in *MUTYH*-defective tumors, suggesting that *MUTYH* deficiency could cause somatic inactivation of MMR genes, leading to a somatic MMR-D phenotype (CASTILLEJO et al., 2014).

The catalytic subunits of replicative DNA polymerases epsilon (Pol ε) and delta (Pol δ), which are encoded by the genes *POLE* and *POLD1*, respectively, maintain the genome fidelity via their proofreading activity which allows for the detection and repair of most mismatches generating during DNA replication (BARBARI; SHCHERBAKOVA, 2017). Somatic missense mutations in the exonuclease domain of *POLE* and *POLD1* disrupt the proofreading activity of those polymerases and were identified in colorectal and endometrial carcinomas exhibiting an ultramutator phenotype (CANCER GENOME ATLAS NETWORK, 2012; CANCER GENOME ATLAS RESEARCH NETWORK et al., 2013). Individuals with germline heterozygous missense variants in the exonuclease domain of *POLE* and *POLD1* are known to have Polymerase proofreading-associated polyposis (PPAP), an autosomal dominant cancer predisposition syndrome characterized by a predisposition to the development of colonic adenomas and CRC, in addition to a lower risk of endometrial, ovarian, and other cancer types (BRIGGS; TOMLINSON, 2013; HAMZAOUI et al., 2020; PALLES et al., 2013). *POLE* and *POLD1* mutations can also occur in MSS and MSI tumors and, when co-existing with MMR deficiency, are characterized by distinct mutational signatures (BILLINGSLEY et al., 2015;

HARADHVALA et al., 2018; YAMAMOTO; IMAI, 2015). Germline or somatic mutations affecting the exonuclease domain of *POLE* and *POLD1* are found in around 14.5% of LLS manifesting as EC and CRC (JANSEN et al., 2016). Similar to LLS cases harboring *MUTYH* mutations, most LLS tumors with mutations in *POLE/POLD1* genes have inactivating mutations in MMR genes, characterized by nucleotide substitutions related to the defective proofreading activity of Pol ε/δ, suggesting that the ultramutator phenotype generated by mutations in *POLE/POLD1* may be an alternative cause of MMR deficiency by the consequent inactivation of MMR genes (BILLINGSLEY et al., 2015; HARALDSDOTTIR et al., 2014; KONSTANTINOPOULOS; MATULONIS, 2015).

SETD2 is another gene potentially implicated in the etiology of some LLS cases. This gene encodes a histone trimethyltranferase that incorporates an epigenetic histone mark (H3K36me3), which was found to be required for the recruitment of the MutSα heterodimer on the chromatin in the first step of the MMR pathway (LI et al., 2013a). Importantly, cells lacking *SETD2* display an MMR-D phenotype without mutations in the MMR genes. As for cell lines, human tumors with mutations in *SETD2* genes exhibit higher scores of MSI than tumors without mutations in this gene (LU et al., 2021). Vargas-Barra *et al*. detected germline variants in *SETD2* in 5% of their cohort of LLS cases. However, the pathogenicity of those mutations was inconclusive (VARGAS-PARRA et al., 2017). More recently, Dámaso *et al*. investigated the occurrence of germline variants in *SETD2* and other genes in 115 LLS cases but did not detect any mutation in this gene (DÁMASO et al., 2020). Therefore, despite its role in the MMR pathway, the involvement of *SETD2* in the etiology of LLS remains to be elucidated.

In addition to *MUTYH*, *POLE*, *POLD1*, and *SETD2*, germline variants in other genes involved with DNA repair pathways, such as *FAN1*, *REV3L*, *BARD1*, *BUB1*, *BUB3*, *WRN*, *PCNA*, and other genes of the MMR pathway, have been identified in LLS cases (MARTÍNEZ-ROCA et al., 2022; XAVIER et al., 2019). However, unlike *MUTYH, POLE/POLD1*, and *SETD2*, there is no evidence that the inactivation of those genes could lead to an MMR-D phenotype.

The WD Repeat And HMG-Box DNA Binding Protein 1 gene (WDHD1) encodes a protein that plays an essential role in eukaryotic DNA replication by assembling several replisome proteins, including DNA helicases and polymerases, to the replication fork (VILLA et al., 2016). By conducting a proteomic analysis, Chen *et al.* revealed that WDHD1 interacts with MSH2 (CHEN et al., 2016). However, the authors did not investigate the impacts of this interaction on the repair activity of the MMR pathway. The role of *WDHD1* in driving MMR deficiency was investigated in **Chapter 4** of this thesis.

4 Relevance of the study

As discussed in the Introduction of this thesis, MMR deficiency is a marker with several clinical implications in cancer, including in the screening for the identification of individuals at risk of having Lynch syndrome. Lynch syndrome carriers have a high lifetime risk of developing several cancers. Therefore, the early diagnosis of Lynch syndrome is critical to improving a patient's prognosis through the adoption of cancer surveillance protocols, such as regular colonoscopy and surveillance of extra-colonic tumors. Upon the identification of a Lynch syndrome case, genetic testing can also be offered to its relatives, and clinical management can be extended to the positive cases.

Despite being a hallmark of Lynch syndrome tumors, most tumors with MMR deficiency are caused by somatic inactivation of MMR genes. Therefore, defining the genetic etiology of MMR deficiency is essential for offering appropriate genetic counseling and clinical protocols to patients with MMR-deficient tumors, avoiding intensive Lynch syndrome management in individuals with sporadic tumors, and directing specific health care to those with a hereditary cancer condition.

In addition to cases with germline and somatic inactivation of MMR genes, the etiology of a fraction of MMR-defective tumors remains to be elucidated. Those cases are usually named Lynch-like syndrome and, based on their clinical features, are assumed to be a mixture of hereditary and sporadic cases. Non-MMR genes, including *MUTHY*, *POLE*, and *SETD2*, have been proposed as potential causes of MMR deficiency in unexplained MMR-deficient cases, but most remain without an etiology. The identification of non-canonical MMR genes has a direct implication on hereditary cancer by their inclusion in genetic testing.

MMR-deficient tumors are highly responsive to immune checkpoint inhibitors, and limited benefit has been observed in individuals with MMR-proficient cancer. Therefore, the identification of non-canonical MMR genes could be therapeutically explored as alternative routes to trigger MSI in MMR-proficient tumors, which are the majority of cases.

Therefore, the genetic etiologies of mismatch repair deficiency were investigated in this study by different approaches. Endometrial cancer was used as a model to investigate MMR deficiency in the first two chapters of this thesis. In Chapter I, the prevalence of germline mutations in the main MMR genes was explored in a Brazilian cohort of 242 primary endometrial carcinomas. Sporadic cases due to *MLH1* methylation, as well as Lynch and Lynch-like syndrome cases, were identified. In Chapter II, we interrogated the genetic etiology of the Lynch-like syndrome cases identified in Chapter I by a germline and somatic mutational

analysis in a set of 63 genes related to DNA repair and cancer predisposition. Finally, in Chapter III, we explored the role of the *WDHD1* gene as a candidate new MMR component and its potential to generate MMR deficiency in vitro.

REFERENCES

ABEDALTHAGAFI, Malak. Constitutional mismatch repair-deficiency: current problems and emerging therapeutic strategies. **Oncotarget**, *[S. l.]*, v. 9, n. 83, p. 35458–35469, 2018. DOI: 10.18632/oncotarget.26249.

ABU-GHAZALEH, Nadine; KAUSHIK, Varun; GORELIK, Alexandra; JENKINS, Mark; MACRAE, Finlay. Worldwide prevalence of Lynch syndrome in patients with colorectal cancer: Systematic review and meta-analysis. **Genetics in Medicine: Official Journal of the American College of Medical Genetics**, *[S. l.]*, v. 24, n. 5, p. 971–985, 2022. DOI: 10.1016/j.gim.2022.01.014.

ACOG Practice Bulletin No. 147: Lynch syndrome. **Obstetrics and Gynecology**, *[S. l.]*, v. 124, n. 5, p. 1042–1054, 2014. DOI: 10.1097/01.AOG.0000456325.50739.72.

ADAR, Tomer; RODGERS, Linda H.; SHANNON, Kristen M.; YOSHIDA, Makoto; MA, Tianle; MATTIA, Anthony; LAUWERS, Gregory Y.; IAFRATE, Anthony J.; CHUNG, Daniel C. A tailored approach to BRAF and MLH1 methylation testing in a universal screening program for Lynch syndrome. **Modern Pathology: An Official Journal of the United States and Canadian Academy of Pathology, Inc**, *[S. l.]*, v. 30, n. 3, p. 440–447, 2017. DOI: 10.1038/modpathol.2016.211.

ALEXANDROV, Ludmil B. et al. Signatures of mutational processes in human cancer. **Nature**, *[S. l.]*, v. 500, n. 7463, p. 415–421, 2013. DOI: 10.1038/nature12477.

ALEXANDROV, Ludmil B. et al. The repertoire of mutational signatures in human cancer. **Nature**, *[S. l.]*, v. 578, n. 7793, p. 94–101, 2020. DOI: 10.1038/s41586-020-1943-3.

AN, Hee Jung et al. Microsatellite instability in endometrioid type endometrial adenocarcinoma is associated with poor prognostic indicators. **The American Journal of Surgical Pathology**, *[S. l.]*, v. 31, n. 6, p. 846–853, 2007. DOI: 10.1097/01.pas.0000213423.30880.ac.

ARABI, Haitham et al. Impact of microsatellite instability (MSI) on survival in high grade endometrial carcinoma. **Gynecologic Oncology**, *[S. l.]*, v. 113, n. 2, p. 153–158, 2009. DOI: 10.1016/j.ygyno.2009.02.004.

ARONSON, Melyssa et al. Diagnostic criteria for constitutional mismatch repair deficiency (CMMRD): recommendations from the international consensus working group. **Journal of Medical Genetics**, *[S. l.]*, v. 59, n. 4, p. 318–327, 2022. DOI: 10.1136/jmedgenet-2020-107627. Disponível em: https://jmg.bmj.com/lookup/doi/10.1136/jmedgenet-2020-107627. Acesso em: 23 ago. 2022.

BAILEY, Matthew H. et al. Comprehensive Characterization of Cancer Driver Genes and Mutations. **Cell**, *[S. l.]*, v. 173, n. 2, p. 371- 385.e18, 2018. DOI: 10.1016/j.cell.2018.02.060.

BAKRY, Doua et al. Genetic and clinical determinants of constitutional mismatch repair deficiency syndrome: report from the constitutional mismatch repair deficiency consortium. **European Journal of Cancer (Oxford, England: 1990)**, *[S. l.]*, v. 50, n. 5, p. 987–996, 2014. DOI: 10.1016/j.ejca.2013.12.005.

BARBARI, Stephanie R.; SHCHERBAKOVA, Polina V. Replicative DNA polymerase defects in human cancers: Consequences, mechanisms, and implications for therapy. **DNA repair**, *[S. l.]*, v. 56, p. 16–25, 2017. DOI: 10.1016/j.dnarep.2017.06.003.

BARETTI, Marina; LE, Dung T. DNA mismatch repair in cancer. **Pharmacology & Therapeutics**, *[S. l.]*, v. 189, p. 45–62, 2018. DOI: 10.1016/j.pharmthera.2018.04.004.

BATEMAN, Adrian C. DNA mismatch repair proteins: scientific update and practical guide. **Journal of Clinical Pathology**, *[S. l.]*, v. 74, n. 4, p. 264–268, 2021. DOI: 10.1136/jclinpath-2020-207281.

BAYOUMY, Ahmed B.; SIMSEK, Melek; SEINEN, Margien L.; MULDER, Chris J. J.; ANSARI, Azhar; PETERS, Godefridus J.; DE BOER, Nanne K. The continuous rediscovery and the benefit-risk ratio of thioguanine, a comprehensive review. **Expert Opinion on Drug Metabolism & Toxicology**, *[S. l.]*, v. 16, n. 2, p. 111–123, 2020. DOI: 10.1080/17425255.2020.1719996.

BENHAMIDA, Jamal K. et al. Reliable Clinical MLH1 Promoter Hypermethylation Assessment Using a High-Throughput Genome-Wide Methylation Array Platform. **The Journal of molecular diagnostics: JMD**, *[S. l.]*, v. 22, n. 3, p. 368–375, 2020. DOI: 10.1016/j.jmoldx.2019.11.005.

BENUSIGLIO, Patrick R.; COULET, Florence; LEFEBVRE, Alexandra; DUVAL, Alex; NUEL, Gregory. Overcoming the challenges associated with universal screening for Lynch syndrome in colorectal and endometrial cancer. **Genetics in Medicine: Official Journal of the American College of Medical Genetics**, *[S. l.]*, v. 22, n. 8, p. 1422–1423, 2020. DOI: 10.1038/s41436-020-0819-0.

BETTSTETTER, Marcus; DECHANT, Stephan; RUEMMELE, Petra; GRABOWSKI, Monika; KELLER, Gisela; HOLINSKI-FEDER, Elke; HARTMANN, Arndt; HOFSTAEDTER, Ferdinand; DIETMAIER, Wolfgang. Distinction of hereditary nonpolyposis colorectal cancer and sporadic microsatellite-unstable colorectal cancer through quantification of MLH1 methylation by real-time PCR. **Clinical Cancer Research: An Official Journal of the American Association for Cancer Research**, *[S. l.]*, v. 13, n. 11, p. 3221–3228, 2007. DOI: 10.1158/1078-0432.CCR-06-3064.

BILLINGSLEY, Caroline C.; COHN, David E.; MUTCH, David G.; STEPHENS, Julie A.; SUAREZ, Adrian A.; GOODFELLOW, Paul J. Polymerase ε (POLE) mutations in endometrial cancer: clinical outcomes and implications for Lynch syndrome testing. **Cancer**, *[S. l.]*, v. 121, n. 3, p. 386–394, 2015. DOI: 10.1002/cncr.29046.

BOLAND, C. Richard; LYNCH, Henry T. The History of Lynch Syndrome. **Familial cancer**, *[S. l.]*, v. 12, n. 2, p. 145–157, 2013. DOI: 10.1007/s10689-013-9637-8.

BONNEVILLE, Russell; KROOK, Melanie A.; KAUTTO, Esko A.; MIYA, Jharna; WING, Michele R.; CHEN, Hui-Zi; REESER, Julie W.; YU, Lianbo; ROYCHOWDHURY, Sameek. Landscape of Microsatellite Instability Across 39 Cancer Types. **JCO precision oncology**, *[S. l.]*, v. 2017, 2017. DOI: 10.1200/PO.17.00073.

BONORA, Giancarlo; RUBBI, Liudmilla; MORSELLI, Marco; MA, Feiyang; CHRONIS, Constantinos; PLATH, Kathrin; PELLEGRINI, Matteo. DNA methylation estimation using methylation-sensitive restriction enzyme bisulfite sequencing (MREBS). **PloS One**, *[S. l.]*, v. 14, n. 4, p. e0214368, 2019. DOI: 10.1371/journal.pone.0214368.

BOYIADZIS, Michael M.; KIRKWOOD, John M.; MARSHALL, John L.; PRITCHARD, Colin C.; AZAD, Nilofer S.; GULLEY, James L. Significance and implications of FDA approval of pembrolizumab for biomarker-defined disease. **Journal for Immunotherapy of Cancer**, *[S. l.]*, v. 6, n. 1, p. 35, 2018. DOI: 10.1186/s40425-018-0342-x.

BRADY, Samuel W.; GOUT, Alexander M.; ZHANG, Jinghui. Therapeutic and prognostic insights from the analysis of cancer mutational signatures. **Trends in genetics: TIG**, *[S. l.]*, v. 38, n. 2, p. 194–208, 2022. DOI: 10.1016/j.tig.2021.08.007.

BRIGGS, Sarah; TOMLINSON, Ian. Germline and somatic polymerase ε and δ mutations define a new class of hypermutated colorectal and endometrial cancers. **The Journal of Pathology**, *[S. l.]*, v. 230, n. 2, p. 148–153, 2013. DOI: 10.1002/path.4185.

BRUEGL, Amanda S.; DJORDJEVIC, Bojana; URBAUER, Diana L.; WESTIN, Shannon N.; SOLIMAN, Pamela T.; LU, Karen H.; LUTHRA, Rajyalakshmi; BROADDUS, Russell R. Utility of MLH1 methylation analysis in the clinical evaluation of Lynch Syndrome in women with endometrial cancer. **Current Pharmaceutical Design**, *[S. l.]*, v. 20, n. 11, p. 1655–1663, 2014. DOI: 10.2174/13816128113199990538.

BUCHANAN, Daniel D. et al. Tumor mismatch repair immunohistochemistry and DNA MLH1 methylation testing of patients with endometrial cancer diagnosed at age younger than 60 years optimizes triage for population-level germline mismatch repair gene mutation testing. **Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology**, *[S. l.]*, v. 32, n. 2, p. 90–100, 2014. a. DOI: 10.1200/JCO.2013.51.2129.

BUCHANAN, Daniel D.; ROSTY, Christophe; CLENDENNING, Mark; SPURDLE, Amanda B.; WIN, Aung Ko. Clinical problems of colorectal cancer and endometrial cancer cases with unknown cause of tumor mismatch repair deficiency (suspected Lynch syndrome). **The Application of Clinical Genetics**, *[S. l.]*, v. 7, p. 183–193, 2014. b. DOI: 10.2147/TACG.S48625.

BUHARD, Olivier; CATTANEO, Francesca; WONG, Yick Fu; YIM, So Fan; FRIEDMAN, Eitan; FLEJOU, Jean-François; DUVAL, Alex; HAMELIN, Richard. Multipopulation analysis

of polymorphisms in five mononucleotide repeats used to determine the microsatellite instability status of human tumors. **Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology**, *[S. l.]*, v. 24, n. 2, p. 241–251, 2006. DOI: 10.1200/JCO.2005.02.7227.

BUHARD, Olivier; SURAWEERA, Nirosha; LECTARD, Aude; DUVAL, Alex; HAMELIN, Richard. Quasimonomorphic Mononucleotide Repeats for High-Level Microsatellite Instability Analysis. **Disease Markers**, *[S. l.]*, v. 20, n. 4–5, p. 251–257, 2004. DOI: 10.1155/2004/159347.

CAHILL, Daniel P. et al. Loss of the mismatch repair protein MSH6 in human glioblastomas is associated with tumor progression during temozolomide treatment. **Clinical Cancer Research: An Official Journal of the American Association for Cancer Research**, *[S. l.]*, v. 13, n. 7, p. 2038–2045, 2007. DOI: 10.1158/1078-0432.CCR-06-2149.

CAMPANELLA, Nathália C.; BERARDINELLI, Gustavo N.; SCAPULATEMPO-NETO, Cristovam; VIANA, Danilo; PALMERO, Edenir I.; PEREIRA, Rui; REIS, Rui M. Optimization of a pentaplex panel for MSI analysis without control DNA in a Brazilian population: correlation with ancestry markers. **European journal of human genetics: EJHG**, *[S. l.]*, v. 22, n. 7, p. 875–880, 2014. DOI: 10.1038/ejhg.2013.256.

CANCER GENOME ATLAS NETWORK. Comprehensive molecular characterization of human colon and rectal cancer. **Nature**, *[S. l.]*, v. 487, n. 7407, p. 330–337, 2012. DOI: 10.1038/nature11252.

CANCER GENOME ATLAS RESEARCH NETWORK et al. Integrated genomic characterization of endometrial carcinoma. **Nature**, *[S. l.]*, v. 497, n. 7447, p. 67–73, 2013. DOI: 10.1038/nature12113.

CAPORALI, Simona; FALCINELLI, Sabrina; STARACE, Giuseppe; RUSSO, Maria Teresa; BONMASSAR, Enzo; JIRICNY, Josef; D'ATRI, Stefania. DNA damage induced by temozolomide signals to both ATM and ATR: role of the mismatch repair system. **Molecular Pharmacology**, *[S. l.]*, v. 66, n. 3, p. 478–491, 2004. DOI: 10.1124/mol.66.3.

CARETHERS, John M. Differentiating Lynch-like from Lynch Syndrome. **Gastroenterology**, *[S. l.]*, v. 146, n. 3, p. 602–604, 2014. DOI: 10.1053/j.gastro.2014.01.041.

CARETHERS, John M.; STOFFEL, Elena M. Lynch syndrome and Lynch syndrome mimics: The growing complex landscape of hereditary colon cancer. **World Journal of Gastroenterology**, *[S. l.]*, v. 21, n. 31, p. 9253–9261, 2015. DOI: 10.3748/wjg.v21.i31.9253.

CARTA, Roberto et al. Cancer Predisposition Syndromes and Medulloblastoma in the Molecular Era. **Frontiers in Oncology**, *[S. l.]*, v. 10, p. 566822, 2020. DOI: 10.3389/fonc.2020.566822.

CASADEI, Silvia et al. Characterization of splice-altering mutations in inherited predisposition to cancer. **Proceedings of the National Academy of Sciences of the United States of America**, *[S. l.]*, v. 116, n. 52, p. 26798–26807, 2019. DOI: 10.1073/pnas.1915608116.

CASTILLEJO, Adela et al. Prevalence of germline MUTYH mutations among Lynch-like syndrome patients. **European Journal of Cancer (Oxford, England: 1990)**, *[S. l.]*, v. 50, n. 13, p. 2241–2250, 2014. DOI: 10.1016/j.ejca.2014.05.022.

CERCEK, Andrea et al. PD-1 Blockade in Mismatch Repair-Deficient, Locally Advanced Rectal Cancer. **The New England Journal of Medicine**, *[S. l.]*, v. 386, n. 25, p. 2363–2376, 2022. DOI: 10.1056/NEJMoa2201445.

CHANEY, Stephen G.; CAMPBELL, Sharon L.; BASSETT, Ekaterina; WU, Yibing. Recognition and processing of cisplatin- and oxaliplatin-DNA adducts. **Critical Reviews in Oncology/Hematology**, *[S. l.]*, v. 53, n. 1, p. 3–11, 2005. DOI: 10.1016/j.critrevonc.2004.08.008.

CHEN, Liang; YAN, Huai-Xiu; LIU, Xiao-Wei; CHEN, Wen-Xin. Clinical efficacy and safety of 6-thioguanine in the treatment of childhood acute lymphoblastic leukemia: A protocol for systematic review and meta-analysis. **Medicine**, *[S. l.]*, v. 99, n. 18, p. e20082, 2020. DOI: 10.1097/MD.0000000000020082.

CHEN, Zhen; TRAN, Mykim; TANG, Mengfan; WANG, Wenqi; GONG, Zihua; CHEN, Junjie. Proteomic Analysis Reveals a Novel Mutator S (MutS) Partner Involved in Mismatch Repair Pathway. **Molecular & cellular proteomics: MCP**, *[S. l.]*, v. 15, n. 4, p. 1299–1308, 2016. DOI: 10.1074/mcp.M115.056093.

CHENG, Donavan T. et al. Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT). **The Journal of Molecular Diagnostics : JMD**, *[S. l.]*, v. 17, n. 3, p. 251–264, 2015. DOI: 10.1016/j.jmoldx.2014.12.006.

CLENDENNING, Mark et al. Mutation deep within an intron of MSH2 causes Lynch syndrome. **Familial cancer**, *[S. l.]*, v. 10, n. 2, p. 297–301, 2011. DOI: 10.1007/s10689-011-9427-0.

COHEN, Stacey A.; PRITCHARD, Colin C.; JARVIK, Gail P. Lynch Syndrome: From Screening to Diagnosis to Treatment in the Era of Modern Molecular Oncology. **Annual Review of Genomics and Human Genetics**, *[S. l.]*, v. 20, n. 1, p. 293–307, 2019. DOI: 10.1146/annurev-genom-083118-015406.

COHEN, Stephanie A.; LEININGER, Anna. The genetic basis of Lynch syndrome and its implications for clinical practice and risk management. **The Application of Clinical Genetics**, *[S. l.]*, v. 7, p. 147–158, 2014. DOI: 10.2147/TACG.S51483.

CORTES-CIRIANO, Isidro; LEE, Sejoon; PARK, Woong-Yang; KIM, Tae-Min; PARK, Peter J. A molecular portrait of microsatellite instability across multiple cancers. **Nature Communications**, *[S. l.]*, v. 8, p. 15180, 2017. DOI: 10.1038/ncomms15180.

CORTEZ, David. Replication-Coupled DNA Repair. **Molecular Cell**, *[S. l.]*, v. 74, n. 5, p. 866–876, 2019. DOI: 10.1016/j.molcel.2019.04.027.

CRAIN, Philip R.; ZEPP, Jamilyn M.; GILLE, Sara; JENKINS, Lindsay; KAUFFMAN, Tia L.; SHUSTER, Elizabeth; GODDARD, Katrina A. B.; WILFOND, Benjamin S.; HUNTER, Jessica Ezzell. Identifying patients with Lynch syndrome using a universal tumor screening program in an integrated healthcare system. **Hereditary Cancer in Clinical Practice**, *[S. l.]*, v. 20, n. 1, p. 17, 2022. DOI: 10.1186/s13053-022-00217-1.

CRISAFULLI, Giovanni et al. Temozolomide Treatment Alters Mismatch Repair and Boosts Mutational Burden in Tumor and Blood of Colorectal Cancer Patients. **Cancer Discovery**, *[S. l.]*, v. 12, n. 7, p. 1656–1675, 2022. DOI: 10.1158/2159-8290.CD-21-1434.

CURTIUS, Kit; GUPTA, Samir; BOLAND, C. Richard. Review article: Lynch Syndrome-a mechanistic and clinical management update. **Alimentary Pharmacology & Therapeutics**, *[S. l.]*, v. 55, n. 8, p. 960–977, 2022. DOI: 10.1111/apt.16826.

DÁMASO, Estela et al. Primary constitutional MLH1 epimutations: a focal epigenetic event. **British Journal of Cancer**, *[S. l.]*, v. 119, n. 8, p. 978–987, 2018. DOI: 10.1038/s41416-018-0019-8.

DÁMASO, Estela et al. Comprehensive Constitutional Genetic and Epigenetic Characterization of Lynch-Like Individuals. **Cancers**, *[S. l.]*, v. 12, n. 7, p. E1799, 2020. DOI: 10.3390/cancers12071799.

DAMIA, Giovanna; BROGGINI, Massimo. Platinum Resistance in Ovarian Cancer: Role of DNA Repair. **Cancers**, *[S. l.]*, v. 11, n. 1, p. 119, 2019. DOI: 10.3390/cancers11010119.

DANAHER, Patrick; WARREN, Sarah; ONG, SuFey; ELLIOTT, Nathan; CESANO, Alessandra; FERREE, Sean. A gene expression assay for simultaneous measurement of microsatellite instability and anti-tumor immune activity. **Journal for Immunotherapy of Cancer**, *[S. l.]*, v. 7, n. 1, p. 15, 2019. DOI: 10.1186/s40425-018-0472-1.

DE, Subhajyoti. Somatic mosaicism in healthy human tissues. **Trends in genetics: TIG**, *[S. l.]*, v. 27, n. 6, p. 217–223, 2011. DOI: 10.1016/j.tig.2011.03.002.

DECORSIÈRE, Adrien et al. Decreased efficiency of MSH6 mRNA polyadenylation linked to a 20-base-pair duplication in Lynch syndrome families. **Cell Cycle (Georgetown, Tex.)**, *[S. l.]*, v. 11, n. 13, p. 2578–2580, 2012. DOI: 10.4161/cc.20625.

DEDEURWAERDERE, Franceska; CLAES, Kathleen Bm; VAN DORPE, Jo; ROTTIERS, Isabelle; VAN DER MEULEN, Joni; BREYNE, Joke; SWAERTS, Koen; MARTENS, Geert. Comparison of microsatellite instability detection by immunohistochemistry and molecular techniques in colorectal and endometrial cancer. **Scientific Reports**, *[S. l.]*, v. 11, n. 1, p. 12880, 2021. DOI: 10.1038/s41598-021-91974-x.

DENG, G.; CHEN, A.; HONG, J.; CHAE, H. S.; KIM, Y. S. Methylation of CpG in a small region of the hMLH1 promoter invariably correlates with the absence of gene expression. **Cancer Research**, *[S. l.]*, v. 59, n. 9, p. 2029–2033, 1999.

DESHPANDE, Madhura; ROMANSKI, Phillip A.; ROSENWAKS, Zev; GERHARDT, Jeannine. Gynecological Cancers Caused by Deficient Mismatch Repair and Microsatellite Instability. **Cancers**, *[S. l.]*, v. 12, n. 11, p. E3319, 2020. DOI: 10.3390/cancers12113319.

DEXHEIMER, Philipp J.; COCHELLA, Luisa. MicroRNAs: From Mechanism to Organism. **Frontiers in Cell and Developmental Biology**, *[S. l.]*, v. 8, p. 409, 2020. DOI: 10.3389/fcell.2020.00409.

DHAR, Ruby; SEETHY, Ashikh; SINGH, Sunil; PETHUSAMY, Karthikeyan; SRIVASTAVA, Tryambak; TALUKDAR, Joyeeta; RATH, Goura Kishor; KARMAKAR, Subhradip. Cancer immunotherapy: Recent advances and challenges. **Journal of Cancer Research and Therapeutics**, *[S. l.]*, v. 17, n. 4, p. 834–844, 2021. DOI: 10.4103/jcrt.JCRT_1241_20.

DI MARCO, Marco et al. Which Lynch syndrome screening programs could be implemented in the “real world”? A systematic review of economic evaluations. **Genetics in Medicine**, *[S. l.]*, v. 20, n. 10, p. 1131–1144, 2018. DOI: 10.1038/gim.2017.244.

DOMINGUEZ-VALENTIN, Mev et al. Cancer risks by gene, age, and gender in 6350 carriers of pathogenic mismatch repair variants: findings from the Prospective Lynch Syndrome Database. **Genetics in Medicine: Official Journal of the American College of Medical Genetics**, *[S. l.]*, v. 22, n. 1, p. 15–25, 2020. DOI: 10.1038/s41436-019-0596-9.

DOS SANTOS, Wellington; DE ANDRADE, Edilene Santos; GARCIA, Felipe Antonio de Oliveira; CAMPACCI, Natália; SÁBATO, Cristina da Silva; MELENDEZ, Matias Eliseo; REIS, Rui Manuel; GALVÃO, Henrique de Campos Reis; PALMERO, Edenir Inez. Whole-Exome Sequencing Identifies Pathogenic Germline Variants in Patients with Lynch-Like Syndrome. **Cancers**, *[S. l.]*, v. 14, n. 17, p. 4233, 2022. DOI: 10.3390/cancers14174233.

DUDLEY, Jonathan C.; LIN, Ming-Tseh; LE, Dung T.; ESHLEMAN, James R. Microsatellite Instability as a Biomarker for PD-1 Blockade. **Clinical Cancer Research: An Official Journal of the American Association for Cancer Research**, *[S. l.]*, v. 22, n. 4, p. 813–820, 2016. DOI: 10.1158/1078-0432.CCR-15-1678.

DURATURO, Francesca; LICCARDO, Raffaella; DE ROSA, Marina; IZZO, Paola. Genetics, diagnosis and treatment of Lynch syndrome: Old lessons and current challenges. **Oncology Letters**, *[S. l.]*, v. 17, n. 3, p. 3048–3054, 2019. DOI: 10.3892/ol.2019.9945.

DURNO, Carol et al. Survival Benefit for Individuals With Constitutional Mismatch Repair Deficiency Undergoing Surveillance. **Journal of Clinical Oncology**, *[S. l.]*, 2021. DOI: 10.1200/JCO.20.02636.

EDWARDS, Penelope; MONAHAN, Kevin J. Diagnosis and management of Lynch syndrome. **Frontline Gastroenterology**, *[S. l.]*, v. 13, n. e1, p. e80–e87, 2022. DOI: 10.1136/flgastro-2022-102123.

EGOAVIL, Cecilia et al. Prevalence of Lynch Syndrome among Patients with Newly Diagnosed Endometrial Cancers. **PLOS ONE**, *[S. l.]*, v. 8, n. 11, p. e79737, 2013. a. DOI: 10.1371/journal.pone.0079737.

EGOAVIL, Cecilia et al. Prevalence of Lynch Syndrome among Patients with Newly Diagnosed Endometrial Cancers. **PLoS ONE**, *[S. l.]*, v. 8, n. 11, p. e79737, 2013. b. DOI: 10.1371/journal.pone.0079737.

EIKENBOOM, Ellis L. et al. Universal Immunohistochemistry for Lynch Syndrome: A Systematic Review and Meta-analysis of 58,580 Colorectal Carcinomas. **Clinical Gastroenterology and Hepatology: The Official Clinical Practice Journal of the American Gastroenterological Association**, *[S. l.]*, v. 20, n. 3, p. e496–e507, 2022. DOI: 10.1016/j.cgh.2021.04.021.

ENGEL, Kelly B.; MOORE, Helen M. Effects of preanalytical variables on the detection of proteins by immunohistochemistry in formalin-fixed, paraffin-embedded tissue. **Archives of Pathology & Laboratory Medicine**, *[S. l.]*, v. 135, n. 5, p. 537–543, 2011. DOI: 10.5858/2010-0702-RAIR.1.

EVALUATION OF GENOMIC APPLICATIONS IN PRACTICE AND PREVENTION (EGAPP) WORKING GROUP. Recommendations from the EGAPP Working Group: genetic testing strategies in newly diagnosed individuals with colorectal cancer aimed at reducing morbidity and mortality from Lynch syndrome in relatives. **Genetics in Medicine: Official Journal of the American College of Medical Genetics**, *[S. l.]*, v. 11, n. 1, p. 35–41, 2009. DOI: 10.1097/GIM.0b013e31818fa2ff.

EVRARD, Camille; ALEXANDRE, Jérôme. Predictive and Prognostic Value of Microsatellite Instability in Gynecologic Cancer (Endometrial and Ovarian). **Cancers**, *[S. l.]*, v. 13, n. 10, p. 2434, 2021. DOI: 10.3390/cancers13102434.

FANG, Hu; ZHU, Xiaoqiang; YANG, Haocheng; OH, Jieun; BARBOUR, Jayne A.; WONG, Jason W. H. Deficiency of replication-independent DNA mismatch repair drives a 5-methylcytosine deamination mutational signature in cancer. **Science Advances**, *[S. l.]*, v. 7, n. 45, p. eabg4398, 2021. DOI: 10.1126/sciadv.abg4398.

FEDERICI, Giulia; SODDU, Silvia. Variants of uncertain significance in the era of high-throughput genome sequencing: a lesson from breast and ovary cancers. **Journal of experimental & clinical cancer research: CR**, *[S. l.]*, v. 39, n. 1, p. 46, 2020. DOI: 10.1186/s13046-020-01554-6.

FUKUI, Kenji. DNA Mismatch Repair in Eukaryotes and Bacteria. **Journal of Nucleic Acids**, *[S. l.]*, v. 2010, p. 260512, 2010. DOI: 10.4061/2010/260512.

FULK, Kelly; TURNER, Morgan; EPPOLITO, Amanda; KRUKENBERG, Rebekah. RNA sequencing uncovers clinically actionable germline intronic MSH2 variants in previously unresolved Lynch syndrome families. **BMJ case reports**, *[S. l.]*, v. 15, n. 4, p. e249580, 2022. DOI: 10.1136/bcr-2022-249580.

GANESA, Sachita; SULE, Amrita; SUNDARAM, Ranjini K.; BINDRA, Ranjit S. Mismatch repair proteins play a role in ATR activation upon temozolomide treatment in MGMT-methylated glioblastoma. **Scientific Reports**, *[S. l.]*, v. 12, n. 1, p. 5827, 2022. DOI: 10.1038/s41598-022-09614-x.

GARBER, Judy E.; OFFIT, Kenneth. Hereditary cancer predisposition syndromes. **Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology**, *[S. l.]*, v. 23, n. 2, p. 276–292, 2005. DOI: 10.1200/JCO.2005.10.042.

GAUSACHS, Mireia et al. MLH1 promoter hypermethylation in the analytical algorithm of Lynch syndrome: a cost-effectiveness study. **European journal of human genetics: EJHG**, *[S. l.]*, v. 20, n. 7, p. 762–768, 2012. DOI: 10.1038/ejhg.2011.277.

GENG, Hui; DU, Chunwei; CHEN, Siying; SALERNO, Vincenzo; MANFREDI, Candela; HSIEH, Peggy. In vitro studies of DNA mismatch repair proteins. **Analytical Biochemistry**, *[S. l.]*, v. 413, n. 2, p. 179–184, 2011. DOI: 10.1016/j.ab.2011.02.017.

GEURTS-GIELE, Willemina R. R. et al. Somatic aberrations of mismatch repair genes as a cause of microsatellite-unstable cancers. **The Journal of Pathology**, *[S. l.]*, v. 234, n. 4, p. 548–559, 2014. DOI: 10.1002/path.4419.

GEURTS-GIELE, Willemina R.; ROSENBERG, Efraim H.; VAN RENS, Anja; VAN LEERDAM, Monique E.; DINJENS, Winand N.; BLEEKER, Fonnet E. Somatic mosaicism by a de novo MLH1 mutation as a cause of Lynch syndrome. **Molecular Genetics & Genomic Medicine**, *[S. l.]*, v. 7, n. 7, p. e00699, 2019. DOI: 10.1002/mgg3.699.

GIARDIELLO, Francis M. et al. Guidelines on genetic evaluation and management of Lynch syndrome: a consensus statement by the US Multi-Society Task Force on colorectal cancer. **Gastroenterology**, *[S. l.]*, v. 147, n. 2, p. 502–526, 2014. DOI: 10.1053/j.gastro.2014.04.001.

GOLUBICKI, Mariano et al. Comprehensive Genomic Characterization of Fifteen Early-Onset Lynch-Like Syndrome Colorectal Cancers. **Cancers**, *[S. l.]*, v. 13, n. 6, p. 1259, 2021. DOI: 10.3390/cancers13061259.

GREEN, R. C.; GREEN, A. G.; SIMMS, M.; PATER, A.; ROBB, J. D.; GREEN, J. S. Germline hMLH1 promoter mutation in a Newfoundland HNPCC kindred. **Clinical Genetics**, *[S. l.]*, v. 64, n. 3, p. 220–227, 2003. DOI: 10.1034/j.1399-0004.2003.t01-1-00110.x.

GROOTHUIZEN, Flora S.; SIXMA, Titia K. The conserved molecular machinery in DNA mismatch repair enzyme structures. **DNA repair**, *[S. l.]*, v. 38, p. 14–23, 2016. DOI: 10.1016/j.dnarep.2015.11.012.

GUILLERM, Erell; SVRCEK, Magali; BARDIER-DUPAS, Armelle; BASSET, Noémie; COULET, Florence; COLAS, Chrystelle. Molecular tumor testing in patients with Lynch-like syndrome reveals a de novo mosaic variant of a mismatch repair gene transmitted to offspring. **European Journal of Human Genetics**, *[S. l.]*, v. 28, n. 11, p. 1624–1628, 2020. DOI: 10.1038/s41431-020-0689-6.

GUILLOTIN, Delphine; MARTIN, Sarah A. Exploiting DNA mismatch repair deficiency as a therapeutic strategy. **Experimental Cell Research**, *[S. l.]*, v. 329, n. 1, p. 110–115, 2014. DOI: 10.1016/j.yexcr.2014.07.004.

GUPTA, Dipika; HEINEN, Christopher D. The mismatch repair-dependent DNA damage response: Mechanisms and implications. **DNA repair**, *[S. l.]*, v. 78, p. 60–69, 2019. DOI: 10.1016/j.dnarep.2019.03.009.

GURIN, C. C.; FEDERICI, M. G.; KANG, L.; BOYD, J. Causes and consequences of microsatellite instability in endometrial carcinoma. **Cancer Research**, *[S. l.]*, v. 59, n. 2, p. 462–466, 1999.

HALL, Geoffrey; CLARKSON, Adele; SHI, Amanda; LANGFORD, Eileen; LEUNG, Helen; ECKSTEIN, Robert P.; GILL, Anthony J. Immunohistochemistry for PMS2 and MSH6 alone can replace a four antibody panel for mismatch repair deficiency screening in colorectal adenocarcinoma. **Pathology**, *[S. l.]*, v. 42, n. 5, p. 409–413, 2010. DOI: 10.3109/00313025.2010.493871.

HAMPEL, Heather et al. Screening for Lynch syndrome (hereditary nonpolyposis colorectal cancer) among endometrial cancer patients. **Cancer Research**, *[S. l.]*, v. 66, n. 15, p. 7810–7817, 2006. DOI: 10.1158/0008-5472.CAN-06-1114.

HAMZAOUI, Nadim et al. Genetic, structural, and functional characterization of POLE polymerase proofreading variants allows cancer risk prediction. **Genetics in Medicine: Official Journal of the American College of Medical Genetics**, *[S. l.]*, v. 22, n. 9, p. 1533–1541, 2020. DOI: 10.1038/s41436-020-0828-z.

HAN, Leng; ZHAO, Zhongming. CpG islands or CpG clusters: how to identify functional GC-rich regions in a genome? **BMC bioinformatics**, *[S. l.]*, v. 10, p. 65, 2009. DOI: 10.1186/1471-2105-10-65.

HAN, Xinyin et al. MSIsensor-ct: microsatellite instability detection using cfDNA sequencing data. **Briefings in Bioinformatics**, *[S. l.]*, v. 22, n. 5, p. bbaa402, 2021. DOI: 10.1093/bib/bbaa402. Disponível em: https://doi.org/10.1093/bib/bbaa402. Acesso em: 13 set. 2022.

HARADHVALA, N. J. et al. Distinct mutational signatures characterize concurrent loss of polymerase proofreading and mismatch repair. **Nature Communications**, *[S. l.]*, v. 9, n. 1, p. 1746, 2018. DOI: 10.1038/s41467-018-04002-4.

HARALDSDOTTIR, Sigurdis; HAMPEL, Heather; TOMSIC, Jerneja; FRANKEL, Wendy L.; PEARLMAN, Rachel; DE LA CHAPELLE, Albert; PRITCHARD, Colin C. Colon and Endometrial Cancers With Mismatch Repair Deficiency Can Arise From Somatic, Rather Than Germline, Mutations. **Gastroenterology**, *[S. l.]*, v. 147, n. 6, p. 1308- 1316.e1, 2014. DOI: 10.1053/j.gastro.2014.08.041.

HEMPELMANN, Jennifer A.; SCROGGINS, Sheena M.; PRITCHARD, Colin C.; SALIPANTE, Stephen J. MSIplus for Integrated Colorectal Cancer Molecular Testing by Next-Generation Sequencing. **The Journal of molecular diagnostics: JMD**, *[S. l.]*, v. 17, n. 6, p. 705–714, 2015. DOI: 10.1016/j.jmoldx.2015.05.008.

HERKERT, Johanna C. et al. Paediatric intestinal cancer and polyposis due to bi-allelic PMS2 mutations: Case series, review and follow-up guidelines. **European Journal of Cancer**, *[S. l.]*, v. 47, n. 7, p. 965–982, 2011. DOI: 10.1016/j.ejca.2011.01.013.

HERMAN, J. G. et al. Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. **Proceedings of the National Academy of Sciences of the United States of America**, *[S. l.]*, v. 95, n. 12, p. 6870–6875, 1998. DOI: 10.1073/pnas.95.12.6870.

HESSON, Luke B. et al. Lynch Syndrome Associated with Two MLH1 Promoter Variants and Allelic Imbalance of MLH1 Expression. **Human Mutation**, *[S. l.]*, v. 36, n. 6, p. 622–630, 2015. DOI: 10.1002/humu.22785.

HEWISH, Madeleine; LORD, Christopher J.; MARTIN, Sarah A.; CUNNINGHAM, David; ASHWORTH, Alan. Mismatch repair deficient colorectal cancer in the era of personalized treatment. **Nature Reviews. Clinical Oncology**, *[S. l.]*, v. 7, n. 4, p. 197–208, 2010. a. DOI: 10.1038/nrclinonc.2010.18.

HEWISH, Madeleine; LORD, Christopher J.; MARTIN, Sarah A.; CUNNINGHAM, David; ASHWORTH, Alan. Mismatch repair deficient colorectal cancer in the era of personalized treatment. **Nature Reviews Clinical Oncology**, *[S. l.]*, v. 7, n. 4, p. 197–208, 2010. b. DOI: 10.1038/nrclinonc.2010.18.

HITCHINS, M. P.; WARD, R. L. Constitutional (germline) MLH1 epimutation as an aetiological mechanism for hereditary non-polyposis colorectal cancer. **Journal of Medical Genetics**, *[S. l.]*, v. 46, n. 12, p. 793–802, 2009. DOI: 10.1136/jmg.2009.068122.

HITCHINS, Megan P. Constitutional epimutation as a mechanism for cancer causality and heritability? **Nature Reviews. Cancer**, *[S. l.]*, v. 15, n. 10, p. 625–634, 2015. DOI: 10.1038/nrc4001.

HOTEIT, Mira; ONEISSI, Zeina; REDA, Ranim; WAKIM, Fadi; ZAIDAN, Amar; FARRAN, Mohammad; ABI-KHALIL, Elie; EL-SIBAI, Mirvat. Cancer immunotherapy: A comprehensive appraisal of its modes of application. **Oncology Letters**, *[S. l.]*, v. 22, n. 3, p. 655, 2021. DOI: 10.3892/ol.2021.12916.

HUANG, Dongqing; SAVAGE, Sara R.; CALINAWAN, Anna P.; LIN, Chenwei; ZHANG, Bing; WANG, Pei; STARR, Timothy K.; BIRRER, Michael J.; PAULOVICH, Amanda G. A highly annotated database of genes associated with platinum resistance in cancer. **Oncogene**, *[S. l.]*, v. 40, n. 46, p. 6395–6405, 2021. DOI: 10.1038/s41388-021-02055-2.

HUNTER, Chris et al. A hypermutation phenotype and somatic MSH6 mutations in recurrent human malignant gliomas after alkylator chemotherapy. **Cancer Research**, *[S. l.]*, v. 66, n. 8, p. 3987–3991, 2006. DOI: 10.1158/0008-5472.CAN-06-0127.

IWAIZUMI, Moriya; TSENG-ROGENSKI, Stephanie; CARETHERS, John M. DNA mismatch repair proficiency executing 5-fluorouracil cytotoxicity in colorectal cancer cells. **Cancer Biology & Therapy**, *[S. l.]*, v. 12, n. 8, p. 756–764, 2011. DOI: 10.4161/cbt.12.8.17169.

JACOBS, Angelika L.; SCHÄR, Primo. DNA glycosylases: in DNA repair and beyond. **Chromosoma**, *[S. l.]*, v. 121, n. 1, p. 1–20, 2012. DOI: 10.1007/s00412-011-0347-4.

JAFFRELOT, Marion et al. An unusual phenotype occurs in 15% of mismatch repair-deficient tumors and is associated with non-colorectal cancers and genetic syndromes. **Modern Pathology**, *[S. l.]*, v. 35, n. 3, p. 427–437, 2022. DOI: 10.1038/s41379-021-00918-3.

JANSEN, Anne ML et al. Combined mismatch repair and POLE/POLD1 defects explain unresolved suspected Lynch syndrome cancers. **European Journal of Human Genetics**, *[S. l.]*, v. 24, n. 7, p. 1089–1092, 2016. DOI: 10.1038/ejhg.2015.252.

JASPERSON, Kw; SAMOWITZ, Ws; BURT, Rw. Constitutional mismatch repair-deficiency syndrome presenting as colonic adenomatous polyposis: clues from the skin. **Clinical Genetics**, *[S. l.]*, v. 80, n. 4, p. 394–397, 2011. DOI: 10.1111/j.1399-0004.2010.01543.x.

JASS, Jeremy R. Hereditary Non-Polyposis Colorectal Cancer: the rise and fall of a confusing term. **World Journal of Gastroenterology**, *[S. l.]*, v. 12, n. 31, p. 4943–4950, 2006. DOI: 10.3748/wjg.v12.i31.4943.

JIA, Peng; YANG, Xiaofei; GUO, Li; LIU, Bowen; LIN, Jiadong; LIANG, Hao; SUN, Jianyong; ZHANG, Chengsheng; YE, Kai. MSIsensor-pro: Fast, Accurate, and Matched-normal-sample-free Detection of Microsatellite Instability. **Genomics, Proteomics & Bioinformatics**, *[S. l.]*, v. 18, n. 1, p. 65–71, 2020. DOI: 10.1016/j.gpb.2020.02.001.

JOOST, Patrick; VEURINK, Nynke; HOLCK, Susanne; KLARSKOV, Louise; BOJESEN, Anders; HARBO, Maria; BALDETORP, Bo; RAMBECH, Eva; NILBERT, Mef. Heterogenous mismatch-repair status in colorectal cancer. **Diagnostic Pathology**, *[S. l.]*, v. 9, p. 126, 2014. DOI: 10.1186/1746-1596-9-126.

JOVER, Rodrigo et al. The efficacy of adjuvant chemotherapy with 5-fluorouracil in colorectal cancer depends on the mismatch repair status. **European Journal of Cancer (Oxford, England: 1990)**, *[S. l.]*, v. 45, n. 3, p. 365–373, 2009. DOI: 10.1016/j.ejca.2008.07.016.

JU, Jennifer Y.; MILLS, Anne M.; MAHADEVAN, Mani S.; FAN, Jinbo; CULP, Stephen H.; THOMAS, Martha H.; CATHRO, Helen P. Universal Lynch Syndrome Screening Should be Performed in All Upper Tract Urothelial Carcinomas. **The American Journal of Surgical Pathology**, *[S. l.]*, v. 42, n. 11, p. 1549–1555, 2018. DOI: 10.1097/PAS.0000000000001141.

KAIRUPAN, Carla; SCOTT, Rodney J. Base excision repair and the role of MUTYH. **Hereditary Cancer in Clinical Practice**, *[S. l.]*, v. 5, n. 4, p. 199–209, 2007. DOI: 10.1186/1897-4287-5-4-199.

KANG, Sanghee; NA, Younghyun; JOUNG, Sung Yup; LEE, Sun Il; OH, Sang Cheul; MIN, Byung Wook. The significance of microsatellite instability in colorectal cancer after controlling for clinicopathological factors. **Medicine**, *[S. l.]*, v. 97, n. 9, p. e0019, 2018. DOI: 10.1097/MD.0000000000010019.

KANG, So Young; KIM, Deok Geun; AHN, Soomin; HA, Sang Yun; JANG, Kee-Taek; KIM, Kyoung-Mee. Comparative analysis of microsatellite instability by next-generation sequencing, MSI PCR and MMR immunohistochemistry in 1942 solid cancers. **Pathology, Research and Practice**, *[S. l.]*, v. 233, p. 153874, 2022. DOI: 10.1016/j.prp.2022.153874.

KANOPIENĖ, Daiva; SMAILYTĖ, Giedrė; VIDUGIRIENĖ, Jolanta; BACHER, Jeff. Impact of microsatellite instability on survival of endometrial cancer patients. **Medicina (Kaunas, Lithuania)**, *[S. l.]*, v. 50, n. 4, p. 216–221, 2014. DOI: 10.1016/j.medici.2014.09.002.

KANOPIENE, Daiva; VIDUGIRIENE, Jolanta; VALUCKAS, Konstantinas Povilas; SMAILYTE, Giedre; ULECKIENE, Saule; BACHER, Jeff. Endometrial cancer and microsatellite instability status. **Open Medicine**, *[S. l.]*, v. 10, n. 1, p. 70–76, 2014. DOI: 10.1515/med-2015-0005.

KASPER, Edwige et al. Detecting inversions in routine molecular diagnosis in MMR genes. **Familial Cancer**, *[S. l.]*, 2022. DOI: 10.1007/s10689-021-00287-5.

KAUTTO, Esko A.; BONNEVILLE, Russell; MIYA, Jharna; YU, Lianbo; KROOK, Melanie A.; REESER, Julie W.; ROYCHOWDHURY, Sameek. Performance evaluation for rapid detection of pan-cancer microsatellite instability with MANTIS. **Oncotarget**, *[S. l.]*, v. 8, n. 5, p. 7452–7463, 2016. DOI: 10.18632/oncotarget.13918.

KAWAKAMI, Hisato; ZAANAN, Aziz; SINICROPE, Frank A. Implications of mismatch repair-deficient status on management of early stage colorectal cancer. **Journal of Gastrointestinal Oncology**, *[S. l.]*, v. 6, n. 6, p. 676–684, 2015. DOI: 10.3978/j.issn.2078-6891.2015.065.

KEEGAN, Niall P.; WILTON, Steve D.; FLETCHER, Sue. Analysis of Pathogenic Pseudoexons Reveals Novel Mechanisms Driving Cryptic Splicing. **Frontiers in Genetics**, *[S. l.]*, v. 12, p. 806946, 2022. DOI: 10.3389/fgene.2021.806946.

KEMPERS, Marlies JE et al. HIGH COLORECTAL AND LOW ENDOMETRIAL CANCER RISK IN EPCAM DELETION-POSITIVE LYNCH SYNDROME: A COHORT STUDY. **The lancet oncology**, *[S. l.]*, v. 12, n. 1, p. 49–55, 2011. DOI: 10.1016/S1470-2045(10)70265-5.

KIM, Chang Gon et al. Effects of microsatellite instability on recurrence patterns and outcomes in colorectal cancers. **British Journal of Cancer**, *[S. l.]*, v. 115, n. 1, p. 25–33, 2016. a. DOI: 10.1038/bjc.2016.161.

KIM, Jung Ho; BAE, Jeong Mo; CHO, Nam-Yun; KANG, Gyeong Hoon. Distinct features between MLH1-methylated and unmethylated colorectal carcinomas with the CpG island methylator phenotype: implications in the serrated neoplasia pathway. **Oncotarget**, *[S. l.]*, v. 7, n. 12, p. 14095–14111, 2016. b. DOI: 10.18632/oncotarget.7374.

KIYOZUMI, Yoshimi; MATSUBAYASHI, Hiroyuki; HORIUCHI, Yasue; OISHI, Takuma; ABE, Masato; OHNAMI, Sumiko; NARUOKA, Akane; KUSUHARA, Masatoshi; YAMAGUCHI, Ken. A novel MLH1 intronic variant in a young Japanese patient with Lynch syndrome. **Human Genome Variation**, *[S. l.]*, v. 5, p. 3, 2018. DOI: 10.1038/s41439-018-0002-1.

KOHONEN-CORISH, Maija R. J. et al. Low microsatellite instability is associated with poor prognosis in stage C colon cancer. **Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology**, *[S. l.]*, v. 23, n. 10, p. 2318–2324, 2005. DOI: 10.1200/JCO.2005.00.109.

KONSTANTINOPOULOS, Panagiotis A.; MATULONIS, Ursula A. POLE mutations as an alternative pathway for microsatellite instability in endometrial cancer: implications for Lynch syndrome testing. **Cancer**, *[S. l.]*, v. 121, n. 3, p. 331–334, 2015. DOI: 10.1002/cncr.29057.

KUIPER, Roland P. et al. Recurrence and variability of germline EPCAM deletions in Lynch syndrome. **Human Mutation**, *[S. l.]*, v. 32, n. 4, p. 407–414, 2011. DOI: 10.1002/humu.21446.

KUNKEL, Thomas A.; ERIE, Dorothy A. Eukaryotic Mismatch Repair in Relation to DNA Replication. **Annual review of genetics**, *[S. l.]*, v. 49, p. 291–313, 2015. DOI: 10.1146/annurev-genet-112414-054722.

KUNNACKAL JOHN, George; DAS VILLGRAN, Vipin; CAUFIELD-NOLL, Christine; GIARDIELLO, Francis M. Comparison of universal screening in major lynch-associated tumors: a systematic review of literature. **Familial Cancer**, *[S. l.]*, v. 21, n. 1, p. 57–67, 2022. DOI: 10.1007/s10689-020-00226-w.

LANCASTER, Johnathan M. et al. Society of Gynecologic Oncologists Education Committee statement on risk assessment for inherited gynecologic cancer predispositions. **Gynecologic Oncology**, *[S. l.]*, v. 107, n. 2, p. 159–162, 2007. DOI: 10.1016/j.ygyno.2007.09.031.

LANCASTER, Johnathan M.; POWELL, C. Bethan; CHEN, Lee-May; RICHARDSON, Debra L.; SGO CLINICAL PRACTICE COMMITTEE. Society of Gynecologic Oncology statement on risk assessment for inherited gynecologic cancer predispositions. **Gynecologic Oncology**, *[S. l.]*, v. 136, n. 1, p. 3–7, 2015. DOI: 10.1016/j.ygyno.2014.09.009.

LARSEN, Rikke Hebo et al. Increments in DNA-thioguanine level during thiopurine-enhanced maintenance therapy of acute lymphoblastic leukemia. **Haematologica**, *[S. l.]*, v. 106, n. 11, p. 2824–2833, 2021. DOI: 10.3324/haematol.2020.278166.

LATHAM, Alicia et al. Microsatellite Instability Is Associated With the Presence of Lynch Syndrome Pan-Cancer. **Journal of Clinical Oncology**, *[S. l.]*, v. 37, n. 4, p. 286–295, 2019. DOI: 10.1200/JCO.18.00283.

LAWRENCE, Jessica; RICHER, Lara; ARSENEAU, Jocelyne; ZENG, Xing; CHONG, George; WEBER, Evan; FOULKES, William; PALMA, Laura. Mismatch Repair Universal Screening of Endometrial Cancers (MUSE) in a Canadian Cohort. **Current Oncology**, *[S. l.]*, v. 28, n. 1, p. 509–522, 2021. DOI: 10.3390/curroncol28010052.

LE, Dung T. et al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. **The New England Journal of Medicine**, *[S. l.]*, v. 372, n. 26, p. 2509–2520, 2015. DOI: 10.1056/NEJMoa1500596.

LE, Dung T. et al. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. **Science (New York, N.Y.)**, *[S. l.]*, v. 357, n. 6349, p. 409–413, 2017. DOI: 10.1126/science.aan6733.

LECLERC, Julie; VERMAUT, Catherine; BUISINE, Marie-Pierre. Diagnosis of Lynch Syndrome and Strategies to Distinguish Lynch-Related Tumors from Sporadic MSI/dMMR Tumors. **Cancers**, *[S. l.]*, v. 13, n. 3, p. 467, 2021. DOI: 10.3390/cancers13030467.

LEE, Petra W. C. et al. Evaluating the impact of universal Lynch syndrome screening in a publicly funded healthcare system. **Cancer Medicine**, *[S. l.]*, v. 9, n. 18, p. 6507–6514, 2020. DOI: 10.1002/cam4.3279.

LEE, Soo Young et al. Low-Level Microsatellite Instability as a Potential Prognostic Factor in Sporadic Colorectal Cancer. **Medicine**, *[S. l.]*, v. 94, n. 50, p. e2260, 2015. DOI: 10.1097/MD.0000000000002260.

LEFOL, Cedrick; SOHIER, Emilie; BAUDET, Christian; NAÏBO, Pierre; RUANO, Eric; GRAND-MASSON, Chloé; VIARI, Alain; WANG, Qing. Acquired somatic MMR deficiency is a major cause of MSI tumor in patients suspected for "Lynch-like syndrome" including young patients. **European Journal of Human Genetics**, *[S. l.]*, v. 29, n. 3, p. 482–488, 2021. DOI: 10.1038/s41431-020-00778-6.

LEGIUS, Eric et al. Revised diagnostic criteria for neurofibromatosis type 1 and Legius syndrome: an international consensus recommendation. **Genetics in Medicine**, *[S. l.]*, v. 23, n. 8, p. 1506–1513, 2021. DOI: 10.1038/s41436-021-01170-5.

LI, Feng; MAO, Guogen; TONG, Dan; HUANG, Jian; GU, Liya; YANG, Wei; LI, Guo-Min. The histone mark H3K36me3 regulates human DNA mismatch repair through its interaction with MutSα. **Cell**, *[S. l.]*, v. 153, n. 3, p. 590–600, 2013. a. DOI: 10.1016/j.cell.2013.03.025.

LI, Jianfeng; KOCZOR, Christopher A.; SAVILLE, Kate M.; HAYAT, Faisal; BEISER, Alison; MCCLELLAN, Steven; MIGAUD, Marie E.; SOBOL, Robert W. Overcoming Temozolomide Resistance in Glioblastoma via Enhanced NAD+ Bioavailability and Inhibition of Poly-ADP-Ribose Glycohydrolase. **Cancers**, *[S. l.]*, v. 14, n. 15, p. 3572, 2022. DOI: 10.3390/cancers14153572.

LI, Kai; LUO, Haiqing; HUANG, Lianfang; LUO, Hui; ZHU, Xiao. Microsatellite instability: a review of what the oncologist should know. **Cancer Cell International**, *[S. l.]*, v. 20, p. 16, 2020. a. DOI: 10.1186/s12935-019-1091-8.

LI, Lin; FENG, Qiushi; WANG, Xiaosheng. PreMSIm: An R package for predicting microsatellite instability from the expression profiling of a gene panel in cancer. **Computational and Structural Biotechnology Journal**, *[S. l.]*, v. 18, p. 668–675, 2020. DOI: 10.1016/j.csbj.2020.03.007.

LI, Xia et al. MLH1 promoter methylation frequency in colorectal cancer patients and related clinicopathological and molecular features. **PloS One**, *[S. l.]*, v. 8, n. 3, p. e59064, 2013. b. DOI: 10.1371/journal.pone.0059064.

LI, Yirong; SALO-MULLEN, Erin; VARGHESE, Anna; TROTTIER, Magan; STADLER, Zsofia K.; ZHANG, Liying. Insertion of an Alu-like element in MLH1 intron 7 as a novel cause of Lynch syndrome. **Molecular Genetics & Genomic Medicine**, *[S. l.]*, v. 8, n. 12, p. e1523, 2020. b. DOI: 10.1002/mgg3.1523.

LI, Zhongdao; PEARLMAN, Alexander H.; HSIEH, Peggy. DNA mismatch repair and the DNA damage response. **DNA repair**, *[S. l.]*, v. 38, p. 94–101, 2016. DOI: 10.1016/j.dnarep.2015.11.019.

LICCARDO, Raffaella; SESSA, Raffaele; TROMBETTI, Silvia; DE ROSA, Marina; IZZO, Paola; GROSSO, Michela; DURATURO, Francesca. MiR-137 Targets the 3’ Untranslated Region of MSH2: Potential Implications in Lynch Syndrome-Related Colorectal Cancer. **Cancers**, *[S. l.]*, v. 13, n. 18, p. 4662, 2021. DOI: 10.3390/cancers13184662.

LIGTENBERG, Marjolijn J. L. et al. Heritable somatic methylation and inactivation of MSH2 in families with Lynch syndrome due to deletion of the 3′ exons of TACSTD1. **Nature Genetics**, *[S. l.]*, v. 41, n. 1, p. 112–117, 2009. DOI: 10.1038/ng.283.

LIU, Dekang; KEIJZERS, Guido; RASMUSSEN, Lene Juel. DNA mismatch repair and its many roles in eukaryotic cells. **Mutation Research. Reviews in Mutation Research**, *[S. l.]*, v. 773, p. 174–187, 2017. DOI: 10.1016/j.mrrev.2017.07.001.

LIU, Qing; HESSON, Luke B.; NUNEZ, Andrea C.; PACKHAM, Deborah; WILLIAMS, Rachel; WARD, Robyn L.; SLOANE, Mathew A. A cryptic paracentric inversion of MSH2 exons 2-6 causes Lynch syndrome. **Carcinogenesis**, *[S. l.]*, v. 37, n. 1, p. 10–17, 2016. DOI: 10.1093/carcin/bgv154.

LOCKE, Warwick J.; GUANZON, Dominic; MA, Chenkai; LIEW, Yi Jin; DUESING, Konsta R.; FUNG, Kim Y. C.; ROSS, Jason P. DNA Methylation Cancer Biomarkers: Translation to the Clinic. **Frontiers in Genetics**, *[S. l.]*, v. 10, p. 1150, 2019. DOI: 10.3389/fgene.2019.01150.

LOUGHREY, Maurice B. et al. Identifying mismatch repair-deficient colon cancer: near-perfect concordance between immunohistochemistry and microsatellite instability testing in a large, population-based series. **Histopathology**, *[S. l.]*, v. 78, n. 3, p. 401–413, 2021. DOI: 10.1111/his.14233.

LOUKOVAARA, Mikko; PASANEN, Annukka; BÜTZOW, Ralf. Mismatch repair protein and MLH1 methylation status as predictors of response to adjuvant therapy in endometrial cancer. **Cancer Medicine**, *[S. l.]*, v. 10, n. 3, p. 1034–1042, 2021. DOI: 10.1002/cam4.3691.

LU, A.-Lien. DNA repair | DNA Mismatch Repair in Bacteria☆. *Em*: JEZ, Joseph (org.). **Encyclopedia of Biological Chemistry III (Third Edition)**. Oxford: Elsevier, 2021. p. 188–196. DOI: 10.1016/B978-0-12-819460-7.00174-2.

LU, Mingdong; ZHAO, Bin; LIU, Mengshan; WU, Le; LI, Yingying; ZHAI, Yingna; SHEN, Xian. Pan-cancer analysis of SETD2 mutation and its association with the efficacy of immunotherapy. **NPJ Precision Oncology**, *[S. l.]*, v. 5, p. 51, 2021. DOI: 10.1038/s41698-021-00193-0.

LYNCH, H. T. et al. Hereditary nonpolyposis colorectal cancer (Lynch syndromes I and II). I. Clinical description of resource. **Cancer**, *[S. l.]*, v. 56, n. 4, p. 934–938, 1985. DOI: 10.1002/1097-0142(19850815)56:4<934::aid-cncr2820560439>3.0.co;2-i.

LYNCH, H. T.; KRUSH, A. J. Cancer family "G" revisited: 1895-1970. **Cancer**, *[S. l.]*, v. 27, n. 6, p. 1505–1511, 1971. DOI: 10.1002/1097-0142(197106)27:6<1505::aid-cncr2820270635>3.0.co;2-l.

LYNCH, H. T.; SHAW, M. W.; MAGNUSON, C. W.; LARSEN, A. L.; KRUSH, A. J. Hereditary factors in cancer. Study of two large midwestern kindreds. **Archives of Internal Medicine**, *[S. l.]*, v. 117, n. 2, p. 206–212, 1966.

LYNCH, Henry T.; SNYDER, Carrie L.; SHAW, Trudy G.; HEINEN, Christopher D.; HITCHINS, Megan P. Milestones of Lynch syndrome: 1895–2015. **Nature Reviews Cancer**, *[S. l.]*, v. 15, n. 3, p. 181–194, 2015. DOI: 10.1038/nrc3878.

LYNCH, HT; LYNCH, PM; LANSPA, SJ; SNYDER, CL; LYNCH, JF; BOLAND, CR. Review of the Lynch syndrome: history, molecular genetics, screening, differential diagnosis, and medicolegal ramifications. **Clinical genetics**, *[S. l.]*, v. 76, n. 1, p. 1–18, 2009. DOI: 10.1111/j.1399-0004.2009.01230.x.

MACHIN, Pilar et al. Microsatellite instability and immunostaining for MSH-2 and MLH-1 in cutaneous and internal tumors from patients with the Muir-Torre syndrome. **Journal of Cutaneous Pathology**, *[S. l.]*, v. 29, n. 7, p. 415–420, 2002. DOI: 10.1034/j.1600-0560.2002.290705.x.

MACKAY, Helen J.; GALLINGER, Steven; TSAO, Ming S.; MCLACHLIN, C. Meg; TU, Dongsheng; KEISER, Katharina; EISENHAUER, Elizabeth A.; OZA, Amit M. Prognostic value of microsatellite instability (MSI) and PTEN expression in women with endometrial cancer: results from studies of the NCIC Clinical Trials Group (NCIC CTG). **European Journal of Cancer (Oxford, England: 1990)**, *[S. l.]*, v. 46, n. 8, p. 1365–1373, 2010. DOI: 10.1016/j.ejca.2010.02.031.

MALOBERTI, Thais et al. BRAF and MLH1 Analysis Algorithm for the Evaluation of Lynch Syndrome Risk in Colorectal Carcinoma Patients: Evidence-Based Data from the Analysis of 100 Consecutive Cases. **Journal of Molecular Pathology**, *[S. l.]*, v. 3, n. 3, p. 115–124, 2022. DOI: 10.3390/jmp3030011.

MANHART, Carol M. DNA repair | DNA Mismatch Repair in Mammals☆. *Em*: JEZ, Joseph (org.). **Encyclopedia of Biological Chemistry III (Third Edition)**. Oxford: Elsevier, 2021. p. 197–206. DOI: 10.1016/B978-0-12-819460-7.00234-6.

MANHART, Carol M.; ALANI, Eric. Roles for mismatch repair family proteins in promoting meiotic crossing over. **DNA repair**, *[S. l.]*, v. 38, p. 84–93, 2016. DOI: 10.1016/j.dnarep.2015.11.024.

MARTIN, Lynn M.; MARPLES, Brian; COFFEY, Mary; LAWLER, Mark; LYNCH, Thomas H.; HOLLYWOOD, Donal; MARIGNOL, Laure. DNA mismatch repair and the DNA damage response to ionizing radiation: Making sense of apparently conflicting data. **Cancer Treatment Reviews**, *[S. l.]*, v. 36, n. 7, p. 518–527, 2010. DOI: 10.1016/j.ctrv.2010.03.008.

MARTÍNEZ-ROCA, Alejandro; GINER-CALABUIG, Mar; MURCIA, Oscar; CASTILLEJO, Adela; SOTO, José Luis; GARCÍA-HEREDIA, Anabel; JOVER, Rodrigo. Lynch-like Syndrome: Potential Mechanisms and Management. **Cancers**, *[S. l.]*, v. 14, n. 5, p. 1115, 2022. DOI: 10.3390/cancers14051115.

MCCARTHY, Aoife J.; CAPO-CHICHI, Jose-Mario; SPENCE, Tara; GRENIER, Sylvie; STOCKLEY, Tracy; KAMEL-REID, Suzanne; SERRA, Stefano; SABATINI, Peter; CHETTY, Runjan. Heterogenous loss of mismatch repair (MMR) protein expression: a challenge for immunohistochemical interpretation and microsatellite instability (MSI) evaluation. **The Journal of Pathology. Clinical Research**, *[S. l.]*, v. 5, n. 2, p. 115–129, 2019. DOI: 10.1002/cjp2.120.

MCCONECHY, M. K.; TALHOUK, A.; LI-CHANG, H. H.; LEUNG, S.; HUNTSMAN, D. G.; GILKS, C. B.; MCALPINE, J. N. Detection of DNA mismatch repair (MMR) deficiencies by immunohistochemistry can effectively diagnose the microsatellite instability (MSI) phenotype in endometrial carcinomas. **Gynecologic Oncology**, *[S. l.]*, v. 137, n. 2, p. 306–310, 2015. DOI: 10.1016/j.ygyno.2015.01.541.

MCGEE, Rose B.; NICHOLS, Kim E. Introduction to cancer genetic susceptibility syndromes. **Hematology: the American Society of Hematology Education Program**, *[S. l.]*, v. 2016, n. 1, p. 293–301, 2016.

MCMEEKIN, D. Scott et al. Clinicopathologic Significance of Mismatch Repair Defects in Endometrial Cancer: An NRG Oncology/Gynecologic Oncology Group Study. **Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology**, *[S. l.]*, v. 34, n. 25, p. 3062–3068, 2016. DOI: 10.1200/JCO.2016.67.8722.

MEIER, Bettina; VOLKOVA, Nadezda V.; HONG, Ye; SCHOFIELD, Pieta; CAMPBELL, Peter J.; GERSTUNG, Moritz; GARTNER, Anton. Mutational signatures of DNA mismatch repair deficiency in C. elegans and human cancers. **Genome Research**, *[S. l.]*, v. 28, n. 5, p. 666–675, 2018. DOI: 10.1101/gr.226845.117.

MENG, Chun-Feng; DAI, Dong-Qiu; GUO, Ke-Jun. [Effects of 5-Aza-2'-deoxycytidine and trichostatin A on DNA methylation and expression of hMLH1 in ovarian cancer cell line COC1/DDP]. **Ai Zheng = Aizheng = Chinese Journal of Cancer**, *[S. l.]*, v. 27, n. 12, p. 1251–1255, 2008.

MENSENKAMP, Arjen R. et al. Somatic mutations in MLH1 and MSH2 are a frequent cause of mismatch-repair deficiency in Lynch syndrome-like tumors. **Gastroenterology**, *[S. l.]*, v. 146, n. 3, p. 643- 646.e8, 2014. DOI: 10.1053/j.gastro.2013.12.002.

MEYER, Claus; BRIEGER, Angela; PLOTZ, Guido; WEBER, Nicole; PASSMANN, Sandra; DINGERMANN, Theo; ZEUZEM, Stefan; TROJAN, Joerg; MARSCHALEK, Rolf. An interstitial deletion at 3p21.3 results in the genetic fusion of MLH1 and ITGA9 in a Lynch syndrome family. **Clinical Cancer Research: An Official Journal of the American**

Association for Cancer Research, *[S. l.]*, v. 15, n. 3, p. 762–769, 2009. DOI: 10.1158/1078-0432.CCR-08-1908.

MEYER, Larissa A.; BROADDUS, Russell R.; LU, Karen H. Endometrial Cancer and Lynch Syndrome: Clinical and Pathologic Considerations. **Cancer control : journal of the Moffitt Cancer Center**, *[S. l.]*, v. 16, n. 1, p. 14–22, 2009.

MIYAKURA, Yasuyuki et al. Methylation profile of the MLH1 promoter region and their relationship to colorectal carcinogenesis. **Genes, Chromosomes & Cancer**, *[S. l.]*, v. 36, n. 1, p. 17–25, 2003. DOI: 10.1002/gcc.10134.

MOJTAHED, Amirkaveh; SCHRIJVER, Iris; FORD, James M.; LONGACRE, Teri A.; PAI, Reetesh K. A two-antibody mismatch repair protein immunohistochemistry screening approach for colorectal carcinomas, skin sebaceous tumors, and gynecologic tract carcinomas. **Modern Pathology: An Official Journal of the United States and Canadian Academy of Pathology, Inc**, *[S. l.]*, v. 24, n. 7, p. 1004–1014, 2011. DOI: 10.1038/modpathol.2011.55.

MOORE, Lisa D.; LE, Thuc; FAN, Guoping. DNA methylation and its basic function. **Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology**, *[S. l.]*, v. 38, n. 1, p. 23–38, 2013. DOI: 10.1038/npp.2012.112.

MORAK, Monika et al. Biallelic MLH1 SNP cDNA expression or constitutional promoter methylation can hide genomic rearrangements causing Lynch syndrome. **Journal of Medical Genetics**, *[S. l.]*, v. 48, n. 8, p. 513–519, 2011. DOI: 10.1136/jmedgenet-2011-100050.

MORAK, Monika; HEIDENREICH, Barbara; KELLER, Gisela; HAMPEL, Heather; LANER, Andreas; DE LA CHAPELLE, Albert; HOLINSKI-FEDER, Elke. Biallelic MUTYH mutations can mimic Lynch syndrome. **European Journal of Human Genetics**, *[S. l.]*, v. 22, n. 11, p. 1334–1337, 2014. DOI: 10.1038/ejhg.2014.15.

MORALES-BURGOS, Adisbeth; SÁNCHEZ, Jorge L.; FIGUEROA, Luz D.; DE JESÚS-MONGE, Wilfredo E.; CRUZ-CORREA, Marcia R.; GONZÁLEZ-KEELAN, Carmen; NAZARIO, Cruz María. MSH-2 and MLH-1 protein expression in Muir Torre syndrome-related and sporadic sebaceous neoplasms. **Puerto Rico Health Sciences Journal**, *[S. l.]*, v. 27, n. 4, p. 322–327, 2008.

MOREIRA, Leticia et al. Prevalence of somatic mutl homolog 1 promoter hypermethylation in Lynch syndrome colorectal cancer. **Cancer**, *[S. l.]*, v. 121, n. 9, p. 1395–1404, 2015. DOI: 10.1002/cncr.29190.

MORK, Maureen E.; RODRIGUEZ, Andrea; TAGGART, Melissa W.; RODRIGUEZ-BIGAS, Miguel A.; LYNCH, Patrick M.; BANNON, Sarah A.; YOU, Y. Nancy; VILAR, Eduardo. Identification of MSH2 inversion of exons 1-7 in clinical evaluation of families with suspected Lynch syndrome. **Familial Cancer**, *[S. l.]*, v. 16, n. 3, p. 357–361, 2017. DOI: 10.1007/s10689-016-9960-y.

NARINE, Kelly A. D.; KEULING, Angela M.; GOMBOS, Randi; TRON, Victor A.; ANDREW, Susan E.; YOUNG, Leah C. Defining the DNA mismatch repair-dependent apoptotic pathway in primary cells: evidence for p53-independence and involvement of centrosomal caspase 2. **DNA repair**, *[S. l.]*, v. 9, n. 2, p. 161–168, 2010. DOI: 10.1016/j.dnarep.2009.11.010.

NAZEMALHOSSEINI MOJARAD, Ehsan et al. Low Level of Microsatellite Instability Correlates with Poor Clinical Prognosis in Stage II Colorectal Cancer Patients. **Journal of Oncology**, *[S. l.]*, v. 2016, p. 2196703, 2016. DOI: 10.1155/2016/2196703.

NELL, Rogier J.; VAN STEENDEREN, Debby; MENGER, Nino V.; WEITERING, Thomas J.; VERSLUIS, Mieke; VAN DER VELDEN, Pieter A. Quantification of DNA methylation independent of sodium bisulfite conversion using methylation-sensitive restriction enzymes and digital PCR. **Human Mutation**, *[S. l.]*, v. 41, n. 12, p. 2205–2216, 2020. DOI: 10.1002/humu.24111.

NÉMETH, Eszter; LOVRICS, Anna; GERVAI, Judit Z.; SEKI, Masayuki; ROSPO, Giuseppe; BARDELLI, Alberto; SZÜTS, Dávid. Two main mutational processes operate in the absence of DNA mismatch repair. **DNA repair**, *[S. l.]*, v. 89, p. 102827, 2020. DOI: 10.1016/j.dnarep.2020.102827.

NEWTON, K.; JORGENSEN, NM; WALLACE, AJ; BUCHANAN, DD; LALLOO, F.; MCMAHON, RFT; HILL, J.; EVANS, DG. Tumour MLH1 promoter region methylation testing is an effective pre-screen for Lynch Syndrome (HNPCC). **Journal of medical genetics**, *[S. l.]*, v. 51, n. 12, p. 789–796, 2014. DOI: 10.1136/jmedgenet-2014-102552.

NIKITIN, Aleksey G. et al. Lynch Syndrome Germline Mutations in Breast Cancer: Next Generation Sequencing Case-Control Study of 1,263 Participants. **Frontiers in Oncology**, *[S. l.]*, v. 10, p. 666, 2020. DOI: 10.3389/fonc.2020.00666.

NISHIYAMA, Atsuya; NAKANISHI, Makoto. Navigating the DNA methylation landscape of cancer. **Trends in genetics: TIG**, *[S. l.]*, v. 37, n. 11, p. 1012–1027, 2021. DOI: 10.1016/j.tig.2021.05.002.

O'BRIEN, Jacob; HAYDER, Heyam; ZAYED, Yara; PENG, Chun. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. **Frontiers in Endocrinology**, *[S. l.]*, v. 9, p. 402, 2018. DOI: 10.3389/fendo.2018.00402.

OFFMAN, Judith et al. Defective DNA mismatch repair in acute myeloid leukemia/myelodysplastic syndrome after organ transplantation. **Blood**, *[S. l.]*, v. 104, n. 3, p. 822–828, 2004. DOI: 10.1182/blood-2003-11-3938.

OLDFIELD, Leslie E. et al. An Integrative DNA Sequencing and Methylation Panel to Assess Mismatch Repair Deficiency. **The Journal of molecular diagnostics: JMD**, *[S. l.]*, v. 23, n. 2, p. 242–252, 2021. DOI: 10.1016/j.jmoldx.2020.11.006.

PAČÍNKOVÁ, Anna; POPOVICI, Vlad. Cross-platform Data Analysis Reveals a Generic Gene Expression Signature for Microsatellite Instability in Colorectal Cancer. **BioMed Research International**, *[S. l.]*, v. 2019, p. 6763596, 2019. DOI: 10.1155/2019/6763596.

PAL, Tuya; PERMUTH-WEY, Jenny; SELLERS, Thomas A. A Review of the Clinical Relevance of Mismatch-Repair Deficiency in Ovarian Cancer. **Cancer**, *[S. l.]*, v. 113, n. 4, p. 733–742, 2008. DOI: 10.1002/cncr.23601.

PALLES, Claire et al. Germline mutations affecting the proofreading domains of POLE and POLD1 predispose to colorectal adenomas and carcinomas. **Nature Genetics**, *[S. l.]*, v. 45, n. 2, p. 136–144, 2013. DOI: 10.1038/ng.2503.

PASTRELLO, Chiara; FORNASARIG, Mara; PIN, Elisa; BERTO, Eleonora; PIVETTA, Barbara; VIEL, Alessandra. Somatic mosaicism in a patient with Lynch syndrome. **American Journal of Medical Genetics. Part A**, *[S. l.]*, v. 149A, n. 2, p. 212–215, 2009. DOI: 10.1002/ajmg.a.32620.

PEARLMAN, Rachel et al. Prevalence and Spectrum of Germline Cancer Susceptibility Gene Mutations Among Patients With Early-Onset Colorectal Cancer. **JAMA oncology**, *[S. l.]*, v. 3, n. 4, p. 464–471, 2017. DOI: 10.1001/jamaoncol.2016.5194.

PEĆINA-ŠLAUS, Nives; KAFKA, Anja; SALAMON, Iva; BUKOVAC, Anja. Mismatch Repair Pathway, Genome Stability and Cancer. **Frontiers in Molecular Biosciences**, *[S. l.]*, v. 7, 2020.

PENG, Yong; CROCE, Carlo M. The role of MicroRNAs in human cancer. **Signal Transduction and Targeted Therapy**, *[S. l.]*, v. 1, p. 15004, 2016. DOI: 10.1038/sigtrans.2015.4.

PÉREZ-CABORNERO, Lucia et al. Characterization of new founder Alu-mediated rearrangements in MSH2 gene associated with a Lynch syndrome phenotype. **Cancer Prevention Research (Philadelphia, Pa.)**, *[S. l.]*, v. 4, n. 10, p. 1546–1555, 2011. DOI: 10.1158/1940-6207.CAPR-11-0227.

PÉREZ-RUIZ, Elisabeth; MELERO, Ignacio; KOPECKA, Joanna; SARMENTO-RIBEIRO, Ana Bela; GARCÍA-ARANDA, Marilina; DE LAS RIVAS, Javier. Cancer immunotherapy resistance based on immune checkpoints inhibitors: Targets, biomarkers, and remedies. **Drug Resistance Updates: Reviews and Commentaries in Antimicrobial and Anticancer Chemotherapy**, *[S. l.]*, v. 53, p. 100718, 2020. DOI: 10.1016/j.drup.2020.100718.

PETERSEN, Sanne M. et al. Functional examination of MLH1, MSH2, and MSH6 intronic mutations identified in Danish colorectal cancer patients. **BMC medical genetics**, *[S. l.]*, v. 14, p. 103, 2013. DOI: 10.1186/1471-2350-14-103.

PLUMB, J. A.; STRATHDEE, G.; SLUDDEN, J.; KAYE, S. B.; BROWN, R. Reversal of drug resistance in human tumor xenografts by 2'-deoxy-5-azacytidine-induced demethylation of the hMLH1 gene promoter. **Cancer Research**, *[S. l.]*, v. 60, n. 21, p. 6039–6044, 2000.

PONTI, G.; MANFREDINI, M.; TOMASI, A.; PELLACANI, G. Muir-Torre Syndrome and founder mismatch repair gene mutations: A long gone historical genetic challenge. **Gene**, *[S. l.]*, v. 589, n. 2, p. 127–132, 2016. DOI: 10.1016/j.gene.2015.06.078.

POPE, Bernard J. et al. **Germline and Tumor Whole Genome Sequencing as a Diagnostic Tool to Resolve Suspected Lynch Syndrome**. medRxiv, , 2020. DOI: 10.1101/2020.03.12.20034991.

PORKKA, Noora; LAHTINEN, Laura; AHTIAINEN, Maarit; BÖHM, Jan P.; KUOPIO, Teijo; ELDFORS, Samuli; MECKLIN, Jukka-Pekka; SEPPÄLÄ, Toni T.; PELTOMÄKI, Päivi. Epidemiological, clinical and molecular characterization of Lynch-like syndrome: A population-based study. **International Journal of Cancer**, *[S. l.]*, v. 145, n. 1, p. 87–98, 2019. DOI: 10.1002/ijc.32085.

POULOGIANNIS, George; FRAYLING, Ian M.; ARENDS, Mark J. DNA mismatch repair deficiency in sporadic colorectal cancer and Lynch syndrome. **Histopathology**, *[S. l.]*, v. 56, n. 2, p. 167–179, 2010. DOI: 10.1111/j.1365-2559.2009.03392.x.

PRITCHARD, Colin C. et al. ColoSeq Provides Comprehensive Lynch and Polyposis Syndrome Mutational Analysis Using Massively Parallel Sequencing. **The Journal of Molecular Diagnostics : JMD**, *[S. l.]*, v. 14, n. 4, p. 357–366, 2012. DOI: 10.1016/j.jmoldx.2012.03.002.

PUTNAM, Christopher D. Strand discrimination in DNA mismatch repair. **DNA repair**, *[S. l.]*, v. 105, p. 103161, 2021. DOI: 10.1016/j.dnarep.2021.103161.

QUEZADA-DIAZ, Felipe; GOMEZ, Javier; FULLE, Angelo; IRARRAZAVAL, María Jesús; TORRES, Javiera; BELLOLIO, Felipe. Results of an immunohistochemistry-based universal screening strategy for Lynch syndrome in patients with colorectal cancer treated in a public hospital from Latin-America. **Surgery Open Digestive Advance**, *[S. l.]*, v. 5, p. 100041, 2022. DOI: 10.1016/j.soda.2022.100041.

RAFFONE, Antonio; TRAVAGLINO, Antonio; CERBONE, Marco; GENCARELLI, Annarita; MOLLO, Antonio; INSABATO, Luigi; ZULLO, Fulvio. Diagnostic Accuracy of Immunohistochemistry for Mismatch Repair Proteins as Surrogate of Microsatellite Instability Molecular Testing in Endometrial Cancer. **Pathology oncology research: POR**, *[S. l.]*, v. 26, n. 3, p. 1417–1427, 2020. DOI: 10.1007/s12253-020-00811-5.

RATNER, Mark. Approval of first tumor gene panel sends shockwaves through labs. **Nature Biotechnology**, *[S. l.]*, v. 36, n. 2, p. 124–125, 2018. DOI: 10.1038/nbt0218-124. Disponível em: https://www.nature.com/articles/nbt0218-124. Acesso em: 13 set. 2022.

RHEES, Jennifer; ARNOLD, Mildred; BOLAND, C. Richard. Inversion of exons 1–7 of the MSH2 gene is a frequent cause of unexplained Lynch syndrome in one local population. **Familial cancer**, *[S. l.]*, v. 13, n. 2, p. 219–225, 2014. DOI: 10.1007/s10689-013-9688-x.

RICCIARDONE, M. D. et al. Human MLH1 deficiency predisposes to hematological malignancy and neurofibromatosis type 1. **Cancer Research**, *[S. l.]*, v. 59, n. 2, p. 290–293, 1999.

RICHMAN, Susan. Deficient mismatch repair: Read all about it (Review). **International Journal of Oncology**, *[S. l.]*, v. 47, n. 4, p. 1189–1202, 2015. DOI: 10.3892/ijo.2015.3119.

RING, Kari L.; BRUEGL, Amanda S.; ALLEN, Brian A.; ELKIN, Eric P.; SINGH, Nanda; HARTMAN, Anne-Renee; DANIELS, Molly S.; BROADDUS, Russell R. Germline multi-gene hereditary cancer panel testing in an unselected endometrial cancer cohort. **Modern Pathology: An Official Journal of the United States and Canadian Academy of Pathology, Inc**, *[S. l.]*, v. 29, n. 11, p. 1381–1389, 2016. DOI: 10.1038/modpathol.2016.135.

RODRIGUEZ-BIGAS, M. A. et al. A National Cancer Institute Workshop on Hereditary Nonpolyposis Colorectal Cancer Syndrome: meeting highlights and Bethesda guidelines. **Journal of the National Cancer Institute**, *[S. l.]*, v. 89, n. 23, p. 1758–1762, 1997. DOI: 10.1093/jnci/89.23.1758.

RODRÍGUEZ-SOLER, María et al. Risk of cancer in cases of suspected lynch syndrome without germline mutation. **Gastroenterology**, *[S. l.]*, v. 144, n. 5, p. 926- 932.e1; quiz e13-14, 2013. DOI: 10.1053/j.gastro.2013.01.044.

ROLDÁN-ARJONA, Teresa; ARIZA, Rafael R.; CÓRDOBA-CAÑERO, Dolores. DNA Base Excision Repair in Plants: An Unfolding Story With Familiar and Novel Characters. **Frontiers in Plant Science**, *[S. l.]*, v. 10, p. 1055, 2019. DOI: 10.3389/fpls.2019.01055.

RUMILLA, Kandelaria et al. Frequency of Deletions of EPCAM (TACSTD1) in MSH2-Associated Lynch Syndrome Cases. **The Journal of Molecular Diagnostics : JMD**, *[S. l.]*, v. 13, n. 1, p. 93–99, 2011. DOI: 10.1016/j.jmoldx.2010.11.011.

RYAN, N. A. J.; GLAIRE, M. A.; BLAKE, D.; CABRERA-DANDY, M.; EVANS, D. G.; CROSBIE, E. J. The proportion of endometrial cancers associated with Lynch syndrome: a systematic review of the literature and meta-analysis. **Genetics in Medicine**, *[S. l.]*, v. 21, n. 10, p. 2167–2180, 2019. DOI: 10.1038/s41436-019-0536-8.

SAWANT, Akshada; KOTHANDAPANI, Anbarasi; ZHITKOVICH, Anatoly; SOBOL, Robert W.; PATRICK, Steve M. Role of mismatch repair proteins in the processing of cisplatin interstrand cross-links. **DNA repair**, *[S. l.]*, v. 35, p. 126–136, 2015. DOI: 10.1016/j.dnarep.2015.10.003.

SCARPA, Melania et al. MLH1 Deficiency Down-Regulates TLR4 Expression in Sporadic Colorectal Cancer. **Frontiers in Molecular Biosciences**, *[S. l.]*, v. 8, p. 624873, 2021. DOI: 10.3389/fmolb.2021.624873.

SHCHERBAKOVA, Polina V.; BEBENEK, Katarzyna; KUNKEL, Thomas A. Functions of eukaryotic DNA polymerases. **Science of aging knowledge environment: SAGE KE**, *[S. l.]*, v. 2003, n. 8, p. RE3, 2003. DOI: 10.1126/sageke.2003.8.re3.

SHIMOZAKI, Keitaro et al. Concordance analysis of microsatellite instability status between polymerase chain reaction based testing and next generation sequencing for solid tumors. **Scientific Reports**, *[S. l.]*, v. 11, n. 1, p. 20003, 2021. DOI: 10.1038/s41598-021-99364-z.

SHIN, Ki-Hyuk; SHIN, Joo-Ho; KIM, Jung-Hwa; PARK, Jae-Gahb. Mutational analysis of promoters of mismatch repair genes hMSH2 and hMLH1 in hereditary nonpolyposis colorectal cancer and early onset colorectal cancer patients: identification of three novel germ-line mutations in promoter of the hMSH2 gene. **Cancer Research**, *[S. l.]*, v. 62, n. 1, p. 38–42, 2002.

SHLIEN, Adam et al. Combined hereditary and somatic mutations of replication error repair genes result in rapid onset of ultra-hypermutated cancers. **Nature Genetics**, *[S. l.]*, v. 47, n. 3, p. 257–262, 2015. DOI: 10.1038/ng.3202.

SIJMONS, Rolf H.; GREENBLATT, Marc S.; GENUARDI, Maurizio. Gene variants of unknown clinical significance in Lynch syndrome. An introduction for clinicians. **Familial Cancer**, *[S. l.]*, v. 12, n. 2, p. 181–187, 2013. DOI: 10.1007/s10689-013-9629-8.

SIMPKINS, S. B.; BOCKER, T.; SWISHER, E. M.; MUTCH, D. G.; GERSELL, D. J.; KOVATICH, A. J.; PALAZZO, J. P.; FISHEL, R.; GOODFELLOW, P. J. MLH1 promoter methylation and gene silencing is the primary cause of microsatellite instability in sporadic endometrial cancers. **Human Molecular Genetics**, *[S. l.]*, v. 8, n. 4, p. 661–666, 1999. DOI: 10.1093/hmg/8.4.661.

SINGH, Ashish Kumar; OLSEN, Maren Fridtjofsen; LAVIK, Liss Anne Solberg; VOLD, Trine; DRABLØS, Finn; SJURSEN, Wenche. Detecting copy number variation in next generation sequencing data from diagnostic gene panels. **BMC Medical Genomics**, *[S. l.]*, v. 14, n. 1, p. 214, 2021. DOI: 10.1186/s12920-021-01059-x.

SOROKIN, Maksim et al. Experimental and Meta-Analytic Validation of RNA Sequencing Signatures for Predicting Status of Microsatellite Instability. **Frontiers in Molecular Biosciences**, *[S. l.]*, v. 8, p. 737821, 2021. DOI: 10.3389/fmolb.2021.737821.

SOURROUILLE, Isabelle; COULET, Florence; LEFEVRE, Jeremie H.; COLAS, Chrystelle; EYRIES, Mélanie; SVRCEK, Magali; BARDIER-DUPAS, Armelle; PARC, Yann; SOUBRIER, Florent. Somatic mosaicism and double somatic hits can lead to MSI colorectal tumors. **Familial Cancer**, *[S. l.]*, v. 12, n. 1, p. 27–33, 2013. DOI: 10.1007/s10689-012-9568-9.

SOUTH, Christopher D.; HAMPEL, Heather; COMERAS, Ilene; WESTMAN, Judith A.; FRANKEL, Wendy L.; DE LA CHAPELLE, Albert. The Frequency of Muir-Torre Syndrome Among Lynch Syndrome Families. **JNCI: Journal of the National Cancer Institute**, *[S. l.]*, v. 100, n. 4, p. 277–281, 2008. DOI: 10.1093/jnci/djm291.

STEELE, Christopher D.; PILLAY, Nischalan; ALEXANDROV, Ludmil B. An overview of mutational and copy number signatures in human cancer. **The Journal of Pathology**, *[S. l.]*, v. 257, n. 4, p. 454–465, 2022. DOI: 10.1002/path.5912.

STOJIC, Lovorka; MOJAS, Nina; CEJKA, Petr; DI PIETRO, Massimiliano; FERRARI, Stefano; MARRA, Giancarlo; JIRICNY, Josef. Mismatch repair-dependent G2 checkpoint induced by low doses of SN1 type methylating agents requires the ATR kinase. **Genes & Development**, *[S. l.]*, v. 18, n. 11, p. 1331–1344, 2004. DOI: 10.1101/gad.294404.

STRATHDEE, G.; MACKEAN, M. J.; ILLAND, M.; BROWN, R. A role for methylation of the hMLH1 promoter in loss of hMLH1 expression and drug resistance in ovarian cancer. **Oncogene**, *[S. l.]*, v. 18, n. 14, p. 2335–2341, 1999. DOI: 10.1038/sj.onc.1202540.

STRATIGOPOULOU, Maria; VAN DAM, Tijmen P.; GUIKEMA, Jeroen E. J. Base Excision Repair in the Immune System: Small DNA Lesions With Big Consequences. **Frontiers in Immunology**, *[S. l.]*, v. 11, p. 1084, 2020. DOI: 10.3389/fimmu.2020.01084.

SUN, Sibo et al. Identification of Germline Mismatch Repair Gene Mutations in Lung Cancer Patients With Paired Tumor-Normal Next Generation Sequencing: A Retrospective Study. **Frontiers in Oncology**, *[S. l.]*, v. 9, p. 550, 2019. DOI: 10.3389/fonc.2019.00550.

SURAWEERA, Nirosha; DUVAL, Alex; REPERANT, Maryline; VAURY, Christelle; FURLAN, Daniela; LEROY, Karen; SERUCA, Raquel; IACOPETTA, Barry; HAMELIN, Richard. Evaluation of tumor microsatellite instability using five quasimonomorphic mononucleotide repeats and pentaplex PCR. **Gastroenterology**, *[S. l.]*, v. 123, n. 6, p. 1804–1811, 2002. DOI: 10.1053/gast.2002.37070.

SYNGAL, Sapna; BRAND, Randall E.; CHURCH, James M.; GIARDIELLO, Francis M.; HAMPEL, Heather L.; BURT, Randall W.; AMERICAN COLLEGE OF GASTROENTEROLOGY. ACG clinical guideline: Genetic testing and management of hereditary gastrointestinal cancer syndromes. **The American Journal of Gastroenterology**, *[S. l.]*, v. 110, n. 2, p. 223–262; quiz 263, 2015. DOI: 10.1038/ajg.2014.435.

TALSETH-PALMER, Bente A.; BAUER, Denis C.; SJURSEN, Wenche; EVANS, Tiffany J.; MCPHILLIPS, Mary; PROIETTO, Anthony; OTTON, Geoffrey; SPIGELMAN, Allan D.; SCOTT, Rodney J. Targeted next-generation sequencing of 22 mismatch repair genes identifies Lynch syndrome families. **Cancer Medicine**, *[S. l.]*, v. 5, n. 5, p. 929–941, 2016. DOI: 10.1002/cam4.628.

TANAKAYA, Kohji. Current clinical topics of Lynch syndrome. **International Journal of Clinical Oncology**, *[S. l.]*, v. 24, n. 9, p. 1013–1019, 2019. DOI: 10.1007/s10147-018-1282-7.

TATE, John G. et al. COSMIC: the Catalogue Of Somatic Mutations In Cancer. **Nucleic Acids Research**, *[S. l.]*, v. 47, n. D1, p. D941–D947, 2019. DOI: 10.1093/nar/gky1015.

THOMPSON, Bryony A. et al. Application of a 5-tiered scheme for standardized classification of 2,360 unique mismatch repair gene variants in the InSiGHT locus-specific database. **Nature Genetics**, *[S. l.]*, v. 46, n. 2, p. 107–115, 2014. DOI: 10.1038/ng.2854.

TOGNETTO, Alessia; MICHELAZZO, Maria Benedetta; CALABRÓ, Giovanna Elisa; UNIM, Brigid; DI MARCO, Marco; RICCIARDI, Walter; PASTORINO, Roberta; BOCCIA, Stefania. A Systematic Review on the Existing Screening Pathways for Lynch Syndrome Identification. **Frontiers in Public Health**, *[S. l.]*, v. 5, p. 243, 2017. DOI: 10.3389/fpubh.2017.00243.

TOKSVANG, Linea N. et al. DNA-thioguanine concentration and relapse risk in children and young adults with acute lymphoblastic leukemia: an IPD meta-analysis. **Leukemia**, *[S. l.]*, v. 36, n. 1, p. 33–41, 2022. DOI: 10.1038/s41375-021-01182-9.

TORSHIZI ESFAHANI, Amir; SEYEDNA, Seyed Yoosef; NAZEMALHOSSEINI MOJARAD, Ehsan; MAJD, Ahmad; ASADZADEH AGHDAEI, Hamid. MSI-L/EMAST is a predictive biomarker for metastasis in colorectal cancer patients. **Journal of Cellular Physiology**, *[S. l.]*, v. 234, n. 8, p. 13128–13136, 2019. DOI: 10.1002/jcp.27983.

TUTLEWSKA, Katarzyna; LUBINSKI, Jan; KURZAWSKI, Grzegorz. Germline deletions in the EPCAM gene as a cause of Lynch syndrome – literature review. **Hereditary Cancer in Clinical Practice**, *[S. l.]*, v. 11, n. 1, p. 9, 2013. DOI: 10.1186/1897-4287-11-9.

UMAR, Asad et al. Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. **Journal of the National Cancer Institute**, *[S. l.]*, v. 96, n. 4, p. 261–268, 2004. DOI: 10.1093/jnci/djh034.

VALERI, Nicola et al. Modulation of mismatch repair and genomic stability by miR-155. **Proceedings of the National Academy of Sciences of the United States of America**, *[S. l.]*, v. 107, n. 15, p. 6982–6987, 2010. a. DOI: 10.1073/pnas.1002472107.

VALERI, Nicola et al. MicroRNA-21 induces resistance to 5-fluorouracil by down-regulating human DNA MutS homolog 2 (hMSH2). **Proceedings of the National Academy of Sciences of the United States of America**, *[S. l.]*, v. 107, n. 49, p. 21098–21103, 2010. b. DOI: 10.1073/pnas.1015541107.

VAN DER KLIFT, Heleen M.; JANSEN, Anne M. L.; VAN DER STEENSTRATEN, Niki; BIK, Elsa C.; TOPS, Carli M. J.; DEVILEE, Peter; WIJNEN, Juul T. Splicing analysis for exonic and intronic mismatch repair gene variants associated with Lynch syndrome confirms high concordance between minigene assays and patient RNA analyses. **Molecular Genetics & Genomic Medicine**, *[S. l.]*, v. 3, n. 4, p. 327–345, 2015. DOI: 10.1002/mgg3.145.

VARGAS-PARRA, Gardenia M. et al. Elucidating the molecular basis of MSH2-deficient tumors by combined germline and somatic analysis. **International Journal of Cancer**, *[S. l.]*, v. 141, n. 7, p. 1365–1380, 2017. DOI: 10.1002/ijc.30820.

VASEN, H. F.; MECKLIN, J. P.; KHAN, P. M.; LYNCH, H. T. The International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer (ICG-HNPCC). **Diseases of the Colon and Rectum**, *[S. l.]*, v. 34, n. 5, p. 424–425, 1991. DOI: 10.1007/BF02053699.

VASEN, H. F.; WATSON, P.; MECKLIN, J. P.; LYNCH, H. T. New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC. **Gastroenterology**, *[S. l.]*, v. 116, n. 6, p. 1453–1456, 1999. DOI: 10.1016/s0016-5085(99)70510-x.

VENESIO, Tiziana; BALSAMO, Antonella; D'AGOSTINO, Vito G.; RANZANI, Guglielmina N. MUTYH-associated polyposis (MAP), the syndrome implicating base excision repair in inherited predisposition to colorectal tumors. **Frontiers in Oncology**, *[S. l.]*, v. 2, p. 83, 2012. DOI: 10.3389/fonc.2012.00083.

VILAR, Eduardo; GRUBER, Stephen B. Microsatellite instability in colorectal cancer—the stable evidence. **Nature Reviews Clinical Oncology**, *[S. l.]*, v. 7, n. 3, p. 153–162, 2010. DOI: 10.1038/nrclinonc.2009.237.

VILLA, Fabrizio; SIMON, Aline C.; ORTIZ BAZAN, Maria Angeles; KILKENNY, Mairi L.; WIRTHENSOHN, David; WIGHTMAN, Mel; MATAK-VINKOVÍC, Dijana; PELLEGRINI, Luca; LABIB, Karim. Ctf4 Is a Hub in the Eukaryotic Replisome that Links Multiple CIP-Box Proteins to the CMG Helicase. **Molecular Cell**, *[S. l.]*, v. 63, n. 3, p. 385–396, 2016. DOI: 10.1016/j.molcel.2016.06.009.

VOLKOVA, Nadezda V. et al. Mutational signatures are jointly shaped by DNA damage and repair. **Nature Communications**, *[S. l.]*, v. 11, n. 1, p. 2169, 2020. DOI: 10.1038/s41467-020-15912-7.

WAGNER, Anja et al. A 10-Mb paracentric inversion of chromosome arm 2p inactivates MSH2 and is responsible for hereditary nonpolyposis colorectal cancer in a North-American kindred. **Genes, Chromosomes & Cancer**, *[S. l.]*, v. 35, n. 1, p. 49–57, 2002. DOI: 10.1002/gcc.10094.

WALDMAN, Alex D.; FRITZ, Jill M.; LENARDO, Michael J. A guide to cancer immunotherapy: from T cell basic science to clinical practice. **Nature Reviews Immunology**, *[S. l.]*, v. 20, n. 11, p. 651–668, 2020. DOI: 10.1038/s41577-020-0306-5.

WANG, Bingyan; LI, Fei; ZHOU, Xin; MA, Yanpeng; FU, Wei. Is microsatellite instability-high really a favorable prognostic factor for advanced colorectal cancer? A meta-analysis. **World Journal of Surgical Oncology**, *[S. l.]*, v. 17, n. 1, p. 169, 2019. DOI: 10.1186/s12957-019-1706-5.

WANG, Q.; LASSET, C.; DESSEIGNE, F.; FRAPPAZ, D.; BERGERON, C.; NAVARRO, C.; RUANO, E.; PUISIEUX, A. Neurofibromatosis and early onset of cancers in hMLH1-deficient children. **Cancer Research**, *[S. l.]*, v. 59, n. 2, p. 294–297, 1999.

WANG, Shuyue; XIE, Kun; LIU, Tengfei. Cancer Immunotherapies: From Efficacy to Resistance Mechanisms - Not Only Checkpoint Matters. **Frontiers in Immunology**, *[S. l.]*, v. 12, p. 690112, 2021. DOI: 10.3389/fimmu.2021.690112.

WANG, Yang; SHI, Chanjuan; EISENBERG, Rosana; VNENCAK-JONES, Cindy L. Differences in Microsatellite Instability Profiles between Endometrioid and Colorectal Cancers. **The Journal of Molecular Diagnostics : JMD**, *[S. l.]*, v. 19, n. 1, p. 57–64, 2017. DOI: 10.1016/j.jmoldx.2016.07.008.

WARTHIN, ALDRED SCOTT. HEREDITY WITH REFERENCE TO CARCINOMA: AS SHOWN BY THE STUDY OF THE CASES EXAMINED IN THE PATHOLOGICAL LABORATORY OF THE UNIVERSITY OF MICHIGAN, 1895-1913. **Archives of Internal Medicine**, *[S. l.]*, v. XII, n. 5, p. 546–555, 1913. DOI: 10.1001/archinte.1913.00070050063006.

WATSON, Patrice; RILEY, Bronson. The Tumor Spectrum in the Lynch Syndrome. **Familial Cancer**, *[S. l.]*, v. 4, n. 3, p. 245–248, 2005. DOI: 10.1007/s10689-004-7994-z.

WIMMER, Katharina et al. Diagnostic criteria for constitutional mismatch repair deficiency syndrome: suggestions of the European consortium 'Care for CMMRD' (C4CMMRD). **Journal of Medical Genetics**, *[S. l.]*, v. 51, n. 6, p. 355–365, 2014. DOI: 10.1136/jmedgenet-2014-102284.

WIMMER, Katharina; ETZLER, Julia. Constitutional mismatch repair-deficiency syndrome: have we so far seen only the tip of an iceberg? **Human Genetics**, *[S. l.]*, v. 124, n. 2, p. 105–122, 2008. DOI: 10.1007/s00439-008-0542-4.

.

WIMMER, Katharina; KRATZ, Christian P. Constitutional mismatch repair-deficiency syndrome. **Haematologica**, *[S. l.]*, v. 95, n. 5, p. 699–701, 2010. DOI: 10.3324/haematol.2009.021626.

WONG, Yick Fu; CHEUNG, Tak Hong; LO, Keith Wing Kit; YIM, So Fan; CHAN, Loucia Kit Yin; BUHARD, Olivier; DUVAL, Alex; CHUNG, Tony Kwok Hung; HAMELIN, Richard. Detection of microsatellite instability in endometrial cancer: advantages of a panel of five mononucleotide repeats over the National Cancer Institute panel of markers. **Carcinogenesis**, *[S. l.]*, v. 27, n. 5, p. 951–955, 2006. DOI: 10.1093/carcin/bgi333.

WYATT, M. D.; WILSON, D. M. Participation of DNA repair in the response to 5-fluorouracil. **Cellular and molecular life sciences: CMLS**, *[S. l.]*, v. 66, n. 5, p. 788–799, 2009. DOI: 10.1007/s00018-008-8557-5.

XAVIER, Alexandre; OLSEN, Maren Fridtjofsen; LAVIK, Liss A.; JOHANSEN, Jostein; SINGH, Ashish Kumar; SJURSEN, Wenche; SCOTT, Rodney J.; TALSETH-PALMER, Bente A. Comprehensive mismatch repair gene panel identifies variants in patients with Lynch-like syndrome. **Molecular Genetics & Genomic Medicine**, *[S. l.]*, v. 7, n. 8, p. e850, 2019. DOI: 10.1002/mgg3.850.

XIAO, Jian et al. A next-generation sequencing-based strategy combining microsatellite instability and tumor mutation burden for comprehensive molecular diagnosis of advanced colorectal cancer. **BMC cancer**, *[S. l.]*, v. 21, n. 1, p. 282, 2021. DOI: 10.1186/s12885-021-07942-1.

XICOLA, Rosa M. et al. Implication of DNA repair genes in Lynch-like syndrome. **Familial cancer**, *[S. l.]*, v. 18, n. 3, p. 331–342, 2019. DOI: 10.1007/s10689-019-00128-6.

XU, Weizhi; LI, Guo-Min. DNA repair | DNA Mismatch Repair and the DNA Damage Response. *Em*: JEZ, Joseph (org.). **Encyclopedia of Biological Chemistry III (Third Edition)**. Oxford: Elsevier, 2021. p. 232–235. DOI: 10.1016/B978-0-12-819460-7.00238-3.

XU, Yun; HUANG, Zonghao; LI, Cong; ZHU, Congcong; ZHANG, Yuqin; GUO, Tian'an; LIU, Fangqi; XU, Ye. Comparison of Molecular, Clinicopathological, and Pedigree Differences Between Lynch-Like and Lynch Syndromes. **Frontiers in Genetics**, *[S. l.]*, v. 11, 2020. a.

XU, Yun; HUANG, Zonghao; LI, Cong; ZHU, Congcong; ZHANG, Yuqin; GUO, Tian'an; LIU, Fangqi; XU, Ye. Comparison of Molecular, Clinicopathological, and Pedigree Differences Between Lynch-Like and Lynch Syndromes. **Frontiers in Genetics**, *[S. l.]*, v. 11, 2020. b.

YAMAMOTO, Hiroyuki; IMAI, Kohzoh. Microsatellite instability: an update. **Archives of Toxicology**, *[S. l.]*, v. 89, n. 6, p. 899–921, 2015. DOI: 10.1007/s00204-015-1474-0.

YAMANE, Kazuhiko; TAYLOR, Kerri; KINSELLA, Timothy J. Mismatch repair-mediated G2/M arrest by 6-thioguanine involves the ATR-Chk1 pathway. **Biochemical and Biophysical Research Communications**, *[S. l.]*, v. 318, n. 1, p. 297–302, 2004. DOI: 10.1016/j.bbrc.2004.04.030.

YAN, Tao; BERRY, Suzanne E.; DESAI, Anand B.; KINSELLA, Timothy J. DNA mismatch repair (MMR) mediates 6-thioguanine genotoxicity by introducing single-strand breaks to signal a G2-M arrest in MMR-proficient RKO cells. **Clinical Cancer Research: An Official Journal of the American Association for Cancer Research**, *[S. l.]*, v. 9, n. 6, p. 2327–2334, 2003.

YANG, M.; HSIEH, P. Chapter 18 - DNA Mismatch Repair in Mammals. *Em*: KOVALCHUK, Igor; KOVALCHUK, Olga (org.). **Genome Stability**. Boston: Academic Press, 2016. p. 303–319. DOI: 10.1016/B978-0-12-803309-8.00018-5.

YANG, Ming; OLAOBA, Olamide Tosin; ZHANG, Chunye; KIMCHI, Eric T.; STAVELEY-O'CARROLL, Kevin F.; LI, Guangfu. Cancer Immunotherapy and Delivery System: An Update. **Pharmaceutics**, *[S. l.]*, v. 14, n. 8, p. 1630, 2022. DOI: 10.3390/pharmaceutics14081630.

YANG, Zihui; LIU, Xinyu; YANG, Xi; LIAO, Qin-Ping. Screening and identification of Lynch syndrome: a systematic review of the frequency of Lynch syndrome-associated clinicopathologic and molecular characteristics in Lynch syndrome gynecologic cancers. **Translational Cancer Research**, *[S. l.]*, v. 10, n. 10, p. 4523–4531, 2021. DOI: 10.21037/tcr-21-677.

YARCHOAN, Mark; JOHNSON, Burles A.; LUTZ, Eric R.; LAHERU, Daniel A.; JAFFEE, Elizabeth M. Targeting neoantigens to augment antitumour immunity. **Nature Reviews. Cancer**, *[S. l.]*, v. 17, n. 4, p. 209–222, 2017. DOI: 10.1038/nrc.2016.154.

YIP, Stephen; MIAO, Jiangyong; CAHILL, Daniel P.; IAFRATE, A. John; ALDAPE, Ken; NUTT, Catherine L.; LOUIS, David N. MSH6 mutations arise in glioblastomas during temozolomide therapy and mediate temozolomide resistance. **Clinical Cancer Research: An Official Journal of the American Association for Cancer Research**, *[S. l.]*, v. 15, n. 14, p. 4622–4629, 2009. DOI: 10.1158/1078-0432.CCR-08-3012.

YURGELUN, Matthew B.; HAMPEL, Heather. Recent Advances in Lynch Syndrome: Diagnosis, Treatment, and Cancer Prevention. **American Society of Clinical Oncology Educational Book. American Society of Clinical Oncology. Annual Meeting**, *[S. l.]*, v. 38, p. 101–109, 2018. DOI: 10.1200/EDBK_208341.

ZHAO, Chuchu; LI, Saisai; ZHAO, Menghuang; ZHU, Haiyan; ZHU, Xueqiong. Prognostic values of DNA mismatch repair genes in ovarian cancer patients treated with platinum-based chemotherapy. **Archives of Gynecology and Obstetrics**, *[S. l.]*, v. 297, n. 1, p. 153–159, 2018. DOI: 10.1007/s00404-017-4563-x.

ZHAO, Pengfei; LI, Li; JIANG, Xiaoyue; LI, Qin. Mismatch repair deficiency/microsatellite instability-high as a predictor for anti-PD-1/PD-L1 immunotherapy efficacy. **Journal of Hematology & Oncology**, *[S. l.]*, v. 12, n. 1, p. 54, 2019. DOI: 10.1186/s13045-019-0738-1.

ZHENG, Kai et al. A novel NGS-based microsatellite instability (MSI) status classifier with 9 loci for colorectal cancer patients. **Journal of Translational Medicine**, *[S. l.]*, v. 18, n. 1, p. 215, 2020. DOI: 10.1186/s12967-020-02373-1.

ZIGHELBOIM, Israel; GOODFELLOW, Paul J.; GAO, Feng; GIBB, Randall K.; POWELL, Matthew A.; RADER, Janet S.; MUTCH, David G. Microsatellite instability and epigenetic inactivation of MLH1 and outcome of patients with endometrial carcinomas of the endometrioid type. **Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology**, *[S. l.]*, v. 25, n. 15, p. 2042–2048, 2007. DOI: 10.1200/JCO.2006.08.2107.

OBJECTIVES

The overall aim of this thesis is to investigate the genetic etiologies of mismatch repair deficiency in cancer. To achieve this goal, several specific objectives were explored in three chapters, as described below:

CHAPTER I

- To assess the MMR proficiency in a Brazilian cohort of endometrial adenocarcinoma.
- To characterize the germline variants in *MLH1*, *MSH2*, *MSH6*, *PMS2*, and *EPCAM* in patients with MMR-deficient tumors.
- To determine the prevalence of Lynch and Lynch-like syndromes in tumors with MMR deficiency.

CHAPTER II

- To describe the germline variants in genes related to DNA repair and to hereditary endometrial cancer among Lynch-Like patients.
- To characterize somatic driver variants in genes related to cancer-related genes in endometrial tumors of patients with Lynch-like syndrome.
- To assess the MMR proficiency in Lynch-like syndrome tumors by mutational signatures and microsatellite instability scores.

CHAPTER III

- To determine the prevalence of *WDHD1* somatic variants in tumors with unexplained MMR deficiency.
- To generate a *Wdhd1* mutant cell model lacking the domain for interaction between MSH2 and WDHD1 proteins.
- To characterize the mutational profile of *Wdhd1*-mutant cells.
- To evaluate the sensitivity of *Wdhd1*-mutant cells to 6-thioguanine.
- To characterize the impact of 6-thioguanine on the cell cycle of *Wdhd1*- mutant cells.

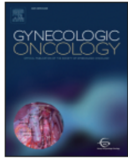
CHAPTER I: Lynch syndrome identification in a Brazilian cohort of endometrial cancer screened by a universal approach

Gynecologic Oncology 159 (2020) 229–238

Contents lists available at ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno



Lynch syndrome identification in a Brazilian cohort of endometrial cancer screened by a universal approach

Reginaldo Cruz Alves Rosa [a,b], Jessica Oliveira Santis [a,b], Lorena Alves Teixeira [c], Greice Andreotti Molfetta [a,b], Jennifer Thalita Targino dos Santos [d], Vanessa dos Santos Ribeiro [e], Fernando Chahud [f], Alfredo Ribeiro-Silva [f], Mariângela Ottoboni Brunaldi [f], Wilson Araújo Silva Jr [a,b], Victor Evangelista de Faria Ferraz [a,g,*]

[a] *Department of Genetics, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil*
[b] *Regional Blood Center at University Hospital of the Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil*
[c] *Department of Gynecology and Obstetrics, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil*
[d] *Post-graduate Program in Public Health Nursing, Ribeirão Preto College of Nursing, University of São Paulo, Ribeirão Preto, Brazil*
[e] *Inter-institutional Doctoral Program in Nursing, Ribeirão Preto College of Nursing, University of São Paulo, Ribeirão Preto, Brazil*
[f] *Department of Pathology and Legal Medicine, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil*
[g] *Center for Medical Genomics at Clinical Hospital of the Ribeirão Preto Medical School, University of São Paulo, Brazil*

HIGHLIGHTS

- Universal screening for LS in women with endometrial cancer is poorly performed in Latin America, including Brazil.
- At least 4.1% of Brazilian women diagnosed with EC are LS carriers.
- LLS individuals had EC at intermediate age between LS and sporadic cases.
- Most LS patients did not meet Amsterdam II Criteria and Revised Bethesda Guidelines.

ARTICLE INFO

Article history:
Received 3 June 2020
Accepted 7 July 2020
Available online 18 July 2020

Keywords:
Brazil
Endometrial cancer
Lynch syndrome
Lynch-like syndrome
Next-generation sequencing
Universal screening

ABSTRACT

Objective. To report the frequency of Lynch syndrome (LS) in a cohort of patients from Southeast Brazil bearing endometrial cancer (EC), using a tumor screening universal approach.

Methods. A total of 242 endometrial carcinomas were screened by immunohistochemistry (IHC) and microsatellite instability (MSI) for detection of DNA mismatch repair deficiency (dMMR). *MLH1* methylation was assessed to identify sporadic cases. Patients with dMMR tumors were recruited for germline variant analysis by next-generation sequencing of the *MLH1*, *MSH2*, *MSH6*, *PMS2*, and *EPCAM* genes.

Results. Ninety-three out of 242 tumors (38.5%) were classified as dMMR based on MSI and IHC results. Of these, 54 cases were selected for germline analysis, and 37/54 (68.5%) were available for sequencing. Ten patients (10/37, 27%) harbored germline pathogenic or likely pathogenic variants, most of them in the *MSH6* gene (4/10, 40%). Seven variants of uncertain significance were found. Eight novel germline variants were identified. The LS prevalence in our cohort was of at least 4.1%. LS patients presented lower mean age at cancer diagnosis compared with patients diagnosed with sporadic EC. Individuals with dMMR tumors, without germline pathogenic variants detected in LS-genes ("Lynch-like" syndrome), had an intermediate mean age at cancer diagnosis between LS and sporadic cases.

Conclusion. This is the first report of the LS prevalence in EC screened by a universal approach in Brazil. Our findings contribute to a better understanding of the mutational landscape of this syndrome in Brazil, which is relevant for improved identification, genetic counseling, prevention and control of cancer in LS.

© 2020 Elsevier Inc. All rights reserved.

* Corresponding author at: Department of Genetics, Ribeirão Preto Medical School, University of São Paulo, 3900 Bandeirantes Avenue, Ribeirao Preto, SP 4049-900, Brazil.
E-mail addresses: reginaldoallves@usp.br (R.C.A. Rosa), jolisantis@gmail.com (J.O. Santis), teixeiralorena.alves@gmail.com (L.A. Teixeira), gamolf@fmrp.usp.br (G.A. Molfetta), thalitatargino@usp.br (J.T.T. dos Santos), vasaribeiro@gmail.com (V.S. Ribeiro), fchahud@fmrp.usp.br (F. Chahud), arsilva@fmrp.usp.br (A. Ribeiro-Silva), mabrunaldi@fmrp.usp.br (M.O. Brunaldi), wilsonjr@usp.br (W.A. Silva Jr), vferraz@usp.br (V.E.F. Ferraz).

https://doi.org/10.1016/j.ygyno.2020.07.013
0090-8258/© 2020 Elsevier Inc. All rights reserved.

1 **Lynch syndrome identification in a Brazilian cohort of endometrial cancer screened by**
2 **a universal approach**
3
4 Reginaldo Cruz Alves Rosa[a,b], Jessica Oliveira Santis[a,b], Lorena Alves Teixeira[c], Greice
5 Andreotti Molfetta[a,b], Jennifer Thalita Targino dos Santos[d], Vanessa dos Santos Ribeiro[e],
6 Fernando Chahud[f], Alfredo Ribeiro-Silva[f], Mariângela Ottoboni Brunaldi[f], Wilson Araújo
7 Silva Jr[a,b], Victor Evangelista de Faria Ferraz[a,g*]
8
9 [a] Department of Genetics, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil
10 [b] Regional Blood Center at University Hospital of the Ribeirão Preto Medical School of University of São Paulo,
11 Ribeirão Preto, SP, Brazil
12 [c] Department of Gynecology and Obstetrics. Ribeirão Preto Medical School, University of São Paulo, Ribeirão
13 Preto, Brazil
14 [d] Post-graduate Program in Public Health Nursing, Ribeirão Preto College of Nursing, University of São Paulo,
15 Ribeirão Preto, Brazil
16 [e] Inter-institutional Doctoral Program in Nursing. Ribeirão Preto College of Nursing, University of São Paulo,
17 Ribeirão Preto, Brazil
18 [f] Department of Pathology and Legal Medicine, Ribeirão Preto Medical School, University of São Paulo, Ribeirão
19 Preto, Brazil
20 [g] Center for Medical Genomics at Clinical Hospital of the Ribeirão Preto Medical School, University of São Paulo,
21 Brazil
22
23 E-mail addresses:
24 reginaldoallves@usp.br (R.C.A. Rosa); jolisantis@gmail.com (J.O. Santis), teixeiralorena.alves@gmail.com
25 (L.A. Teixeira), thalitatargino@usp.br (J.T.T dos Santos), vasaribeiro@gmail.com (V.S. Ribeiro),
26 fchahud@fmrp.usp.br (F. Chahud), arsilva@fmrp.usp.br (A.R. Silva), mabrunaldi@fmrp.usp.br (M.O. Brunaldi),
27 gamolf@fmrp.usp.br (G. A. Molfetta), wilsonjr@usp.br (W. A. Silva-Jr), vferraz@usp.br (V.E.F. Ferraz).
28
29 * Corresponding author:
30 Name: Dr. Victor Evangelista de Faria Ferraz. Address: Department of Genetics, Ribeirão Preto Medical School,
31 University of São Paulo. 3900 Bandeirantes Avenue, Ribeirao Preto, SP, 4049-900, Brazil. E-mail address:
32 vferraz@usp.br Telephone number: +551633154500 Fax number: +551633150222
33

34 **Abstract**

35

36 **Objective:** To report the frequency of Lynch syndrome (LS) in a cohort of patients from
37 Southeast Brazil bearing endometrial cancer (EC), using a tumor screening universal approach.

38

39 **Methods:** A total of 242 endometrial carcinomas were screened by immunohistochemistry
40 (IHC) and microsatellite instability (MSI) for detection of DNA mismatch repair deficiency
41 (dMMR). *MLH1* methylation was assessed to identify sporadic cases. Patients with dMMR
42 tumors were recruited for germline variant analysis by next-generation sequencing of the
43 *MLH1*, *MSH2*, *MSH6*, *PMS2*, and *EPCAM* genes.

44

45 **Results:** Ninety-three out of 242 tumors (38.5%) were classified as dMMR based on MSI and
46 IHC results. Of these, 54 cases were selected for germline analysis, and 37/54 (68.5%) were
47 available for sequencing. Ten patients (10/37, 27%) harbored germline pathogenic or likely
48 pathogenic variants, most of them in the *MSH6* gene (4/10, 40%). Seven variants of uncertain
49 significance were found. Eight novel germline variants were identified. The LS prevalence in
50 our cohort was of at least 4.1%. LS patients presented lower mean age at cancer diagnosis
51 compared with patients diagnosed with sporadic EC. Individuals with dMMR tumors, without
52 germline pathogenic variants detected in LS genes ("Lynch-like" syndrome), had an
53 intermediate mean age at cancer diagnosis between LS and sporadic cases.

54

55 **Conclusion:** This is the first report of the LS prevalence in EC screened by a universal approach
56 in Brazil. Our findings contribute to a better understanding of the mutational landscape of this
57 syndrome in Brazil, which is relevant for improved identification, genetic counseling,
58 prevention and control of cancer in LS.

59

60 **Keywords:** Brazil. Endometrial cancer. Lynch syndrome. Lynch-like syndrome. Next-
61 generation sequencing. Universal screening.

62 **Highlights**

63

64 • Universal screening for LS in women with endometrial cancer is poorly performed in
65 Latin America, including Brazil.

66

67 • At least 4.1% of Brazilian women diagnosed with EC are LS carriers.

68

69 • LLS individuals had EC at intermediate age between LS and sporadic cases.

70

71 • Most LS patients did not meet Amsterdam II Criteria and Revised Bethesda Guidelines.

Introduction

Lynch syndrome (LS) is the most common cancer susceptibility syndrome associated with hereditary endometrial cancer (EC), which is caused by germline pathogenic variants in genes of the DNA mismatch repair (MMR) pathway (*MLH1*, *MSH2, MSH6*, and *PMS2*) or the epithelial cell adhesion molecule (EPCAM) gene [1,2]. Individuals affected by LS have an increased risk of developing various cancers, mainly EC and colorectal cancer (CRC) [3]. Notably, women with LS present up to 49% lifetime risk of EC development, and an elevated risk for colorectal, ovarian, gastric, and other cancers [4].

The role of DNA MMR is to maintain genomic stability by correcting base mismatches and insertion-deletion mismatches that may arise during DNA replication [5]. Inactivation of MMR genes result in defective repair of DNA replication errors that preferentially accumulate in genomic regions called microsatellites, which are composed of repetitive DNA sequences. Consequently, LS-associated tumors commonly exhibit MMR deficiency (dMMR), as reflected by high-level microsatellite instability (MSI-H) or loss of MMR protein expression, which are the hallmarks of these tumors [6].

DNA sequencing is the gold standard technique for identifying germline variants in LS patients [7]. However, due to its relatively high cost, screening strategies were proposed to offer genetic testing only to those with highly suggestive clinical features of LS [8]. The universal screening, which comprises the molecular testing of all endometrial tumors for the MMR deficiency by immunohistochemistry (IHC) or PCR-based microsatellite instability analysis (MSI), has been recommended by several international institutions as the preferred screening approach for LS testing due to its higher sensitivity to detect LS patients in comparison with screening strategies based on clinical criteria [9, 10]

Most tumors arising in LS carriers present with dMMR; however, this tumor phenotype is not specific for LS since around 70% of endometrial tumors with dMMR do not have any LS-associated pathogenic variants on germline testing [11]. Approximately 15-20% of sporadic endometrial carcinomas with dMMR are primarily caused by somatically acquired hypermethylation of both alleles of the *MLH1* promoter, leading to the loss of MLH1 protein expression [3, 12]. The dMMR tumors not related to *MLH1* methylation and without a germline variant in any of the four MMR genes are termed “Lynch-like syndrome” (LLS) [13]. The underlying cause of dMMR in LLS is not well established, and it has been assumed to be heterogeneous, comprising both hereditary and sporadic cases of cancer, making management decisions for LLS patients and their families more complicated than for LS carriers [14].

106 Although universal tumor screening is preferred for identifying LS individuals in many
107 countries, this approach is still not common in Latin America. Up to 70% of the centers that
108 investigate LS in Latin American countries, including Brazil, perform neither MSI nor IHC on
109 EC and CRC to select patients for genetic testing of LS. Most of these centers use only clinical
110 criteria for screening [15]. Additionally, germline variants testing in Brazil has only recently
111 become available for patients with private health insurance and remains largely unavailable in
112 the public health care system [16]. Therefore, the prevalence of LS and LLS among EC patients
113 in the Brazilian population is still unclear.
114 To elucidate these open questions, we conducted a retrospective study on 242
115 endometrial cancer patients who underwent surgical treatment at a university general hospital
116 in Southeast Brazil from 2005 to 2017. The MMR deficiency was assessed in tumor samples,
117 and next-generation sequencing (NGS) of LS genes was performed when the patient's blood
118 samples were available. Our analysis is the first study providing information about the
119 molecular epidemiology and clinicopathological features in a Brazilian cohort of EC screened
120 by a universal approach.
121

122 **Materials and Methods**

123 *Patients and samples*

124

125 The Scientific and Research Committee of the Ribeirão Preto Medical School of the
126 University of Sao Paulo, Brazil (HC-FMRP-USP) approved the research protocol and consent
127 form of this study (protocol number 1.578.206/2016). A total of 317 patients diagnosed with
128 primary EC were identified between January 2005 and January 2017 at our institution. We
129 included 242 (76%) cases based on the following criteria: patients were 18 years or older at
130 time of EC diagnosis; there was satisfactory amount of tumor material for molecular analysis,
131 and the patients should be alive at time of selection for further family history of cancer
132 assessment and germline variant investigation. We did not include patients that received
133 neoadjuvant treatment. Informed consent was prospectively obtained from all patients selected
134 for germline analyzes. Clinicopathological data were extracted from medical records, including
135 histopathological classification, grading, and staging of the tumors, according to the
136 International Federation of Gynecology and Obstetrics (FIGO) [17].
137

138 *Immunohistochemistry*

139

140 Tumor representative areas were identified on hematoxylin-eosin slides and punched
141 from donor formalin-fixed paraffin-embedded (FFPE) blocks. One tumor tissue core (5 mm^2
142 area) from each EC sample was transferred to a paraffin receptor tissue microarray (TMA)
143 block. Each TMA was composed of 23 tumor cores and one liver tissue core, which was used
144 for posterior sample identification.
145 IHC staining of MLH1, MSH2, MSH6, and PMS2 proteins was performed on 4 μm
146 sections of TMA blocks using the REVEAL Polyvalent HRP-DAB Detection System (Spring
147 Bioscience, Pleasanton, CA), following the manufacturer's protocol. The primary antibodies
148 used for detecting the MMR proteins were anti-MLH1 (dilution 1:100; clone 6168-728; BioSB,
149 Santa Barbara, CA), anti-MSH2 (dilution 1:250; clone 25D12; Leica Biosystems, Buffalo
150 Grove, IL), anti-MSH6 (dilution 1:75; clone 6TBP H-141; Santa Cruz Biotechnology, Santa
151 Cruz, CA), and anti-PMS2 (dilution 1: 25; clone MOR46; Leica Biosystems, Buffalo Grove,
152 IL). IHC slides were independently evaluated by two pathologists (FC and ARS). The complete
153 absence of nuclear staining in tumor cells in the presence of internal positive control (nuclear
154 staining of non-neoplastic cells, such as adjacent normal endometrial cells, lymphocytes, or
155 stromal cells), was considered to determine the loss of expression of MMR proteins in tumors.
156
157 *Microsatellite instability*
158
159 MSI analysis was performed on tumors with the intact expression of all four MMR
160 proteins. Genomic DNA was extracted from tumor and non-neoplastic areas of FFPE tissue
161 sections using the Maxwell Rapid Sample Concentrator System (Promega, Madison – WI).
162 MSI status was performed using multiplexed polymerase chain reaction (PCR) for genotyping
163 of the BAT26, BAT25, NR21, NR24, and NR27 monomorphic microsatellites. Primer
164 sequences and PCR conditions were described elsewhere [18]. Amplicon detection was run on
165 an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) and data were
166 analyzed using the GeneMarker v.1.85 software (SoftGenetics, State College, PA). Tumors
167 were classified as MSI-high (MSI-H) when two or more microsatellite markers showed an
168 altered pattern; MSI-low (MSI-L) when one marker was altered and microsatellite stable (MSS)
169 when none of the markers were present.
170
171 *MLH1* methylation
172

173 Tumors with loss of MLH1 or MLH1/PMS2 protein expression were tested for *MLH1*
174 promoter methylation. Tumor genomic DNA was extracted as described above, and
175 modification of DNA with sodium bisulfite was performed using the EpiTect kit (Qiagen,
176 Valencia, CA), according to the manufacturer's instructions. Methylation-specific polymerase
177 chain reaction (MS-PCR) was performed using primers for the C region of the *MLH1* promoter.
178 Primer sequences and PCR conditions are described elsewhere [19].
179
180 *Next-generation sequencing*
181
182 Samples with loss of expression of MMR proteins and an absence of *MLH1*
183 methylation, as well as MSI-H or MSI-L tumors, were selected for germline variant analysis.
184 Patients were referred to the Cancer Genetic Counseling Service at HCRP-FMRP-USP and,
185 once the patient consented, family cancer records, and peripheral blood samples were
186 prospectively collected.
187 For germline analysis, genomic DNA was extracted from blood using QIAamp DNA
188 Mini Kit (Qiagen) following manufacturer's instructions, and library preparation was
189 performed using the SureSelectQXT Kit (Agilent Technologies, Santa Clara, CA). The coding,
190 canonical splice sites, and both 5' and 3' untranslated regions (UTRs) of *MLH1*, *MSH2*, *MSH6*,
191 *PMS2*, and *EPCAM* genes were sequenced on an Illumina NextSeq 500/550 platform (Illumina,
192 San Diego, CA), on a 2 x 150 bp paired-end mode.
193 The raw files were aligned to the human reference genome GRCh37 and further
194 processed with the Genome Analysis Toolkit (GATK v. 4.0.10.1), according to the GATK Best
195 Practices protocol [20]. Single nucleotide variants (SNV) and indels were identified using the
196 HaplotypeCaller (GATK) and then annotated using ANNOVAR [21]. Copy-number variation
197 (CNV) was assessed with the VisCap algorithm [22].
198
199 *Nomenclature and classification of genetic variants*
200
201 All SNVs were reported at the nucleotide and protein levels according to the Human
202 Genome Variation Society (HGVS) nomenclature recommendations [23]. Classification of
203 clinical significance was based on the American College of Medical Genetics and Genomics
204 and the Association for Molecular Pathology (ACMG/AMP) recommendations [24], using the
205 VarSome variant search Engine [25]. Variants classified as Benign and Likely benign were
206 filtered out from further analysis.

207 *Orthogonal validation*

208

209 For Sanger sequencing validation, we used the BigDye 3.1 sequencing kit (Applied
210 Biosystems, Carlsbad, CA, USA) following manufacturer's protocol, and fragments were
211 analyzed in a 3500xl genetic analyzer (Applied Biosystems). Primer sequences are available
212 upon request.

213 Due to the presence of a large family of highly homologous *PMS2* pseudogenes, *PMS2*
214 variants were validated by Long-range PCR using the LongRange PCR Kit (Qiagen), according
215 to the manufacturer's instructions. Primer sequences and annealing temperatures are described
216 elsewhere [26].

217 CNVs were validated by Multiplex Ligation-dependent Probe Amplification (MLPA).
218 The SALSA MLPA kit P003-D1(MRC Holland, Amsterdam, NH) was used for *MLH1*, *MSH2,*
219 and *EPCAM* genes, P008-C1 kit (MRC Holland) was used for *PMS2* and P072-D1 kit (MRC
220 Holland) for *MSH6*. Amplification products were identified using the 3500xl system (Applied
221 Biosystems). Data analysis was performed using Coffalyser.Net software (MRC Holland).

222

223 *Statistical analysis*

224

225 Mean values and standard deviations were used to describe continuous data, and
226 categorical variables were displayed as totals and frequencies. Categorical variables were
227 compared using Fisher's exact test, and continuous variables were analyzed using ANOVA and
228 Post-hoc analysis with Tukey's Test. *P*-values of <0.05 were considered significant. Statistical
229 analyses were performed using SAS version 9.4.

230

231 **Results**

232 *Clinicopathological features*

233

234 The clinicopathological data of the 242 EC cases are summarized in **Table 1**. The mean
235 age at EC diagnosis was 63 years (range 33-91 years). Nineteen patients (7.9%) were diagnosed
236 under 50 years, 73 patients (30.2%) were diagnosed between 50 and 59 years, and 150 patients
237 (62.0%) were at least 60 years old at EC diagnosis. The majority of tumors were endometrioid
238 adenocarcinomas (92.1%). Most cases were low-grade tumors, with FIGO I (44.2%) and FIGO
239 II (31.4%). In addition, most tumors with available pathological information were restricted to

240 the uterus (77.1%, i.e., FIGO I stage). Angiolymphatic invasion was present in 33 (14.9%) of
241 cases.
242
243

Characteristics	n(%)
Age at diagnosis (in years)	
Mean (SD)	63.0 (10.4)
Median (min-max)	62.0 (33-91)
< 50	19 (7.9)
50-59	73 (30.2)
≥ 60	150 (62.0)
Histology	
Endometrioid	223 (92.1)
Serous	10 (4.1)
Mixed	8 (3.3)
Clear cells	1 (0.4)
FIGO grade	
I	107 (44.2)
II	76 (31.4)
III	59 (24.4)
FIGO stage	
I	185 (77.1)
II	29 (12.1)
III	22 (9.2)
IV	4 (1.7)
Unknown	2
Angiolymphatic invasion	
Absent	189 (85.1)
Present	33 (14.9)
Unknown	20

244 245 246 247 248 249 250 251 252 253 254 255 256 257 258 259 260 261 262 263 264 265
266

267 **Table 1 - Clinical and pathological characteristics of 242 Brazilian cases of endometrial cancer.** SD: standard
268 deviation.
269

270 *Tumor screening*

271

272 Results of IHC, MSI, and *MLH1* methylation are summarized in **Figure 1**. Loss of
273 expression of one or more MMR proteins was detected in 80 out of 242 tumors (33.1%). Most

274 MMR-protein defective tumors had loss of expression of MLH1 protein, thirty-five (14.7%)
275 tumors were negative for the expression of the MLH1/PMS2 dimer, and six tumors (2.4%)
276 showed isolated loss of MLH1. Subsequently, PMS2 protein loss was detected in eleven tumors
277 (4.5%), MSH2/MSH6 loss was observed in ten tumors (4.1%), and MSH6 protein was absent
278 in eighteen tumors (7.4%).

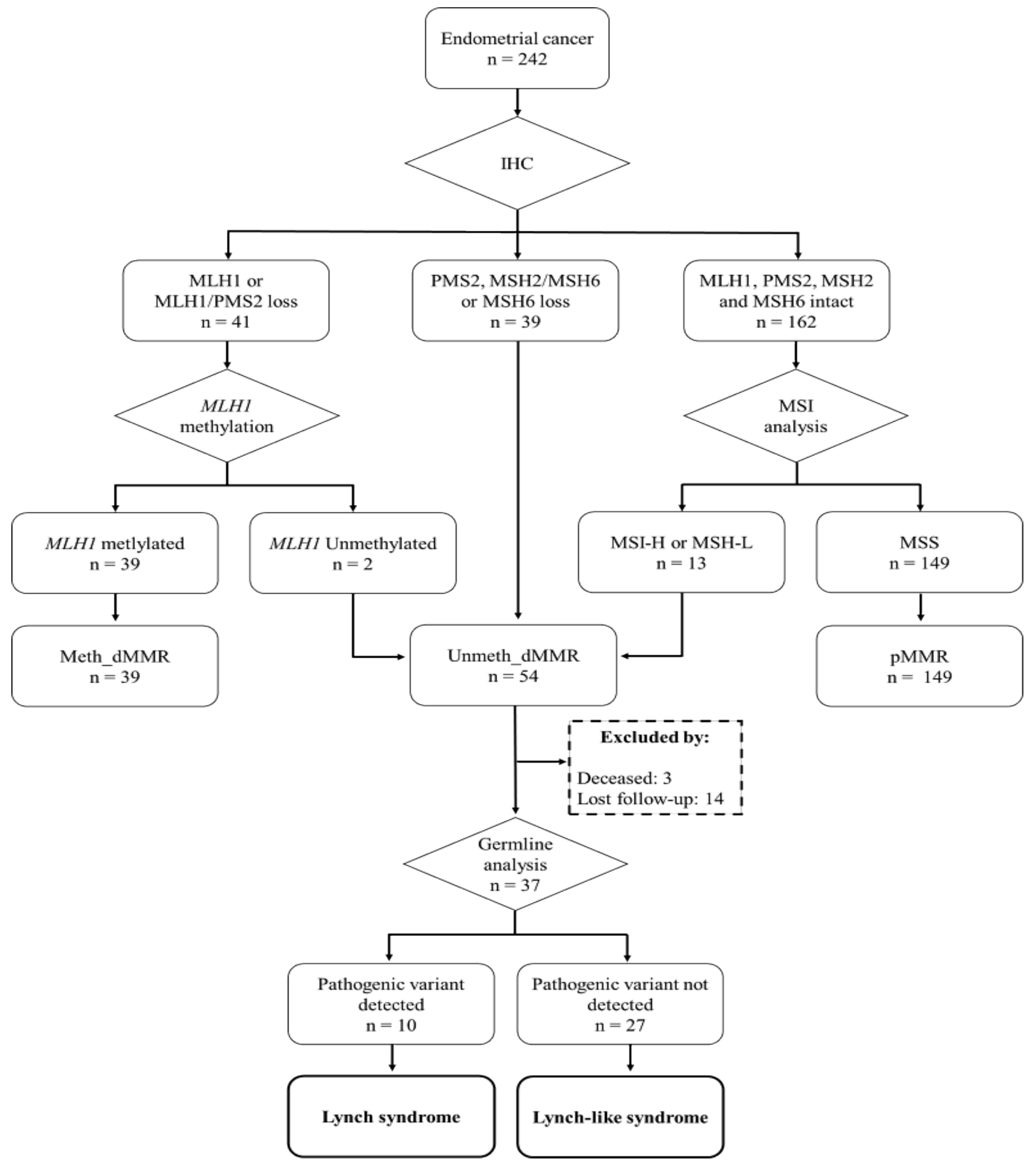


Figure 1 - Flowchart summarizing the result of the strategy used for detection of Lynch syndrome. dMMR: mismatch repair deficiency. IHC: immunohistochemistry, MSI-H: microsatellite instability-high, MSI-L: microsatellite instability-low, MSS: microsatellites stable. Meth_dMMR: dMMR tumors with methylation in *MLH1*. Unmeth_dMMR: dMMR tumors without methylation in *MLH1*. pMMR: mismatch repair proficiency.

279 *MLH1* promoter methylation was analyzed in all 41 tumors showing loss of MLH1
280 protein, and the majority of them (39/41, 95.1%) were methylated. Endometrial tumors with
281 *MLH1* methylation (Meth_dMMR) were considered sporadic and were excluded from the
282 germline analysis.
283 A total of 162/242 endometrial carcinomas (66.9%) showed intact expression of the four
284 MMR proteins and were evaluated by MSI assay. Of these, eleven tumors (6.8%) had MSI-H
285 phenotype, and two tumors (1.2%) were classified as MSI-L. Concordance between IHC and
286 MSI was observed in 149/162 (92.0%) tumors, which were classified as pMMR by both
287 methodologies.
288 Overall, 93/242 (38.5%) EC cases were dMMR. Tumors with MMR protein loss
289 (excluding Meth_dMMR cases), together with MSI-H and MSI-L tumors, were classified as
290 Unmeth_dMMR tumors (54 cases) and were selected for germline variant analysis.
291
292 *Germline variant analysis*
293
294 We carried out germline variant analysis using a subset of 37/54 (68.5%)
295 Unmeth_dMMR cases as we were not able to perform testing in 17/54 (31.5%) patients either
296 due to loss of follow-up (fourteen patients) or death (three patients). The pathogenic and likely
297 pathogenic variants, as well as the VUS, are summarized in **Table 2**. Sixteen SNVs were
298 identified in 14 patients (two patients had two variants each). Eight variants (50%) were
299 identified in the *MSH6* gene (4 missense variants, 3 frameshifts, and 1 in-frame deletion), 4
300 variants (25%) in *MSH2* (3 frameshift variants and 1 missense), 2 variants (12.5%) were
301 detected in *MLH1* (1 nonsense and 1 missense variants) and other 2 variants (12.5%) were
302 identified in the *PMS2* gene (1 frameshift and 1 missense variants). Additionally, one large
303 deletion comprising exons 17-19 of the *MLH1* gene was identified in 1 individual (**Table 2** and
304 **Fig. 2A**). We did not identify any germline variants in the *EPCAM* gene.
305 Ten variants were classified as pathogenic or likely pathogenic, and seven variants were
306 classified as VUS, according to ACMG/AMP guidelines. Three pathogenic variants and one
307 likely pathogenic variant (40%) were detected in *MSH6*, three pathogenic variants (30%) were
308 found in *MSH2*, two pathogenic variants (20%) were identified in *MLH1* (including the exons
309 17-19 deletion), and one pathogenic variant (10%) was detected in *PMS2*. Seven VUS were
310 also identified in *MSH6* (four VUS), *MLH1*, *MSH2,* and *PMS2*. Eight distinct novel variants,
311 i.e., not described in the literature or by variation databases so far, were identified. They
312 comprised: three pathogenic variants and two VUS in *MSH6*, two pathogenic variants in *MSH2*

Patient ID	Gene	Transcript [a]	Nucleotide	Amino acid	Variant type	ACMG/AMP classification	Immunohistochemistry				dbSNP ID	ClinVar	AIIC	RBG
							MLH1	PMS2	MSH2	MSH6				
Pathogenic variants [b]														
042-S	*MLH1*	NM_000249.3	c.1276C>T	p.Gln426Ter	Nonsense	Pathogenic	+	-	+	+	rs63750316	Pathogenic	No	No
063-S	*MLH1*	NM_000249.3	NA	NA	Exon deletion	Pathogenic	-	-	+	+	NA	Pathogenic	Yes	Yes
001-S	*PMS2*	NM_000535.7	c.1055delT	p.Leu352TrpfsTer4	Frameshift	Pathogenic	-	+	+	+	Novel	ND	No	No
062-S	*MSH2*	NM_000251.3	c.174dupC	p.Lys59GlnfsTer23	Frameshift	Pathogenic	+	+	-	-	Novel	ND	Yes	No
075-S	*MSH2*	NM_000251.3	c.174dupC	p.Lys59GlnfsTer23	Frameshift	Pathogenic	+	+	-	-	Novel	ND	Yes	No
065-S	*MSH2*	NM_000251.3	c.820dupA	p.Ile274AsnfsTer10	Frameshift	Pathogenic	+	+	-	-	Novel	ND	Yes	Yes
025-S	*MSH6*	NM_000179.3	c.1517dupA	p.Asp506GlufsTer2	Frameshift	Pathogenic	+	+	+	-	Novel	ND	No	No
017-S	*MSH6*	NM_000179.3	c.3247delG	p.Glu1083LysfsTer7	Frameshift	Pathogenic	+	+	+	-	Novel	ND	Yes	Yes
027-S	*MSH6*	NM_000179.3	c.3847_3850dupATTA	p.Thr1284AsnfsTer6	Frameshift	Pathogenic	+	+	-	-	rs267608128	Pathogenic	No	No
014-S	*MSH6*	NM_000179.3	c.3848_3862del	p.T980_Y985del	In-frame deletion	Likely pathogenic	+	+	-	-	Novel	ND	No	No
Variants of unclear significance														
061_S	*MLH1*	NM_000249.3	c.2152C>T	p.His718Tyr	missense	VUS	+	+	+	-	rs2020873	Benign	Yes	No
064_S	*PMS2*	NM_000535.7	c.1963G>A	p.Gly655Arg	missense	VUS	+	+	+	-	rs1064793236	VUS	Yes	Yes
001_S	*MSH2*	NM_000251.3	c.192C>G	p.Ile64Met	missense	VUS	-	+	+	+	rs1395172053	VUS	No	No
034_S	*MSH6*	NM_000179.3	c.253C>T	p.Pro85Ser	missense	VUS	+	+	+	-	rs779664343	VUS	No	No
020_S	*MSH6*	NM_000179.3	c.1019T>C	p.Phe340Ser	missense	VUS	+	+	-	-	rs61753793	Likely benign	Yes	No
064_S	*MSH6*	NM_000179.3	c.3422C>T	p.Ser1141Phe	missense	VUS	+	+	+	-	Novel	ND	Yes	Yes
033_S	*MSH6*	NM_000179.3	c.3670G>T	p.Gly1224Trp	missense	VUS	+	+	+	-	Novel	ND	No	Yes

Table 2 - Germline variants identified among 37 patients diagnosed with unmethylated dMMR endometrial tumors. [a] reference transcript from NCBI Reference Sequence Database (RefSeq). [b] both pathogenic and likely pathogenic variants, according to ACMG/AMP classification, were considered as Pathogenic, i.e., Lynch syndrome causing. ACMG/AMP: American College of Medical Genetics and Genomics and the Association for Molecular Pathology. AIIC: Amsterdam II Criteria. ND: not described. NA: not applicable. RBG: Revised Bethesda Guidelines, VUS: variant of uncertain significance.

314 (p.Lys59GlnfsTer23 was identified in two unrelated patients), and one pathogenic variant in
315 *PMS2*. The *MLH1* exons 17-19 deletion is reported as pathogenic in ClinVar database.
316 Personal and family history of cancer from individuals carrying pathogenic, likely
317 pathogenic, and VUS was assessed to identify patients who met Amsterdam II Criteria (AIIC)
318 or Revised Bethesda Guidelines (RBG) (**Table 2**). Half of the individuals carrying a pathogenic
319 or likely pathogenic variant in LS genes fulfilled neither AIIC nor any of the RBG. Regarding
320 VUS carriers, one patient (034_S) did not meet any criteria. One patient (001_S) had one
321 pathogenic variant in *PMS2* and one VUS in *MSH2* and met neither AIIC nor RBG.
322 Summarizing, of the 93 dMMR tumors identified, 39 (41.9%) were Meth_dMMR, 10
323 (10.8%) were LS cases, 27 (29.0%) were LLS. We were not able to perform germline analysis
324 in 17 dMMR cases (Not Sequenced dMMR cases - NS) due to loss of follow-up and deaths.
325

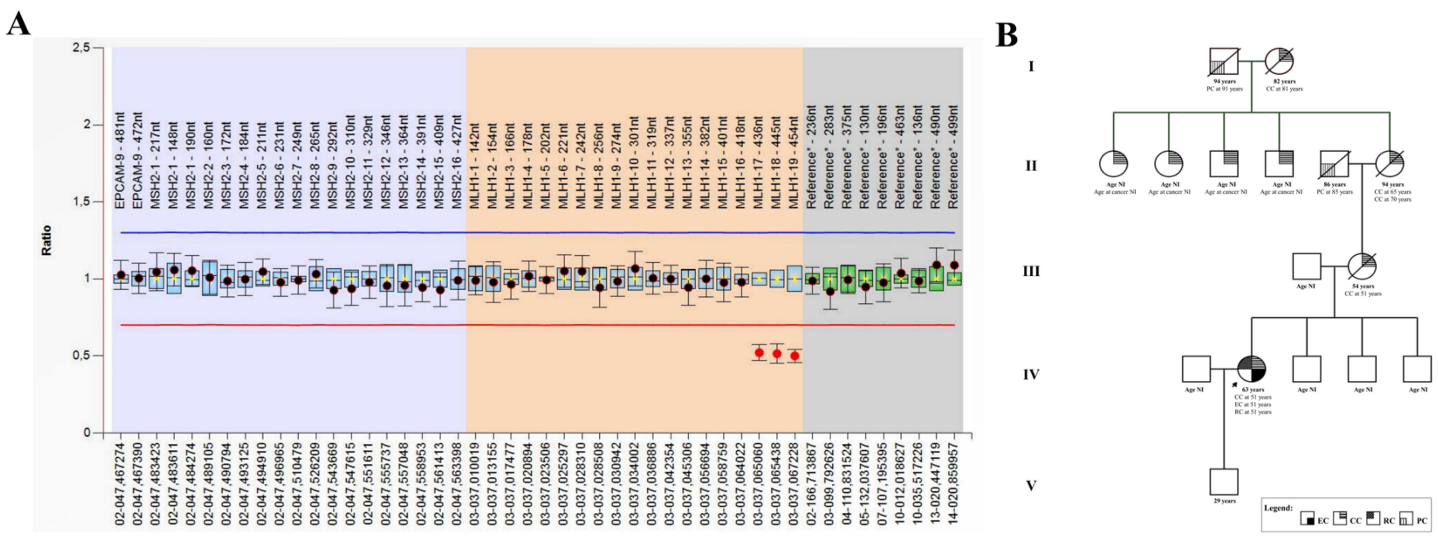


Figure 2 - Molecular and clinical findings of the LS patient carrying a CNV in the MLH1 gene. (A) Normalized MLPA data of the *MLH1* exons 17-19 deletion identified in patient 063_S. Y-axis contains ratio of copies between sample and controls. X-axis has chromosomic coordinates for each probe. Exonic deletions are represented by consecutive red bar charts reaching ratio values around 0.5, compatible with heterozygous deletion. (B) Family pedigree of the patient (indicated with an arrow) with the *MLH1* exons 17-19 deletion. Cancer type, age of patients (bold), and age at cancer diagnosis were recorded. This patient met the Amsterdam II criteria and Revised Bethesda Guidelines. Roman numerals were used for generation identification. CC: colon cancer, EC: endometrial cancer, NI: not informed, PC: prostate cancer, and RC: rectal cancer.

326 *Clinicopathological association*

327

328 Clinical and pathological features of endometrial tumors were compared among the
329 Meth_dMMR, LS, LLS, NS and pMMR groups (**Table 3**). We observed a significant difference
330 in mean age at diagnosis of endometrial carcinoma (*p*-value = 0.0019, ANOVA) among groups.
331 LS group had a lower mean age at the diagnosis compared to Meth_dMMR, NS, and pMMR
332 groups (*p*-value < 0.05, Tukey's test). The mean age at diagnosis observed in the LLS group
333 was similar to the LS group and the other molecular groups (*p*-value >0.05, Tukey's test).
334 We also detected a significant difference after the stratification of cases by categories
335 of age at cancer diagnosis (*p*-value < 0.00001, Fisher's exact test). All LS patients were under
336 60 years at the time of EC diagnosis. On the other hand, more than 60% of patients with pMMR
337 and Meth_dMMR tumors, as well as most patients of the NS group, were at least 60 years at
338 EC diagnosis. LLS patients had intermediate ages of EC diagnosis when compared to LS and
339 the other groups.

340

341 **Discussion**

342

343 For the implementation of successful molecular tests in clinical practice, it is essential
344 to comprehensively understand the genetic variability in the population being served [27]. Our
345 study is the first description of LS and LLS in a Brazilian cohort of EC identified by a universal
346 screening approach. Risk assessment for Lynch Syndrome may be a complex and challenging
347 task in developing countries such as Brazil with limited resources for molecular genetic
348 analyses in the public health sector. We successfully performed universal screening in a total
349 of 242 unrelated EC cases, of which 93 tumors (38.5%) were dMMR based on IHC and MSI
350 results. Thirty tumors were found to be sporadic due to *MLH1* promoter methylation, and 54
351 cases were selected for germline variant analysis. Targeted sequencing of LS-associated genes
352 was successfully performed in 37/54 (68.5%) patients and pathogenic or likely pathogenic
353 variants were identified in ten patients (**Fig. 1**). Clinicopathological features were compared
354 among groups (**Table 3**).
355 We detected a LS prevalence of 4.1% in our cohort through the tumor universal
356 screening approach. This prevalence may be underestimated since we did not test 31.5% of the
357 patients with dMMR endometrial tumors. Previous studies have reported prevalence of LS
358 among EC patients ranging between 2% and 5.9% [21, 28]. Further studies may increase data
359 of LS prevalence among women diagnosed with EC in Brazil. A recent systematic review and

Variables	Meth_dMMR *n* = 39 (16.1%)	Unmeth_dMMR			pMMR *n* = 149 (61.6%)	*p*-value [1]
		LS *n* = 10 (4.1%)	LLS *N* = 27 (11.2%)	NS *n* = 17 (7.0%)		
Mean age at diagnosis (sd)	65.7 (8.3) [a]	53.8 (4.1) [b]	59.1 (8.7) [ab]	66.8 (11.2) [a]	63.1 (10.8) [a]	**0.0019** [2]
Age groups	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	**< 0.00001** [3]
< 50	0 (0.0)	2 (20.0)	2 (7.4)	1 (5.9)	14 (9.4)	
50-59	10 (25.6)	8 (80.0)	14 (51.9)	2 (11.8)	39 (26.2)	
≥ 60	29 (74.4)	0 (0.0)	11 (40.7)	14 (82.3)	96 (64.4)	
Histology	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	**0.1725** [3]
Endometrioid	36 (92.3)	8 (80.0)	27 (100.0)	15 (88.2)	137 (91.9)	
Serous	1 (2.6)	0 (0.0)	0 (0.0)	1 (5.9)	8 (5.4)	
Mixed	2 (5.1)	1 (10.0)	0 (0.0)	1 (5.9)	4 (2.7)	
Clear cells	0 (0.0)	1 (10.0)	0 (0.0)	0 (0.0)	0 (0.0)	
FIGO grade	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	**0.1571** [3]
I	10 (25.6)	3 (30.0)	14 (51.9)	7 (41.2)	73 (49.0)	
II	18 (46.2)	2 (20.0)	7 (25.9)	5 (29.4)	44 (29.5)	
III	11 (28.2)	5 (50.0)	6 (22.2)	5 (29.4)	32 (21.5)	
FIGO stage	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	**0.5255** [3]
I	25 (64.1)	9 (90.0)	20 (74.1)	14 (82.4)	117 (79.6)	
II	7 (17.9)	0 (0.0)	4 (14.8)	3 (17.6)	15 (10.2)	
III	6 (15.4)	1 (10.0)	2 (7.4)	0 (0.0)	13 (8.8)	
IV	1 (2.6)	0 (0.0)	1 (3.7)	0 (0.0)	2 (1.4)	
Unknown	0	0	0	0	2	
Angiolymphatic invasion	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	**0.6442** [3]
Absent	29 (76.3)	8 (88.9)	24 (88.9)	12 (85.7)	115 (85.8)	
Present	9 (23.7)	1 (12.1)	3 (11.1)	2 (14.3)	19 (14.2)	
Unknown	1	1	0	3	15	

Table 3 - Comparison between clinicopathologic variables and tumor MMR status and germline findings. Mean age at the diagnosis of endometrial cancer is described in years. *P*-values of <0.05 were considered significant. [1] *p*-value derived from comparison between variables and the five molecular groups. [2] Mean age was compared among groups using ANOVA and Post-Hoc Analysis with Tukey's Test. [a] and [b] were used to indicate mean age similarities among groups, based on Tukey's test. Groups with the same letter are not significantly different (p > 0.05). [3] categorical variables were compared using the Fisher's exact test. sd: standard deviation. Meth_dMMR: dMMR tumors with methylation in *MLH1*. pMMR: mismatch repair proficiency. LS: Lynch syndrome, LLS: Lynch-like syndrome. NS: *MLH1* unmethylated dMMR tumors that did not undergo germline analysis.

361 meta-analysis suggested that prevalence of LS in EC patients is approximately 3% [29]. A
362 single study assessing the MMR status in a series of EC from the Southern region of Brazil was
363 included in this meta-analysis, but germline variant data was not assessed [30].

364 Most pathogenic or likely pathogenic variants identified in our LS patients were found
365 in the *MSH6* gene (**Table 2**), which is in agreement with the current knowledge about LS and
366 EC. It is known that women who carry *MSH6* pathogenic variants have a 26-fold increased
367 incidence of EC and a six-fold increased incidence of other cancers associated with LS [31,32].
368 Additionally, these women have a cumulative incidence of EC at 75 years of 41% which
369 outweighs the frequency of uterine cancer among women carrying pathogenic variants in *MLH1*
370 or *PMS2* genes [4].

371 Historically, Amsterdam criteria and Bethesda guidelines have been widely used to
372 screen endometrial and CRC for LS genetic testing [3]. However, at least half of the germline-
373 confirmed LS patients fail to fulfill these criteria [28]. Half of the patients carrying pathogenic
374 variants in LS-genes did not meet any of these clinical criteria (**Table 2**), most of them were
375 *MSH6* variant carriers. *MSH6* variant carriers can be challenging to diagnose because they may
376 not entirely fulfill the criteria for LS diagnosis, and their age at cancer onset is often later than
377 for *MLH1* and *MSH2* variant carriers [33]. These findings, together with the higher frequency
378 of *MSH6* pathogenic variants found in EC patients, support the use of the tumor universal
379 screening approach for selection of EC cases for LS testing, rather than methods based on
380 clinical features.

381 Numerous genetic point variants have been identified in LS patients, including
382 frameshift, nonsense, and splicing variants that result in nonsense-mediated mRNA decay of
383 the transcripts, truncated, or altered protein structure [3]. Moreover, the occurrence of missense
384 variants, which lead to single amino acid substitutions, is expressive (about 30–60%) for all
385 four LS-associated genes [34]. A large subset of missense variants detected in LS probands
386 have been termed VUS because the pathogenic effect of these class of variants on MMR genes
387 is unclear as well as their contribution to the disease pathogenesis. [35]. In this study, we
388 identified seven VUS present in five out of 37 patients tested for LS (18.9%). Five patients

389 harboring a VUS in a LS-genes fulfilled the AIIC or at least one of RBG (**Table 2**). Since these
390 criteria, mainly AIIC, are indicative of a possible hereditary origin of the EC, our results suggest
391 that these VUS could have a pathogenic effect on the MMR pathway. These inconclusive
392 classifications are a challenge when it comes to medical management decisions. The functional
393 impact of VUS cannot be inferred from sequence information alone and there are often
394 insufficient clinical and epidemiological data to make clinically meaningful inferences about
395 their association with cancer risks [36]. This issue poses an even bigger challenge in Brazil and
396 other countries where the genetic testing is limited since the spectrum of germline variants and
397 their associated phenotypes are not completely understood [16]. Further analysis, such as
398 segregation analysis, loss of heterozygosity in tumors, and well-established functional assays
399 could contribute to the elucidation of the VUS role on the LS etiology [16, 35].
400 We assessed the MMR status in endometrial tumors by IHC and MSI. Although both
401 methodologies are recommended for detecting MMR deficiency, their results are not always in
402 concordance. In our cohort, MSI was performed in 162 endometrial tumors that exhibited intact
403 expression of the MMR proteins. Discordant results were found in thirteen tumors (8%), which
404 showed MSI-H (11 tumors) or MSI-L (2 tumors) phenotype and intact expression of MLH1,
405 MSH2, MSH6, and PMS2 proteins. Discordance between MSI and IHC is not always a
406 technical issue and may represent the differences in cellular functions detected by the two
407 testing strategies. A fraction of these cases can be explained by the occurrence of missense
408 variants that disrupt the MMR gene function, leading to a MSI phenotype, but maintaining the
409 epitope of the MMR protein intact [28]. Conversely, some pathogenic variants, particularly in
410 *MSH6* and *PMS2*, can result in protein loss by IHC without causing MSI-H [37]. Additionally,
411 since MSI testing assesses the functional status of the MMR mechanism, some tumors with
412 intact IHC could have an MSI phenotype that went undetected. Such events could be explained
413 by pathogenic variants in an MMR gene that are not amenable to current methods of IHC
414 analysis or caused by a yet unidentified MMR gene [38]. We were able to perform germline
415 analysis in 11/13 (85%) MSI/IHC discordant cases and did not identify any VUS or pathogenic
416 variant. A wider genomic approach, such as whole-exome or whole-genome sequencing could
417 identify genes related to MSI in tumors with normal IHC staining for the main MMR proteins.
418 Rare germline CNVs are associated with cancer susceptibility, providing an explanation
419 for part of cancer cases with a missing hereditary cause [39]. Up to 10% of LS cases are related
420 to CNVs in *MLH1*, *MSH2*, *MSH6,* or *PMS2* genes [7]. We identified one LS patient, who self-
421 reported German ancestry, harboring a large deletion encompassing exons 17, 18, and 19 of the
422 *MLH1* gene (**Fig. 2A**). This patient has a strong family history of CRC (**Fig. 2B**), which

423 includes a colon carcinoma at 31 years old and two other tumors, a metachronous rectal and
424 endometrial adenocarcinoma, twenty years later. This large *MLH1* deletion was previously
425 described as a founder variant in Northern Portugal [39] and has been identified in three
426 Brazilian patients diagnosed with CRC [15]. Most patients harboring this *MLH1* deletion were
427 diagnosed with CRC at a median age of 44 years, and a metachronous or synchronous second
428 colorectal tumor was commonly found in half of them [40]. Genotyping of relatives is strongly
429 recommended to identify the LS-carriers for adequate clinical management, and should be
430 offered not only to the relatives of the patient harboring the CNV in *MLH1*, but also for relatives
431 of individuals with pathogenic and likely pathogenic variants in LS-associated genes (**Table**
432 **2**).

433 We did not detect any pathogenic or likely pathogenic variant in 27 cases (73% of all
434 cases with sequencing results). The underlying cause of MMR deficiency in LLS tumors is
435 heterogeneous, comprising both hereditary and sporadic cases [14]. It has been shown that up
436 to 70% of all LLS cases may be explained by biallelic somatic variants in the MMR genes,
437 mainly *MLH1* and *MSH2*, representing a sporadic origin [41]. On the other hand, patients with
438 LLS developing cancer at younger ages, similar to LS, raise the speculation that undiagnosed
439 germline variants may be implicated in at least some of these cases [13]. In our cohort, we did
440 not detect a significant difference of mean ages at onset between LS and LLS patients.
441 Additionally, both LS and LLS patients had lower mean ages at diagnosis when compared to
442 sporadic endometrial tumors (Meth_dMMR).

443 The current study has some limitations. As previously discussed, the prevalence of LS
444 found in this study may be underestimated since we were not able to perform germline analysis
445 in part of the patients with dMMR endometrial tumors. Additionally, only whole exons and
446 adjacent short sequences of introns were sequenced, we cannot exclude the occurrence of
447 pathogenic variants in intronic and non-coding regions in a small portion of the cases. Finally,
448 this study was conducted at a single general hospital in Southeast Brazil, which may not be
449 completely representative of the genetic heterogeneity present in the population of Brazil.
450 Therefore, studies performed based on patient population drawn from other regions of Brazil
451 will add to the knowledge about the mutational profile and prevalence of LS in Brazilian
452 women diagnosed with EC.

453 Although universal screening is strongly recommended for LS identification [9], its
454 broader implementation may be challenged by socio-economic and technical issues in some
455 countries. In Brazil, the vast majority of the population relies on the Public Health Care System,
456 whose resources for hereditary cancer diagnosis and management, including restricted access

457 to molecular tests and genetic counseling, are still unsatisfactory [42]. Alternative screening
458 approaches, such as tumor screening in EC patients selected by age-based cut-off [43] or
459 particular tumor morphology [44], are cost-effective and highly sensitive methods for LS
460 detection and may be helpful to overcome the challenges of universal screening in countries
461 like Brazil. However, studies about the cost-effectiveness of different approaches for LS
462 screening in Brazil are mandatory to define the most cost-effective strategy for LS investigation
463 in our country.

464 In conclusion, this is the first study in a Brazilian cohort of EC screened for LS through
465 a universal tumor screening approach, which revealed a LS prevalence of at least 4.1%.
466 Variants in the *MSH6* gene were detected in most of the LS cases. The prevalence of LLS in
467 endometrial tumors with defective MMR was around 73%. LSS patients had an intermediate
468 age at diagnosis of EC compared to LS and sporadic EC groups. The investigation of LS in
469 other regions of Brazil may help define more cost-effective strategies for LS identification,
470 prevention, and cancer treatment.

Ethics approval and consent to participate

This study was approved by the Ethics and Research Committee of the Clinics Hospital of the Ribeirão Preto Medical School (protocol number 1.578.206/2016). Informed written consent was obtained from all patients included in the study.

Acknowledgments

The authors would like to thank the patients for their kind collaboration and contribution to this study. Also, we thank Dr. Jeremy Squire for his careful review of the manuscript and correction of the English.

Author contributions

VF, WJ, and RR conceived and planned the study design. RR, GM, and JS performed Molecular Biology experiments. LT, JT, and VR helped to collect blood samples from patients and supported family history acquisition. FC, AS, and MB provided the clinicopathological data and carried out IHC experiments and their further analysis. RR, JS, GM, and LT led the writing of the manuscript. JS and LT supported data acquisition and data analysis. VF and WJ provided a critical review of the manuscript. All authors reviewed the final version of the manuscript and gave full consent for publication.

Role of the funding source

Costs of the study design and molecular analyses were funded by the São Paulo Research Foundation (FAPESP grant 2017/04283-8) and National Council for Scientific and Technological Development (CNPq grant 423007/2016-5).

Declaration of competing interest

The authors declared no potential conflicts of interest regarding the research, authorship, and/or publication of this article.

Submission of declaration and verification

505 This manuscript has not been published previously, is not under consideration for
506 publication elsewhere, and this publication is approved by all authors and the responsible
507 authorities where the work has been carried out. This manuscript will not be published
508 elsewhere in the same form.

509

510 **Availability of data**

511

512 The raw data supporting the conclusions of this manuscript will be made available by
513 the corresponding author upon request

514

515 **References**

516

517 [1] L.M. Randall, B. Pothuri, E.M. Swisher, J.P. Diaz, A. Buchanan, C.T. Witkop et al. (2017).
518 Multi-disciplinary summit on genetics services for women with gynecologic cancers: A Society
519 of Gynecologic Oncology White Paper. Gynecol Oncol. 146(2):217-224. doi:
520 10.1016/j.ygyno.2017.06.002

521

522 [2] K. Takahashi, N. Sato, T. Sugawara, A. Kato, T. Sato, D. Shimizu et al. (2017). Clinical
523 characteristics of Lynch-like cases collaterally classified by Lynch syndrome identification
524 strategy using universal screening in endometrial cancer. Gynecol Oncol. 147(2):388-395. doi:
525 10.1016/j.ygyno.2017.08.016

526

527 [3] H.T. Lynch, C.L. Snyder, T.G. Shaw, C.D. Heinen, M.P. Hitchins. (2015). Milestones of
528 Lynch syndrome: 1895-2015. Nat Rev Cancer. 15(3): 181-94. doi: 10.1038/nrc3878

529

530 [4] M. Dominguez-Valentin, J.R. Sampson, T.T. Seppälä, S.W.T. Broeke, J.P. Plazzer, S.
531 Nakken et al. (2020). Cancer Risks by Gene, Age, and Gender in 6350 Carriers of Pathogenic
532 Mismatch Repair Variants: Findings From the Prospective Lynch Syndrome Database. Genet
533 Med. 22(1):15-25. doi: 10.1038/s41436-019-0596-9

534

535 [5] D. Liu, G. Keijzers, L.J. Rasmussen. (2017). DNA mismatch repair and its many roles in
536 eukaryotic cells. Mutat Res. 773: 174-187. doi: 10.1016/j.mrrev.2017.07.001

537

538 [6] V.L. Cox, A.A. Saeed Bamashmos, W.C. Foo, S. Gupta, S. Yedururi, N. Garg, H.C. Kang.
539 (2018). Lynch Syndrome: Genomics Update and Imaging Review. Radiographics. 38(2): 483-
540 499. doi: 10.1148/rg.2018170075

541

542 [7] K. Tamura, M. Kaneda, M. Futagawa, M. Takeshita, S. Kim, M. Nakama, et al. (2019).
543 Genetic and genomic basis of the mismatch repair system involved in Lynch syndrome. Int J
544 Clin Oncol. 24(9): 999-1011. doi: 10.1007/s10147-019-01494-y.

545

546 [8] T. Adar, L.H. Rodgers, K.M. Shannon, M. Yoshida, T. Ma, A. Mattia. (2018). Universal
547 screening of both endometrial and colon cancers increases the detection of Lynch syndrome.
548 Cancer. 124(15): 3145-3153. doi: 10.1002/cncr.31534

549
550 [9] E.J. Crosbie, N.A.J. Ryan, M.J. Arends, T. Bosse, J. Burn, J.M. Cornes et al. (2019). The
551 Manchester International Consensus Group Recommendations for the Management of
552 Gynecological Cancers in Lynch Syndrome. Genet Med. 21(10):2390-2400. doi:
553 10.1038/s41436-019-0489-y
554
555 [10] W. Jiang, M.Y. Cai, S.Y. Li, J.X. Bei, F. Wang, H. Hampel, et al. (2019). Universal
556 screening for Lynch syndrome in a large consecutive cohort of Chinese colorectal cancer
557 patients: High prevalence and unique molecular features. Int J Cancer. 144(9): 2161-2168. doi:
558 10.1002/ijc.32044
559
560 [11] K. Tanakaya. (2019). Current clinical topics of Lynch syndrome. Int J Clin Oncol. 24(9):
561 1013-1019. doi: 10.1007/s10147-018-1282-7.
562
563 [12] A.S. Bruegl, B. Djordjevic, D.L. Urbauer, S.N. Westin, P.T. Soliman, K.H. Lu et al.
564 (2014). Utility of MLH1 Methylation Analysis in the Clinical Evaluation of Lynch Syndrome
565 in Women with Endometrial Cancer. Curr Pharm Des. 20(11): 1655–1663. doi:
566 10.2174/13816128113199990538
567
568 [13] J.M. Carethers, E.M. Stoffel. (2015). Lynch syndrome and Lynch syndrome mimics: The
569 growing complex landscape of hereditary colon cancer. World J Gastroenterol. 21(31): 9253-
570 61. doi: 10.3748/wjg.v21.i31.9253
571
572 [14] D.D. Buchanan, C. Rosty, M. Clendenning, A.B. Spurdle, A.K. Win. (2014). Clinical
573 problems of colorectal cancer and endometrial cancer cases with unknown cause of tumor
574 mismatch repair deficiency (suspected Lynch syndrome). Appl Clin Genet. 7: 183-93. doi:
575 10.2147/TACG.S48625
576
577 [15] A. Della Valle, B.M. Rossi, E.I. Palmero, M. Antelo, C.A. Vaccaro, F. López-Kostner.
578 (2019). A snapshot of current genetic testing practice in Lynch syndrome: The results of a
579 representative survey of 33 Latin American existing centres/registries. Eur J Cancer. 119: 112-
580 121. doi: 10.1016/j.ejca.2019.07.017
581

582 [16] N.B. Schneider, T. Pastor, A.E. Paula, M.I. Achatz, Â.R.D. Santos, F.S.L. Vianna, et al.
583 (2018). Germline MLH1, MSH2 and MSH6 variants in Brazilian patients with colorectal
584 cancer and clinical features suggestive of Lynch Syndrome. Cancer Med. 7(5): 2078-2088. doi:
585 10.1002/cam4.1316
586
587 [17] S. Pecorelli. (2009). Revised FIGO staging for carcinoma of the vulva, cervix, and
588 endometrium. Int J Gynaecol Obstet. 105(2): 103-4. doi: 10.1016/j.ijgo.2009.02.012
589
590 [18] O. Buhard, N. Suraweera, A. Lectard, A. Duval, R. Hamelin. (2004). Quasimonomorphic
591 mononucleotide repeats for high-level microsatellite instability analysis. Dis Markers. 20: 251-
592 257. doi: 10.1155/2004/159347
593
594 [19] S.J. Park, A. Rashid, J.H. Lee, S.G. Kim, S.R. Hamilton, T.T. Wu. (2003). Frequent CpG
595 island methylation in serrated adenomas of the colorectum. Am J Pathol. 162(3): 815-22. doi:
596 10.1016/S0002-9440(10)63878-3
597
598 [20] G.A. Van der Auwera, M.O. Carneiro, C. Hartl, R. Poplin, G. Del Angel, A. Levy-
599 Moonshine, et al. (2013). From FastQ data to high confidence variant calls: the Genome
600 Analysis Toolkit best practices pipeline. Curr. Protoc. Bioinformatics. 11(1110): 11.10.1–
601 11.10.33. doi: 10.1002/0471250953.bi1110s43
602
603 [21] K. Wang, M. Li, H. Hakonarson (2010). ANNOVAR: functional annotation of genetic
604 variants from high-throughput sequencing data. Nucleic Acids Res. 38(16):e164. doi:
605 10.1093/nar/gkq603
606
607 [22] T.J. Pugh, S.S. Amr, M.J. Bowser, S. Gowrisankar, E. Hynes, L.M. Mahanta, et al. (2016).
608 VisCap: inference and visualization of germ-line copy-number variants from targeted clinical
609 sequencing data. Genet Med. 18(7): 712-719. doi: 10.1038/gim.2015.156
610
611 [23] J.T. den Dunnen, R. Dalgleish, D.R. Maglott, R.K. Hart, M.S. Greenblatt, J. McGowan-
612 Jordan, et al. (2016). HGVS Recommendations for the Description of Sequence Variants: 2016
613 Update. Hum Mutat. 37(6): 564-9. doi: 10.1002/humu.22981
614

615 [24] S. Richards, N. Aziz, S. Bale, D. Bick, S. Das, J. Gastier-Foster, et al. (2015). Standards
616 and guidelines for the interpretation of sequence variants: a joint consensus recommendation
617 of the American College of Medical Genetics and Genomics and the Association for Molecular
618 Pathology. Genet Med. 17(5): 405-24. doi: 10.1038/gim.2015.30
619
620 [25] C. Kopanos, V. Tsiolkas, A. Kouris, C.E. Chapple, M. Albarca Aguilera, R. Meyer, et al.
621 (2019). VarSome: the human genomic variant search engine. Bioinformatics. 35(11): 1978. doi:
622 10.1093/bioinformatics/bty897
623
624 [26] M. Clendenning, H. Hampel, J. LaJeunesse, A. Lindblom, J. Lockman, M. Nilbert, et al.
625 (2006). Long-range PCR facilitates the identification of PMS2-specific mutations. Hum Mutat.
626 27(5): 490-5. doi: 10.1002/humu.20318
627
628 [27] N.M. J. Cipriano, A.M. de Brito, E.S. de Oliveira, F.C. de Faria, S. Lemos, N.A. Rodrigues,
629 et al. (2018). Mutation screening of TP53, CHEK2 and BRCA genes in patients at high risk for
630 hereditary breast and ovarian cancer (HBOC) in Brazil. Breast Cancer. 26(3): 397-405. doi:
631 10.1007/s12282-018-00938-z
632
633 [28] S.A. Cohen, C.C. Pritchard, G.P. Jarvik. (2019). Lynch Syndrome: From Screening to
634 Diagnosis to Treatment in the Era of Modern Molecular Oncology. Annu Rev Genomics Hum
635 Genet. 31(20): 293-307. doi: 10.1146/annurev-genom-083118-015406
636
637 [29] N.A.J. Ryan, M.A. Glaire, D. Blake, M. Cabrera-Dandy, D.G. Evans, E.J. Crosbie. (2019).
638 The proportion of endometrial cancers associated with Lynch syndrome: a systematic review
639 of the literature and meta-analysis. Genet Med. 21(10): 2167-2180. doi: 10.1038/s41436-019-
640 0536-8
641
642 [30] S.L. Cossio, P. Koehler-Santos, S.A. Pessini, H. Mónego, M.I. Edelweiss, L. Meurer, et
643 al. (2010). Clinical and histomolecular endometrial tumor characterization of patients at-risk
644 for Lynch syndrome in South of Brazil. Fam Cancer. 9(2): 131-9. doi: 10.1007/s10689-009-
645 9297-x
646

[31] H. Terui, T. Tachikawa, M. Kakuta, Y. Nishimura, T. Yatsuoka, K. Yamaguchi, et al. (2013). Molecular and clinical characteristics of MSH6 germline variants detected in colorectal cancer patients. Oncol Rep. 30(6): 2909-16. doi: 10.3892/or.2013.2781

[32] L. Baglietto, N.M. Lindor, J.G. Dowty, D.M. White, A. Wagner, E.B. Gomez Garcia, et al. (2010). Risks of Lynch syndrome cancers for MSH6 mutation carriers. J Natl Cancer Inst. 102(3): 193-201. doi: 10.1093/jnci/djp473

[33] H. Houlleberghs, A. Goverde, J. Lusseveld, M. Dekker, M.J. Bruno, F.H. Menko, A.R. Mensenkamp, et al. (2017). Suspected Lynch syndrome associated MSH6 variants: A functional assay to determine their pathogenicity. PLoS Genet. 13(5):e1006765. doi: 10.1371/journal.pgen.1006765

[34] P. Peltomäki. (2016). Update on Lynch syndrome genomics. Fam Cancer. 15(3): 385-93. doi: 10.1007/s10689-016-9882-8

[35] A. Rath, A. Mishra, V.D. Ferreira, C. Hu, G. Omerza, K. Kelly, et al. (2019). Functional interrogation of Lynch syndrome-associated MSH2 missense variants via CRISPR-Cas9 gene editing in human embryonic stem cells. Hum Mutat. 40(11): 2044-2056. doi: 10.1002/humu.23848. Epub 2019 Aug 17.

[36] R.L.S. Mesman, F.M.G.R. Calléja, G. Hendriks, B. Morolli, B. Misovic, P. Devilee et al. (2019). The Functional Impact of Variants of Uncertain Significance in BRCA2. Genet Med. (2):293-302. doi: 10.1038/s41436-018-0052-2

[37] M.D. Giráldez, F. Balaguer, L. Bujanda, M. Cuatrecasas, J. Muñoz, et al. (2010). MSH6 and MUTYH deficiency is a frequent event in early-onset colorectal cancer. Clin Cancer Res. 16(22): 5402-13. doi: 10.1158/1078-0432.CCR-10-1491

[38] L. Zhang. (2008). Immunohistochemistry versus microsatellite instability testing for screening colorectal cancer patients at risk for Hereditary Nonpolyposis Colorectal Cancer Syndrome: part II. the utility of microsatellite instability testing. J Mol Diagn. 10(4): 301-7. doi: 10.2353/jmoldx.2008.080062

681 [39] R.A. Villacis, P.M. Miranda, I. Gomy, E.M. Santos, D.M. Carraro, M.I. Achatz, et al.
682 (2016). Contribution of rare germline copy number variations and common susceptibility loci
683 in Lynch syndrome patients negative for mutations in the mismatch repair genes. Int J Cancer.
684 138(8): 1928-35. doi: 10.1002/ijc.29948
685
686 [40] M. Pinheiro, C. Pinto, A. Peixoto, I. Veiga, B. Mesquita, R. Henrique, et al. (2011). A
687 novel exonic rearrangement affecting MLH1 and the contiguous LRRFIP2 is a founder
688 mutation in Portuguese Lynch syndrome families. Genet. Med. 13:895–902. doi:
689 10.1097/GIM.0b013e31821dd525
690
691 [41] S. Haraldsdottir, H. Hampel, J. Tomsic, W.L. Frankel, R. Pearlman, A. de la Chapelle, et
692 al. (2014). Colon and endometrial cancers with mismatch repair deficiency can arise from
693 somatic, rather than germline, mutations. Gastroenterology. 147(60): 1308-1316.e1. doi:
694 10.1053/j.gastro.2014.08.041
695
696 [42] P. Ashton-Prolla, H.N. Seuanez (2016). The Brazilian Hereditary Cancer Network:
697 historical aspects and challenges for clinical cancer genetics in the public health care system in
698 Brazil. Genet Mol Biol. 39(2): 163-165. doi: 10.1590/1678-4685-GMB-2014-0373
699
700 [43] A. Goverde, M.C. Spaander, H.C. van Doorn, H.J. Dubbink, A.M. van den Ouweland,
701 C.M. Tops et al. (2016). Cost-effectiveness of Routine Screening for Lynch Syndrome in
702 Endometrial Cancer Patients Up to 70years of Age. Gynecol Oncol. 143(3):453-459. doi:
703 10.1016/j.ygyno.2016.10.008
704
705 [44] K. Garg, M.M. Leitao Jr, N.D. Kauff, J. Hansen, K. Kosarin, J. Shia et al. (2009). Selection
706 of Endometrial Carcinomas for DNA Mismatch Repair Protein Immunohistochemistry Using
707 Patient Age and Tumor Morphology Enhances Detection of Mismatch Repair Abnormalities.
708 Am J Surg Pathol. 33(6):925-33. doi: 10.1097/PAS.0b013e318197a04

CHAPTER II: Mutational landscape of Lynch-like syndrome in endometrial cancer

The article was written following the guidelines of the Gynecologic Oncology Journal and was under preparation for submission to this Journal by November 2022.

709 **Mutational landscape of Lynch-like syndrome manifesting as endometrial cancer**

710

711 Reginaldo Cruz Alves Rosa[a,b], Andrey Yurchenko[c], Mariângela Ottoboni Brunaldi[d], Fernando
712 Chahud[d], Greice Andreotti Molfetta[a,e], Wilson Araújo Silva-Jr[a,e], Sergey Nikolaev[c], Patricia
713 Kannouche[b], Victor Evangelista de Faria Ferraz[a,f*]

714

715 [a] Department of Genetics, Ribeirão Preto Medical School, University of São Paulo, Ribeirão
716 Preto, Brazil
717 [b] Université Paris-Saclay, CNRS UMR9019, Gustave Roussy Cancer Campus, Villejuif, France
718 [c] Université Paris-Saclay, Inserm U981, Gustave Roussy Cancer Campus, Villejuif, France
719 [d] Department of Pathology and Legal Medicine, Ribeirão Preto Medical School, University of
720 São Paulo, Ribeirão Preto, Brazil
721 [e] Regional Blood Center at University Hospital of the Ribeirão Preto Medical School of
722 University of São Paulo, Ribeirão Preto, SP, Brazil
723 [f] Center for Medical Genomics at Clinical Hospital of the Ribeirão Preto Medical School,
724 University of São Paulo, Brazil

725

726

727 * Corresponding author:

728

729 Name: Dr. Victor Evangelista de Faria Ferraz. Address: Department of Genetics, Ribeirão
730 Preto Medical School, University of São Paulo. 3900, Bandeirantes Avenue, Ribeirao Preto,
731 SP, 4049-900, Brazil. E-mail address: vferraz@usp.br. Telephone number: +551633154500.
732 Fax number: +551633150222

733 **Abstract**

734

735 **Objective:** To characterize the germline and somatic mutational landscape of Lynch-like
736 syndrome (LLS) in patients diagnosed with endometrial cancer (EC).

737

738 **Methods:** Germline and somatic variants from 27 Lynch-like patients from a Brazilian cohort
739 of EC were identified by next-generation sequencing of 63 cancer-related genes. The tumor
740 mutational landscape was characterized in terms of driver mutation discovery, tumor mutation
741 burden (TMB), mutational signatures analysis, replicative strand bias, and microsatellite
742 instability (MSI) on sequencing data.

743

744 **Results:** Seven out of 27 patients (26%) had germline pathogenic variants in *ATM*, *ATR*,
745 *CHEK2*, *FAN1*, and *MUTYH* and were associated with a stronger family history of cancer than
746 patients without germline pathogenic variants. Somatic driver mutations in *MSH6* were present
747 in 45% of tumors and co-occurred with POLE-exonuclease mutations in 20% of cases. Tumors
748 with actionable mutations in *POLE*, *POLD1*, and MMR genes were associated with high TMB
749 and had their mutational profiles explained by mutational signatures typically associated with
750 alterations in those genes. The majority of MSI-high tumors (7/10) had somatic alterations in
751 MMR genes.

752

753 **Conclusion:** Most LLS cases manifesting as EC are sporadic, caused by somatic inactivation
754 of MMR genes. In addition, a subset of LLS might be hereditary due to germline variants in
755 cancer-predisposing genes.

756

757 **Keywords:** Brazil. Germline variants. Mismatch repair. Tumor sequencing.

758 **Highlights**

759

760 ● Germline pathogenic variants in cancer-predisposing genes are associated with familial
761 cases of LLS.

762

763 ● Somatic inactivation of MMR genes, mainly *MSH6*, is the leading cause of MMR
764 deficiency in endometrial tumors of LLS patients.

765

766 ● POLE-exonuclease somatic mutations are frequent drivers of MMR deficiency in LLS
767 tumors, probably resulting in inactivating mutations in MMR genes.

768 **Introduction**

769

770 Endometrial cancer (EC) is one of the most prevalent gynecological malignancies in
771 women worldwide, with an increasing incidence in recent years [1]. In Brazil, EC is the eighth-
772 most incident tumor in women, with about 6,540 new cases expected in 2022 [2]. Several risk
773 factors are associated with EC, including genetic predisposition, which is related to around 5%
774 of cases [3]. Lynch syndrome (LS), an autosomal dominant cancer predisposition disease, is
775 the leading cause of hereditary EC [4]. The lifetime risk for EC in women with LS is superior
776 to their risk for colorectal cancer, and EC is frequently the sentinel tumor [4,5]. In addition to
777 endometrial and colorectal carcinomas, individuals with LS are at increased risk for ovarian,
778 gastric, small bowel, urothelial, and other cancers [6].

779 LS is caused by germline pathogenic variants in one of the four main DNA-mismatch
780 repair (MMR) genes (*MLH1*, *MSH2*, *MSH6*, or *PMS2*), or less frequently, by germline deletion
781 of the 3' region of the *EPCAM* gene that leads to the epigenetic silencing of *MSH2* [7]. The
782 MMR pathway maintains genomic stability by correcting spontaneous single-nucleotide
783 mismatches and small insertion/deletion loops generated during DNA replication [8]. The
784 inactivation of MMR genes leads to the accumulation of mutations, mainly in microsatellite
785 loci. Consequently, LS-associated tumors commonly exhibit microsatellite instability (MSI) or
786 loss of MMR protein expression, which are the hallmarks of these tumors [9].

787 The screening of endometrial tumors for MMR deficiency by PCR-based MSI analysis
788 and/or MMR-protein immunohistochemistry (MMR IHC) is a highly recommended approach
789 for the detection of potential LS cases [10]. However, genetic testing fails to identify a germline
790 variant in MMR genes in up to 70% of cases with MMR-deficient (MMR-D) tumors, indicating
791 that this molecular phenotype is not restricted to LS [11]. Individuals with MMR-D tumors not
792 related to known causes, including somatic *MLH1* methylation and germline mutations in LS-
793 associated genes, are known as having Lynch-like syndrome (LLS) [12].

794 The underlying cause of LLS is not well established and is assumed to be
795 heterogeneous, comprising both hereditary and sporadic causes, making management decisions
796 for LLS patients more challenging than for LS carriers [13]. Potential etiologies for the MMR-D
797 phenotype in LLS tumors include germline mutations in MMR not detected by current
798 sequencing methods, bi-allelic somatic mutations in MMR genes, or false-positive results in
799 the tumor screening [14]. Additionally, mutations in genes not commonly accessed by genetic
800 testing approaches, including genes not directly associated with the MMR pathway but with
801 the potential to drive an MMR-D phenotype, may explain some LLS cases [15].

Here, we advanced into the characterization of LLS by using a targeted-sequencing approach in a panel of genes related to DNA repair and cancer predisposition and report the germline and somatic mutational landscape of LLS in a Brazilian cohort of EC.

Materials and Methods

Cohort and MMR deficiency analysis

We have previously accessed the prevalence of germline variants in LS-associated genes in 37 cases of MMR-D tumors from a Brazilian cohort of 242 primary endometrial carcinomas and identified 27 individuals with LLS [16]. Tumor screening for MMR deficiency was detailed in our previous study [16]. Briefly, we assessed the MMR status from tumor samples by MMR IHC, PCR-based MSI, and *MLH1* methylation. For IHC, the expression of MLH1, MSH2, MSH6, and PMS2 proteins was evaluated from tissue microarray blocks using a standard protocol, and the slides were independently analyzed by two pathologists (F.C. and M.O.B.). MSI analysis was performed by Multiplexed PCR amplification of five mononucleotide microsatellites in normal and tumor DNA. *MLH1* methylation was assessed by Methylation-Specific PCR (MS-PCR) using primers for the C region of the *MLH1* promoter.

To further advance in the molecular characterization of those cases, we obtained approval from the Scientific and Research Committee of the HC-FMRP-USP (protocol number 1.578.206/2016) and informed consent from the LLS patients. In addition, clinicopathological data were obtained from medical records.

Next-generation sequencing

For germline mutation analysis, genomic DNA was extracted from peripheral blood using the QIAamp DNA Mini Kit (Qiagen, Germantown, MD), and genomic libraries were prepared using the SureSelectQXT Kit (Agilent Technologies, Santa Clara, CA). Somatic mutations were evaluated in DNA isolated from representative formalin-fixed paraffin-embedded (FFPE) tumor sections, and the genomic libraries were prepared using the SureSelectXT Kit (Agilent Technologies). We performed targeted sequencing from constitutive and tumor DNA using a panel of 63 genes related to DNA repair and cancer predisposition [17]. The coding sequence, canonical splice sites, and both 5’ and 3’ untranslated regions (UTRs) of the 63 genes were sequenced on an Illumina NextSeq 500/550 platform (Illumina, San Diego, CA) on a 2 x 150 bp paired-end mode.

Bioinformatic analyses

Raw data quality analysis, read mapping, germline and somatic single-nucleotide variant (SNV) calling, as well as variant annotation for both germline and somatic sequencing were performed as previously described [17]. Germline copy-number alterations (CNVs) were accessed using the VisCap algorithm [18], and somatic CNVs were called using FACETS [19].

Germline SNVs were classified for their clinical significance according to the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) recommendations [20]. Germline variants classified as Benign or Likely benign were excluded from further analyses.

Somatic variants with a Mutect2 “PASS” flag and Variant Allele Frequency (VAF) higher than 5% were considered for further analyses. Driver mutations were defined as loss-of-function variants (frameshift, nonsense, or splice-site mutations) in tumor suppressor or DNA repair genes and missense mutations annotated as oncogenic or likely-oncogenic in the OncoKB database [21].

Mutational signatures (COSMIC v.3.3) and replication strand bias were accessed in samples with at least 20 SNVs using the R package Mutational Patterns v.1.11.0 [22]. Microsatellite instability analysis was performed using the MSISensor-Pro software [23] in the normal-tumor mode. Tumors with MSIsensor scores of ≥10 were considered MSI-High, ≥3 to <10 were classified as MSI-Low, and <3 were defined as microsatellite stable (MSS). Tumor mutation burden (TMB), defined as nonsynonymous variants per Megabase pair (Mb), was estimated from the coding region size (0.257 Mbp) of the gene panel.

Statistical analysis

Continuous data were described by mean and standard deviation values, and categorical variables were shown as numbers and percentages. Clinicopathological features between individuals with germline pathogenic or likely pathogenic variants (GPVs) and without GPVs (Non-GPVs) were compared using the Fisher's exact test (qualitative variables) or unpaired *t*-test (quantitative variables). *P*-values of <0.05 were considered significant. Statistical analyses were performed using R (version 4.2.1).

Results

870 The clinicopathological characteristics of the 27 LLS cases are summarized in **Table 1**.
871 All patients were diagnosed with endometrioid adenocarcinoma at a mean age of 59.1 years
872 (SD = 8.7 years). Most patients had their cancer diagnosis under 60 years (59.3%). The majority
873 of cases were low-grade tumors (77.8%), restricted to the uterus (88.9%, i.e., FIGO Stage I or
874 II). Angiolymphatic invasion was present in three (11.1%) cases.
875

Variables	All	GPVs	Non-GPVs	p-value
	***n* = 27 (100%)**	***n* = 7 (25.9%)**	***n* = 20 (74.1%)**	
Mean age at diagnosis (SD)	59.1 (8.7)	59.1 (12.2)	58.8 (7.5)	**0.9812[1]**
Age groups	***n* (%)**	***n* (%)**	***n* (%)**	**0.6618[2]**
< 60	16 (59.3)	5 (71.4)	11 (55.0)	
≥ 60	11 (40.7)	2 (28.6)	9 (45.0)	
Histology	***n* (%)**	***n* (%)**	***n* (%)**	**1.0000[2]**
Endometrioid	27 (100.0)	7 (100.0)	20 (100.0)	
Others	0 (0.0)	0 (0.0)	0 (0.0)	
FIGO grade	***n* (%)**	***n* (%)**	***n* (%)**	**1.0000[2]**
Low (1-2)	21 (77.8)	6 (85.7)	15 (75.0)	
High (3)	6 (22.2)	1 (14.3)	5 (25.0)	
FIGO stage	***n* (%)**	***n* (%)**	***n* (%)**	**1.0000[2]**
I-II	24 (88.9)	6 (85.7)	18 (90.0)	
III-IV	3 (11.1)	1 (14.3)	2 (10.0)	
Angiolymphatic invasion	***n* (%)**	***n* (%)**	***n* (%)**	**0.5453[2]**
Present	3 (11.1)	0 (0.0)	3 (15.0)	
Absent	24 (88.9)	7 (100.0)	17 (85.0)	
Amsterdam II	***n* (%)**	***n* (%)**	***n* (%)**	**0.0312[2]**
Yes	4 (16.0)	3 (50.0)	1 (5.3)	
No	21 (84.0)	3 (50.0)	18 (94.7)	
Unknown	2	1	1	

876
877 **Table 1 – Clinicopathologic features of 27 Lynch-like patients according to the presence of pathogenic**
878 **germline variants**. *P*-values were derived from comparing clinicopathological variables between patients with
879 pathogenic and likely pathogenic germline variants (GPVs) and those without GPVs (Non-GPVs). [1] Mean age
880 was compared among groups using the unpaired *t*-test. [2] Categorical variables were compared using the Fisher's
881 exact test. SD: standard deviation.
882

883 *Germline variants*

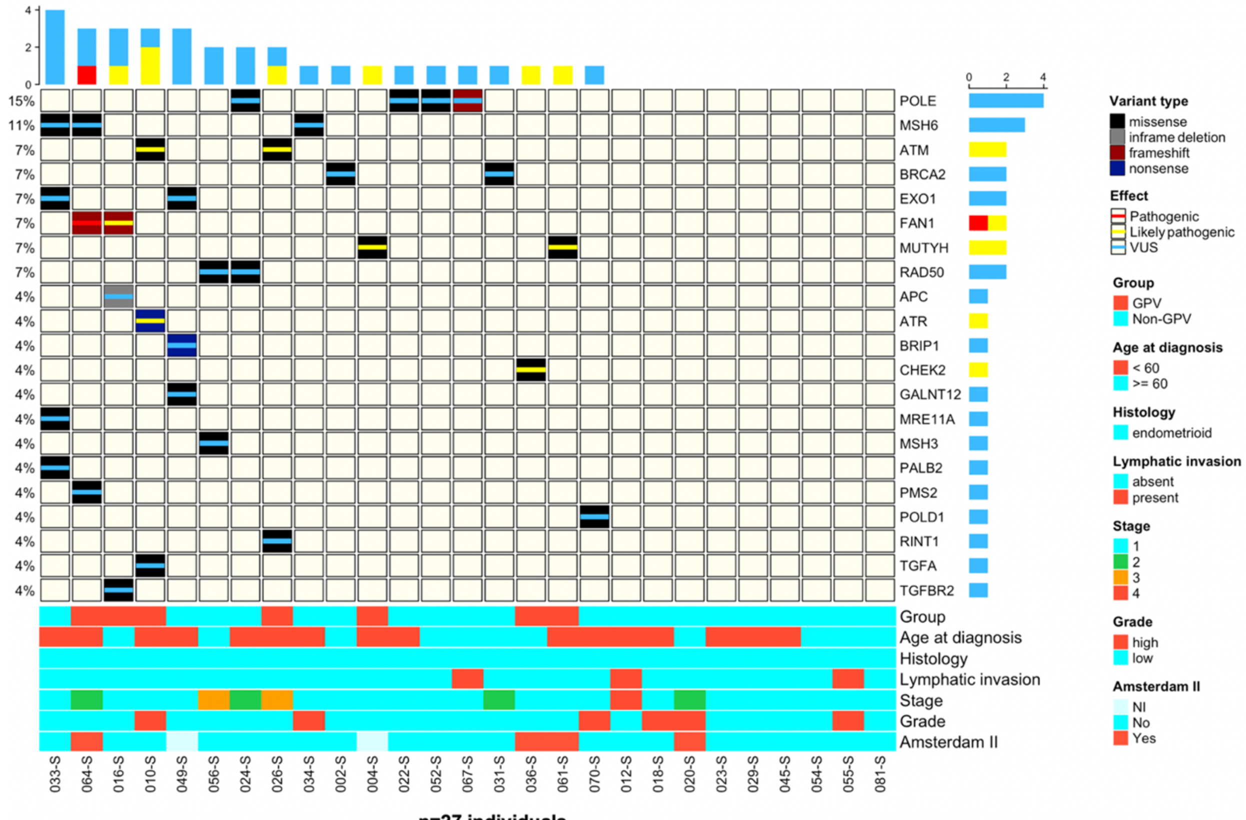
884

885 Targeted sequencing in constitutive DNA revealed a total of 32 heterozygous germline
886 SNVs in 20 out of 63 (31.7%) tested genes, comprising 26 missenses, three frameshifts, two

887 nonsense variants, and one in-frame deletion. Eighteen out of 27 (66.7%) LLS patients had at

888 least one germline variant (**Figure 1**).

889



890

891 **Figure 1 – Oncoprint of germline variants identified in 27 cases of Lynch-like syndrome.** All variants were
892 identified in heterozygosity. The genes are ordered from top to bottom by the decreasing percentage of altered
893 individuals (right panel). The top panel describes the number of germline mutations found in each individual. The
894 bottom panel shows the clinicopathological information of each LLS patient. The number of variants found in
895 each gene is shown in the left panel. Patient identification is provided at the bottom of the bottom panel. GPV:
896 individuals with germline pathogenic or likely pathogenic variants. Non-GPV: individuals without germline
897 pathogenic and likely pathogenic variants. NI: not informed.

898

899

900 Eight pathogenic or likely pathogenic variants were identified in seven patients (**Figure**

901 **1 and Table 2**). The variant c.736G>T (p.Val246Phe) in *MUTYH* was detected in two

902 unrelated individuals, two missense variants were identified in *ATM*, two frameshift variants

903 were observed in *FAN1*, one missense variant was detected in *CHEK2,* and one missense was

904 identified in the *ATR* gene (in the same individual with one likely pathogenic variant in *ATM*).

905 Moreover, 23 variants of uncertain clinical significance (VUS) were identified (**Figure 1 and**

906 **Table 2**). *POLE,* which is responsible for the proofreading function of the DNA Polymerase

907 epsilon, and *MSH6* were the genes most frequently mutated, with four and three VUS,

908 respectively. In addition to *MSH6*, we detected VUS in two other MMR genes: a missense
909 variant in *PMS2* and another in *MSH3.*

910 Eight distinct novel variants, *i.e*., not present neither in the literature nor in variation
911 databases, were identified (**Table 2**). They comprised one likely pathogenic frameshift in *FAN1*
912 and one likely pathogenic nonsense variant in *ATR*, in addition to six distinct VUS, including
913 one in-frame deletion in *APC*, and five missense variants in *MSH6* (two different variants),
914 *TGFA*, *PALB2*, *EXO1* and *GALNT12* (one variant each).

915 Personal and family history of cancer was available for 25/27 (92.3%) LLS patients
916 (**Table 1**). The Amsterdam II Criteria, which indicate a personal and family history of cancer
917 typically observed in Lynch syndrome patients, were more frequently fulfilled in LLS
918 individuals with GPVs compared to patients without GPVs (50% vs. 5.3%, p=0.0312, Fisher's
919 exact test). These results suggest that germline mutations in non-MMR genes may explain, at
920 least partially, the strong family history of cancer observed in a subset of LLS cases.

Patient ID	Gene	Transcript[1]	Nucleotide	Amino acid	Mutation type	ACMG/AMP classification	dbSNP ID	ClinVar	GnomAD AF	AbraOM AF
Patients with GPVs and VUS										
004-S	*MUTYH*	NM_001128425.2	c.736G>T	p.Val246Phe	Missense	Likely pathogenic	rs587780749	Conflicting	0.0000559	0.000427
010-S	*ATM*	NM_000051.4	c.8581A>G	p.Ile2861Val	Missense	Likely pathogenic	rs1555138472	VUS	NA	NA
	ATR	NM_001184.4	c.6265C>T	p.Arg2089Ter	Nonsense	Likely pathogenic	Novel	ND	NA	NA
	TGFA	NM_003236.4	c.451A>G	p.Thr151Ala	Missense	VUS	Novel	ND	NA	NA
016-S	*FAN1*	NM_014967.5	c.154_155insT	p.Pro52LeufsTer2	Frameshift	Likely pathogenic	Novel	ND	NA	NA
	APC	NM_000038.6	c.4847_4852del	p.Lys1616_Leu1618delinsIle	In-frame deletion	VUS	Novel	ND	NA	NA
	TGFBR2	NM_001024847.2	c.1159G>A	p.Val412Met	Missense	VUS	rs35766612	Conflicting	0.00103	0.002135
026-S	*ATM*	NM_000051.4	c.8558C>T	p.Thr2853Met	Missense	Likely pathogenic	rs141534716	VUS	0.0000518	NA
	RINT1	NM_021930.6	c.1088G>A	p.Gly363Asp	Missense	VUS	rs146567094	VUS	0.00000797	NA
036-S	*CHEK2*	NM_001005735.2	c.599T>C	p.Ile200Thr	Missense	Pathogenic	rs17879961	Conflicting	0.00412	0.000427
061-S	*MUTYH*	NM_001128425.2	c.736G>T	p.Val246Phe	Missense	Likely pathogenic	rs587780749	ND	0.0000559	0.000427
064-S	*FAN1*	NM_014967.5	c.560_561delCA	p.Thr187SerfsTer3	Frameshift	Pathogenic	rs772700881	ND	0.0000279	NA
	MSH6	NM_000179.3	c.3422C>T	p.Ser1141Phe	Missense	VUS	Novel	ND	NA	NA
	PMS2	NM_000535.7	c.1963G>A	p.Gly655Arg	Missense	VUS	rs1064793236	VUS	NA	NA
Patients with VUS only										
002-S	*BRCA2*	NM_000059.4	c.8850G>T	p.Lys2950Asn	Missense	VUS	rs28897754	Conflicting	0.000738	0.000854
022-S	*POLE*	NM_006231.4	c.1337G>A	p.Arg446Gln	Missense	VUS	rs151273553	Conflicting	0.000279	0.000427
024-S	*RAD50*	NM_005732.4	c.2548C>T	p.Arg850Cys	Missense	VUS	rs181961360	VUS	0.00028	ND
	POLE	NM_006231.4	c.1337G>A	p.Arg446Gln	Missense	VUS	rs151273553	Conflicting	0.000279	0.000427
031-S	*BRCA2*	NM_000059.4	c.1244A>G	p.His415Arg	Missense	VUS	rs80358417	Conflicting	0.00000802	0.000427
033-S	*MSH6*	NM_000179.3	c.3670G>T	p.Gly1224Trp	Missense	VUS	Novel	ND	ND	ND
	PALB2	NM_024675.4	c.3280G>T	p.Gly1094Trp	Missense	VUS	Novel	ND	ND	ND
	EXO1	NM_130398.4	c.836A>G	p.Asn279Ser	Missense	VUS	rs4149909	ND	0.024	0.019641
	MRE11	NM_005591.4	c.1051C>T	p.Arg351Cys	Missense	VUS	rs757492041	VUS	0.0000358	ND
034-S	*MSH6*	NM_000179.3	c.253C>T	p.Pro85Ser	Missense	VUS	rs779664343	VUS	ND	0.000427
049-S	*BRIP1*	NM_032043.3	c.3651G>A	p.Trp1217Ter	Nonsense	VUS	rs542698396	VUS	0.0000159	0.004697
	EXO1	NM_130398.5	c.409G>C	p.Ala137Ser	Missense	VUS	rs147663824	ND	0.00000399	0.001708
	GALNT12	NM_024642.5	c.1190A>G	p.His397Arg	Missense	VUS	Novel	ND	ND	ND
052-S	*POLE*	NM_006231.4	c.1015G>A	p.Asp339Asn	Missense	VUS	rs149029910	Conflicting	0.0000896	0.000427
056-S	*MSH3*	NM_002439.5	c.2732T>G	p.Leu911Trp	Missense	VUS	rs41545019	Conflicting	0.00239	0.002562
	RAD50	NM_005732.4	c.3882T>G	p.Asp1294Glu	Missense	VUS	rs587781426	VUS	0.00000796	ND
067-S	*POLE*	NM_006231.4	c.806delC	p.Pro269LeufsTer26	Frameshift	Pathogenic	rs1435794183	Conflicting	ND	ND
070-S	*POLD1*	NM_001256849.1	c.946G>C	p.Asp316His	Missense	VUS	rs746087148	VUS	ND	ND

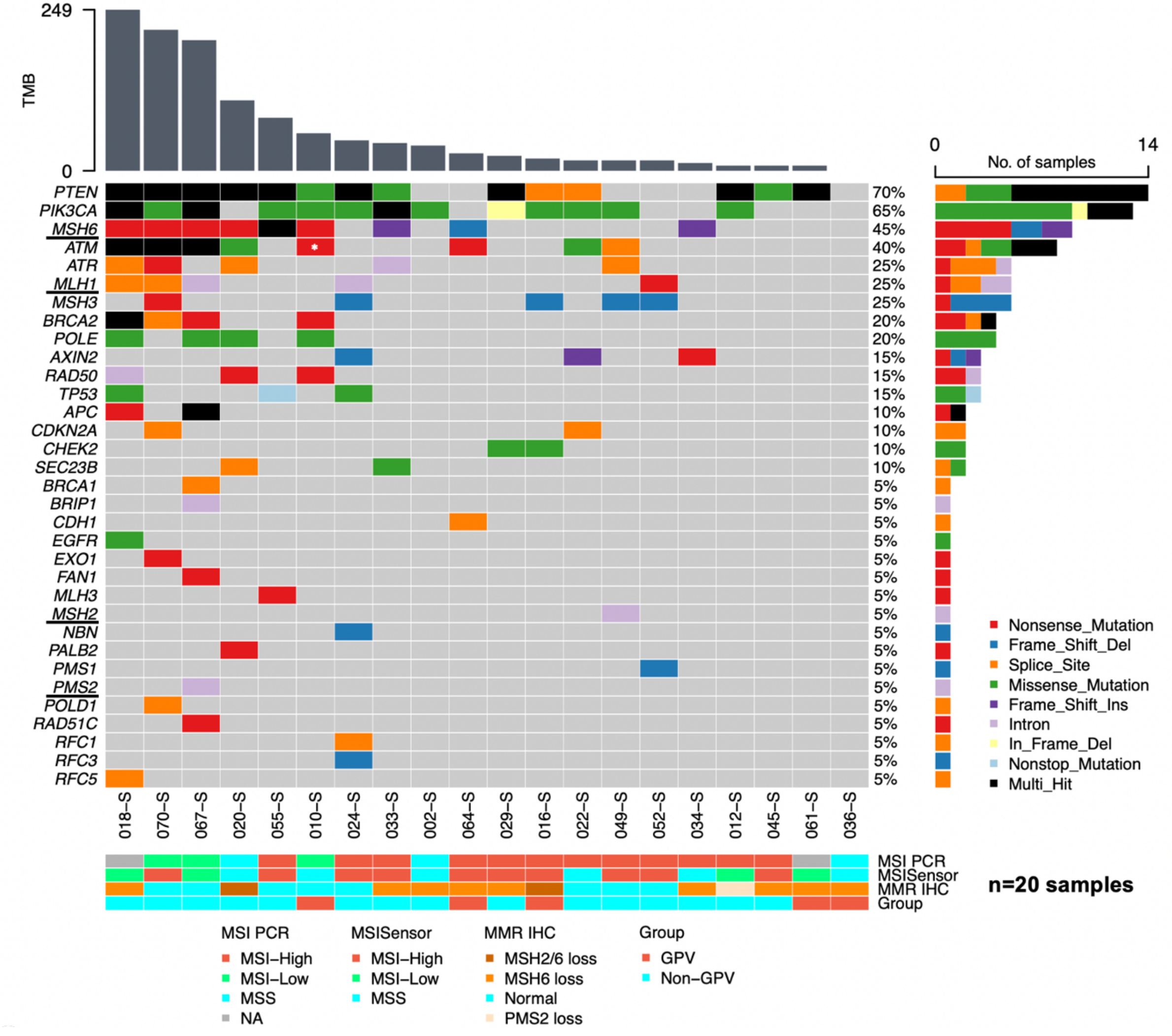
Table 2 – Germline variants, including pathogenic, likely pathogenic, and variants of uncertain significance, identified in the cohort of LLS patients. [1] Reference transcript ID from NCBI Reference Sequence Database (RefSeq). ACMG/AMP: American College of Medical Genetics and Genomics and the Association for Molecular Pathology. GnomAD AF: Total allele frequency in the Genome Aggregation Database. AbraOM AF: Total allele frequency in the Online Archive of Brazilian Mutations. ND: not described. VUS: variant of uncertain significance.

921 *Somatic mutational landscape*

922

923 We performed tumor sequencing in 20/27 (74.1%) LLS cases with sufficient amount 924 and quality of DNA and obtained a median depth of coverage of 558x, ranging from 114x to 925 2,288x. Driver mutations, TMB, MSI status, and MMR IHC findings are shown in **Figure 2**. 926 *PTEN* and *PIK3CA,* well-known drivers of tumorigenesis in EC [24], were the most frequently 927 mutated genes, with actionable mutations in 70% and 65% of the tumors, respectively.

928



929

930

931 **Figure 2 – Oncoplot of somatic driver variants found in 20 Lynch-like tumors.** Driver somatic variants were 932 defined as loss-of-function mutations (frameshifts, nonsense, and splice-site mutations) in tumor suppressor and 933 DNA repair genes, and missense mutations annotated as Oncogenic or Likely oncogenic by the OncoKB database. 934 Samples were sorted according to the decreasing values of TMB. The higher panel shows the TMB values of each 935 tumor indicated as the number of nonsynonymous mutations/Mbp (see the Materials and Methods section for more 936 details). Gene symbols with at least one driver mutation are indicated on the left of the plot, with the main MMR

genes being underlined with a black line. The right bar panel shows the number of samples with alterations in each gene. Tumor identification is present at the bottom of the plot. The white asterisk in the main graph indicates genes with pathogenic germline and driver somatic mutations. PCR-based MSI, MSISensor, MMR IHC, and Group annotations for each LLS tumor are shown at the bottom of the figure. TMB: tumor mutation burden. MSI: microsatellite instability. MSS: microsatellite stable. MMR IHC: immunohistochemistry staining for MLH1, MSH2, MSH6, and PMS2 proteins. GPV: LLS patient with germline pathogenic or likely pathogenic variants. Non-GPV: LLS patients without germline or likely pathogenic variants. NA: missing information.

One of the seven LLS patients with GPVs in cancer-predisposing genes (010-S) presented a somatic inactivating mutation in combination with a germline pathogenic variant in *ATM* (**Figure 2**). These findings indicate that the occurrence of the “second hit” in cancer-associated genes is rarely found by tumor sequencing in LLS tumors, suggesting that it is either not required to confer a cancer predisposition in LLS or that other factors, such as epigenetic mechanisms, might play the role of a second-hit in these cases.

MSH6 was the most mutated MMR gene, with inactivating variants in 45% of cases, one of which (055-S) with two inactivating variants (**Figure 2**, **Supplementary Table S1**). In addition to *MSH6*, we also detected somatic mutations in *MLH1* and *MSH3* in 25% of cases, and *MLH3*, *MSH2*, and *PMS2,* in 5% of cases. Together, these results confirm the high prevalence of inactivating mutations in MMR genes, especially *MSH6*, in endometrial tumors with an MMR-D phenotype.

Four (20%) tumors had missense variants in the exonuclease domain of *POLE* (POLE-exo*) in combination with somatic inactivating mutations in *MSH6* (010-S, 018-S, 020-S, and 067-S). The four *MSH6* variants were characterized by nonsense mutations resulting from G:C>T:A substitutions at the first position of a glutamic acid codon, resulting in a stop codon (GAA > TAA) (**Supplementary Figure S1**). Of note, all *MSH6* mutations had VAF values ≤ the ones of the POLE-exo*variants, suggesting that the *MSH6* mutations most likely resulted from the mutator phenotype triggered by mutations in the exonuclease domain of *POLE.*

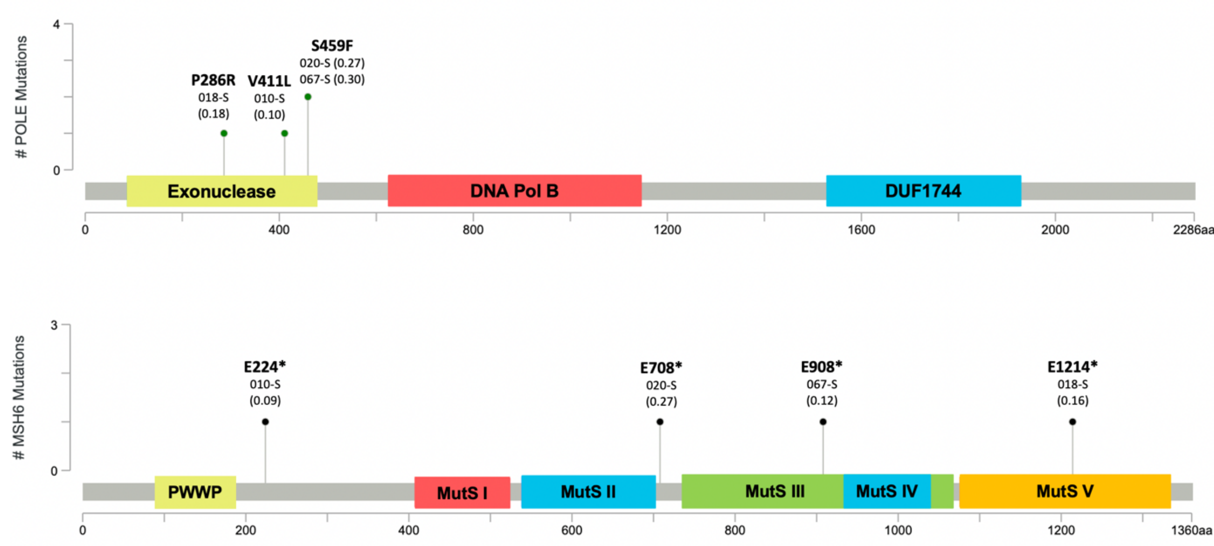
Patient ID	MMR IHC	PCR-based MSI	MSISensor				Tumor Purity (%)	Median depth of coverage (x)	Mutation Load	SNVs	Non-synonymous	TMB (mut/Mb)	Somatic variants			
			Total number of Loci	Loci with somatic instability	Score	Classification							MMR genes (VAF)	POLE (VAF)	POLD1 (VAF)	Others (VAF)[1]
002-S	MSH6 loss	MSS	247	1	0.4	MSS	65	369	53	50	10	39	none	none	none	none
004-S	PMS2 loss	MSI-High	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
010-S	Normal	MSI-Low	266	3	1.1	MSS	30	690	34	31	15	58	MSH6:p.E224* (0.09)	V411L (0.094)	none	ATM:p.E1996* (0.10)
012-S	PMS2 loss	MSI-High	157	7	4.5	MSI-Low	50	114	6	4	2	8	none	none	none	none
016-S	MSH2/6 loss	MSI-High	223	37	16.6	MSI-High	50	262	16	6	5	19	MSH3:p.K383fs (0.35)	none	none	none
018-S	MSH6 loss	NA	264	23	9.1	MSI-Low	40	2234	244	243	64	249	MSH6:p.E1214* (0.16) MLH1:splice site (0.17)	P286R (0.176)	none	none
020-S	MSH2/6 loss	MSS	204	3	1.5	MSS	75	383	133	132	28	109	MSH6:p.E708* (0.27)	S459F (0.355)	none	none
022-S	Normal	MSI-High	232	2	0.9	MSS	45	645	19	11	4	16	none	none	none	none
023-S	PMS2 loss	MSI-High	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
024-S	Normal	MSI-High	233	56	24.0	MSI-High	85	226	37	21	12	47	MSH3:p.K383fs (0.21) MLH1:intronic (0.22)	none	none	none
026-S	MSH6 loss	MSS	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
029-S	MSH6 loss	MSI-High	243	35	14.4	MSI-High	75	383	24	10	6	23	none	none	none	none
031-S	PMS2 loss	MSS	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
033-S	MSH6 loss	MSI-High	241	49	20.3	MSI-High	85	425	27	22	11	43	MSH6:p.T1085fs (0.41)	none	none	none
034-S	MSH6 loss	MSI-High	214	7	3.3	MSS	20	227	17	11	3	12	MSH6:p.I1170fs (0.06) MSH3:p.G260fs (0.06)	none	none	none
036-S	MSH6 loss	MSS	227	5	2.2	MSS	30	729	2	2	0	0	none	none	none	none
045-S	MSH6 loss	MSI-High	284	36	12.7	MSI-High	20	187	3	3	2	8	none	none	none	none
049-S	Normal	MSI-High	254	85	33.5	MSI-High	90	258	20	8	4	16	MSH2:intronic (0.21) MSH3:p.K383fs (0.31)	none	none	none
052-S	Normal	MSI-High	259	107	41.3	MSI-High	65	187	35	18	4	16	MLH1:p.Y384*(0.23) PMS2:intronic (0.25) MSH3:p.L1083fs (0.06)	none	none	none
054-S	PMS2 loss	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

The table continues in the next page.

Patient ID	MMR IHC	PCR-based MSI	MSISensor				Tumor Purity (%)	Median depth of coverage (x)	Mutation Load	SNVs	Non-synonymous	TMB (mut/Mb)	Somatic variants			
			Total number of Loci	Loci with somatic instability	Score	Classification							MMR genes (VAF)	POLE (VAF)	POLD1 (VAF)	Others (VAF)[1]
055-S	Normal	MSI-High	309	100	32.4	MSI-High	55	345	68	51	21	82	MSH6:p.R1172fs (0.59) MSH6 (LOH) MLH3:p.Q1445* (0.06)	none	none	none
056-S	MSH2/6 loss	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
061-S	MSH6 loss	NA	309	18	5.83	MSI-Low	20	430	3	2	2	8	none	none	none	none
064-S	MSH6 loss	MSI-High	227	73	32.16	MSI-High	80	177	26	20	7	27	MSH6:p.F1088fs (0.37)	none	none	none
067-S	Normal	MSI-Low	330	28	8.48	MSI-Low	80	606	191	190	52	202	MSH6:p.E908* (0.12) PMS2:intronic (0.37) MLH1:intronic (0.10)	S459F (0.298)	none	none
070-S	Normal	MSI-Low	370	43	11.62	MSI-High	75	2288	207	202	56	218	MSH6:p.S612* (0.29) MSH3:p.E725* (0.28) MLH1:splice site (0.06)	none	Splice site (0.27)	none
081-S	PMS2 loss	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Supplementary Table S1 – Molecular findings of the endometrial cohort of Lynch-syndrome patients. Results of MMR IHC, PCR-based MSI classification, MSIsensor, and tumor sequencing of the 27 LLS tumors included in this study. MMR IHC: immunohistochemistry staining for MLH1, MSH2, MSH6, and PMS2 proteins. MSI: microsatellite instability. MSS: microsatellite stable. SNVs: single-nucleotide variants. TMB: tumor mutation burden. VAF: variant allele frequency. [1] refers to genes with germline pathogenic variants other than MMR and *POLE/POLD1* genes. NA: missing value.

964



965 **Supplementary Figure S1 – Lollipop plot of somatic variants identified in four tumors with both *POLE*-
966 exo* and *MSH6* mutations.** Protein positions with a mutation were represented by a circle at the top of a vertical
967 line. The length of the lines, as well as the y-axis, represent the frequency of the variants among the tumors. The
968 full length of the protein is represented by a vertical gray bar. Colored boxes represent protein functional domains.
969 Protein consequence of mutations, as well as their respective VAF values (in parentheses), and tumor
970 identification, are represented at the top of the circles. VAF: variant allele frequency.
971

972 LLS tumors exhibited a mean TMB of 60 mutations/Mbp, ranging from 0 to 249
973 mutations/Mb (**Figure 2**, **Supplementary Table S1**). Tumors with somatic *POLE*-exo*
974 mutations, in addition to the tumor with two inactivating events in *MSH6* (055-S), reached some
975 of the highest TMB values. Moreover, the case with the second greatest TMB (070-S) had a
976 somatic splice-site mutation in *POLD1* (implicated in the proofreading activity of the DNA
977 polymerase delta) and a germline mutation in the exonuclease domain of *POLD1* (*POLD1*-
978 exo*) (**Figure 1** and **Figure 2**). These results corroborate the relationship between mutations
979 in the proofreading domain of replicative DNA polymerases, as well as the inactivation of
980 MMR genes, with a mutator phenotype in human tumors.

981 The 96-type mutational profile and refitting mutational signature analyses were
982 performed in 10/20 (50%) tumors with a minimum of 20 SNVs (**Figure 3, Supplementary**
983 **Figure S2**). The mutational profile of all four tumors with POLE-exo* somatic mutations was
984 explained mainly by COSMIC signatures SBS10a and SBS10b, which are associated with
985 defects in the proofreading activity of Polymerase epsilon. We also observed a 2-fold increase
986 of C>T substitutions in the DNA leading strand compared to the lagging strand of tumors 018-S
987 and 067-S (**Supplementary Figure S3**). In addition, 32% of the somatic mutational profile in
988 the case with *POLD1* mutations was explained by the COSMIC SBS10c and SBS10d

signatures, which are associated with POLD1-exo* mutations. COSMIC signatures associated with MMR deficiency (SBS6, SBS15, SBS21, and SBS44) explained more than 65% of mutations in tumor 033-S, a case with a germline VUS, and a somatic frameshift mutation in *MSH6*. MMR-D signatures also explained about 20% of mutations observed in tumors 024-S (with monoallelic driver mutations in *MLH1* and *MSH3*) and 002-S (with neither germline nor somatic variants in MMR genes). Despite the presence of a germline VUS and a somatic frameshift in *MSH6*, COSMIC signatures related to MMR deficiency did not contribute to the somatic mutational profile of 064-S. These findings demonstrate that mutational signatures frequently reflect the presence of driver mutations in *POLE/POLD1* and MMR genes in endometrial cancer.

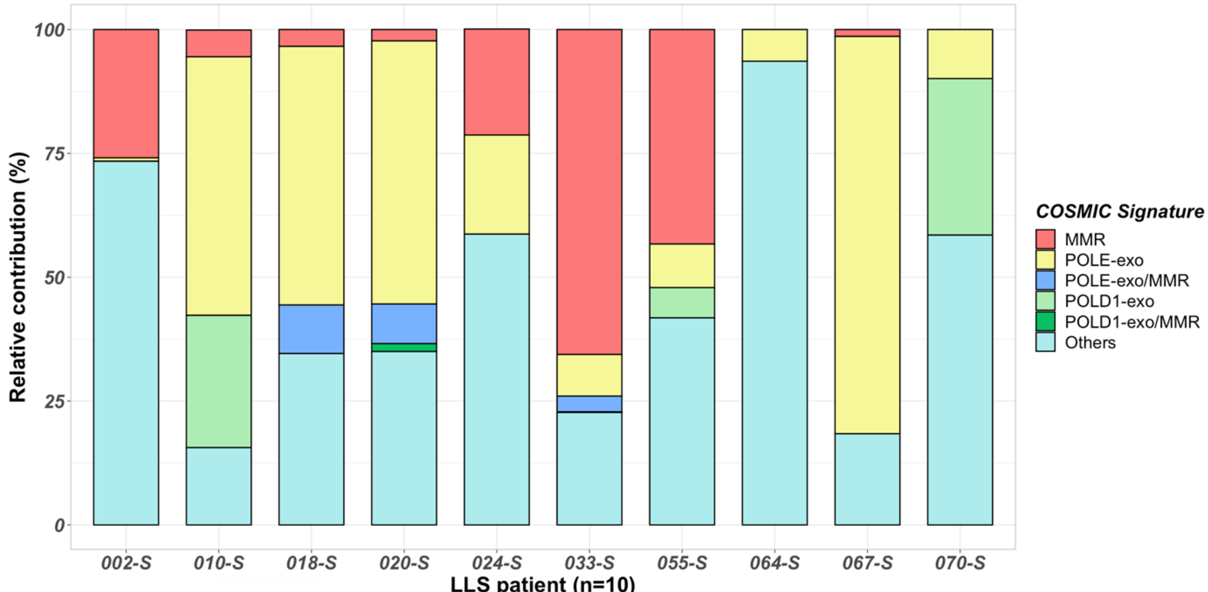
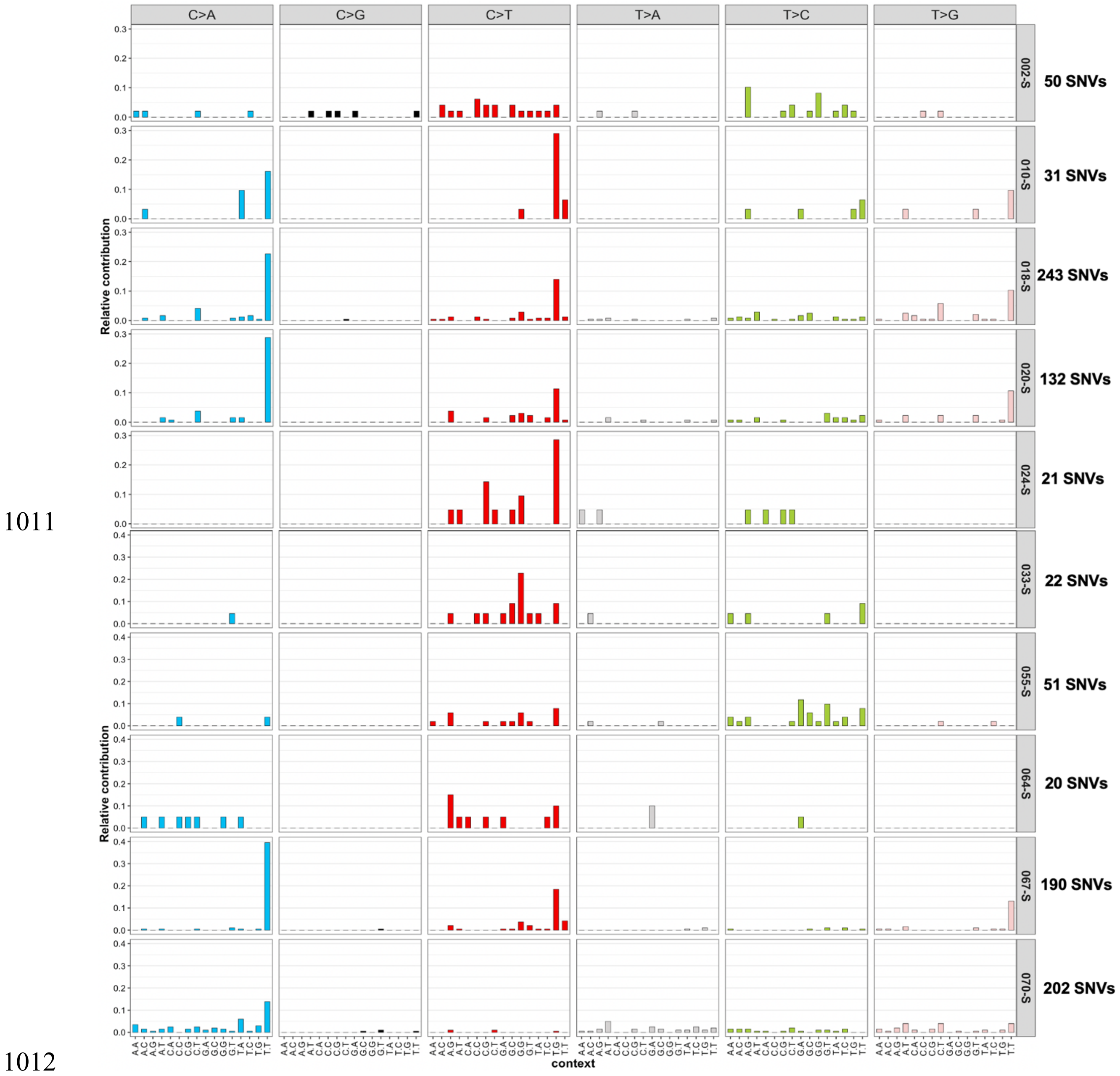


Figure 3 – Relative contribution of COSMIC SBS mutational signatures to the mutational profile of somatic single-nucleotide substitutions observed in LLS tumors. The contribution of COSMIC signatures to the mutational profile of 10 LLS patients with a minimum of 20 single base substitutions (SBS) was quantified by refitting analysis using the MutationalPatters R package. MMR: combined contribution of signatures SBS6, SBS15, SBS21, and SBS44. POLE-exo: contribution of signatures SBS10a and SBS10b. POLE-exo*/MMR: signature SBS14. POLD1-exo*: combined contribution of signatures SBS10c and SBS10d. POLD1-exo*/MMR: signature SBS20.

1011

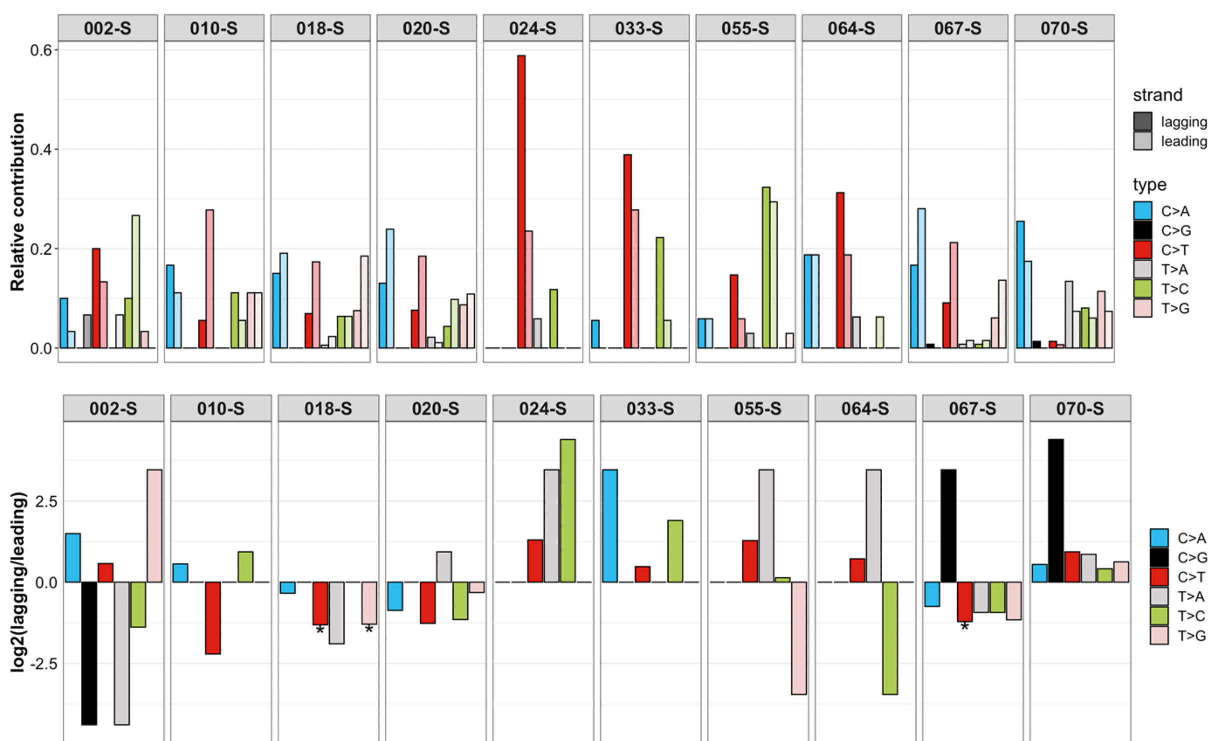


1012

1013 **Supplementary Figure S2 – 96 trinucleotide mutational profile of 10 LLS tumors with a minimum of 20**
1014 **single-nucleotide substitutions**. Trinucleotides (shown in x-axes) are formed based on the six classes of single-
1015 nucleotide substitutions (C>A, C>G, C>T, T>A, T>C, and T>G) and their immediate 5' and 3' nucleotide
1016 contexts. The y-axes indicate the relative contribution of each trinucleotide to the total amount of single-nucleotide
1017 substitutions in each tumor. Tumor identification and the total amount of single-nucleotide variants (SNVs) are
1018 shown at the right of each mutational profile.
1019

1020 Microsatellite instability was evaluated in a set of more than 200 loci by the MSIsensor
1021 tool (**Figure 2** and **Supplementary Table S1**). Ten out of 20 (50%) tumors were classified as
1022 MSI-high, and (7/10, 70%) of them had at least a somatic inactivating mutation in the main
1023 MMR genes (cases 024-S, 033-S, 049-S, 052-S, 055-S, 064-S, and 070-S). Three MSI-High

tumors (016-S, 029-S, and 045-S) had no germline or somatic mutation in the main MMR genes. In addition, 4/20 (20%) LLS tumors were classified as MSI-Low, two of which with somatic POLE-exo* mutations (018-S and 067-S), and the two others (012-S and 061-S) with no mutation in the main MMR genes. Finally, 6/20 (30%) microsatellite stable (MSS) were identified, including two cases with somatic POLE-exo* variants (010-S and 020-S), one case with a germline VUS and a somatic inactivating mutation in *MSH6* (034-S), and three cases with no mutations in the main MMR genes (002-S, 022-S, and 036-S). Together, these results corroborate the association between the inactivation of the main MMR genes with the MSI-High phenotype in endometrial tumors and its absence in tumors with POLE-exo* mutation.



Supplementary Figure S3 – Replicative strand bias. The top panel shows the relative frequency of each base substitution to the mutations in both lagging (solid colors) and leading (opaque colors) DNA strands. The bottom panel shows the Log2 ratio of mutations occurring in each DNA strand. (*): significative difference (p>0.05, based on the Poisson test for strand asymmetry) of mutations in one strand in relation to the other.

We found discordant MSI classification between PCR-based and MSIsensor methods in 5/20 (25%) tumors (**Figure 2**, **Supplementary Table S1**). The tumor 070-S, with a germline POLD1-exo* mutation, was previously classified as MSI-Low by PCR-based MSI and was found to be MSI-High by MSIsensor analysis. Three tumors have been classified as MSI-High

1044 by PCR-MSI but were classified as MSI-Low (012-S) or MSS (022-S and 034-S) by the
1045 MSIsensor tool. Finally, the tumor 010-S was classified as MSI-Low by PCR-based MSI and
1046 was classified as MSS after analysis with MSIsensor. These discordant findings indicate that
1047 the use of the PCR-based MSI analysis in a pentaplex panel of mononucleotide microsatellites
1048 might not be as accurate in classifying LLS tumors for the MMR-deficiency status as it is for
1049 Lynch syndrome and *MLH1*-methylated endometrial tumors.
1050 Finally, we found six tumors with a normal expression of the four main MMR proteins
1051 classified with either MSI-High (070-S, 055-S, 024-S, 049-S, 052-S) or MSI-Low (067-S) by
1052 both MSIsensor and PCR-based MSI analysis (**Figure 2**, **Supplementary Table S1**). Except
1053 for tumor 055-S, which had two somatic inactivating events in *MSH6*, all of these tumors had
1054 a monoallelic driver mutation in at least one of the main MMR genes, indicating that mutation
1055 in MMR genes leading to MMR deficiency is not always correlated to the lack of the protein
1056 in endometrial tumors.
1057
1058 **Discussion**
1059
1060 In this study, we used a targeted sequencing approach to decipher the mutational
1061 landscape of Lynch-like syndrome in a Brazilian cohort of endometrial cancer. Germline
1062 analyses revealed the presence of pathogenic variants in cancer-predisposing genes in 26% of
1063 the cohort. A strong family history of cancer was prevalent in individuals with pathogenic
1064 variants. A "second hit" mutation was identified in the tumor of one patient with a germline
1065 pathogenic variant in *ATM*. Driver mutations in MMR genes, mainly in *MSH6*, were present in
1066 65% of tumors. Co-occurrence of actionable mutations in *POLE* and *MSH6* genes was noticed
1067 in 20% of cases, with results supporting *POLE* as a frequent early driver event in endometrial
1068 tumors from LLS patients. Tumors with *POLE, POLD1,* or MMR mutations presented with
1069 high TMB and mutational signatures, reflecting the mutational status of these genes. MSI
1070 analysis on tumor sequencing data confirmed the association between inactivating mutations in
1071 the main MMR genes and the MSI-High phenotype.
1072 The etiology of Lynch-like syndrome is heterogeneous, with a subset of LLS patients
1073 presenting a strong family of cancer, suggesting a heredity etiology. However, the existence of
1074 genetic factors conferring predisposition to cancer development is not always identified [25].
1075 We detected seven cases of LLS manifesting as endometrial cancer carrying pathogenic

1076 germline variants in DNA damage response and repair, including *ATM*, *ATR*, *CHEK2*, *FAN1*,
1077 and *MUTYH*. Those patients fulfilled the Amsterdam Criteria II, which indicates a family
1078 history of cancer typically observed in Lynch syndrome, more frequently than individuals
1079 without pathogenic variants (**Table 1**). Biallelic germline variants in *MUTYH* [26,27], as well
1080 as monoallelic mutations in *CHEK2*, especially the variant I200T identified in our cohort, have
1081 already been reported in LLS patients with CRC [28,29]. Additionally, heterozygous variants
1082 in *FAN1* and *ATR* have been found in patients with MMR-proficient CRC, positive for
1083 Amsterdam criteria [30,31]. The relation between *ATM* variants and hereditary tumors from the
1084 LS spectrum is rare, with a single polymorphism being associated with an increased risk for
1085 LS-related tumors [32]. Mutations in some of these genes have also been reported in
1086 endometrial cancer. Yurgelun *et al*. have found germline variants in *ATM* and *CHEK2* in
1087 patients diagnosed with endometrial tumors and with an LS-associated family history of cancer.
1088 However, the author did not assess the MMR status tumor testing [33]. Ring *et al*. have also
1089 reported a case of endometrial carcinoma with a germline variant in *ATM* without reporting its
1090 MMR status [34].
1091 The involvement of DNA repair and other tumor suppressor genes in cancer
1092 predisposition are assumed to follow Knudson's two-hit hypothesis, which postulates that
1093 hereditary cancer predisposition is caused by a germline mutation and a somatic inactivation of
1094 the wild-type allele, by a second mutation, loss of heterozygosity (LOH), or epigenetic gene
1095 silencing [35]. However, a somatic event is not always identified in tumors of individuals with
1096 germline pathogenic variants in cancer predisposing genes. In our study, we found a somatic
1097 mutation in *ATM* as a potential second hit in one of the seven patients with GPVs (**Figure 2**).
1098 In agreement with our results, Huang et al. identified pathogenic or likely pathogenic germline
1099 variants in 7.9% of the TCGA cohort comprising 10,389 adult cancers, and the presence of
1100 LOH or somatic mutations was observed in less than 40% of the cases [36]. The absence of a
1101 second hit in a cancer-predisposing gene does not exclude the involvement of those genes with
1102 cancer predisposition, especially for individuals with a strong family history of cancer.
1103 Moreover, we cannot exclude the involvement of epigenetic factors such as promoter
1104 hypermethylation acting as the somatic second hits in tumors of LLS patients with germline
1105 pathogenic variants. Likewise, some missense variants may act as a dominant-negative in some
1106 tissues and be associated with cancer predisposition, even in a monoallelic state [37,38]. This
1107 hypothesis could explain the cancer predisposition observed in two patients with likely

1108 pathogenic missense mutations in *MUTYH* and *CHEK2*, positive for Amsterdam II Criteria
1109 from our cohort.
1110 Somatic inactivation of MMR genes by biallelic mutations or LOH events is known as
1111 the leading cause of LLS tumors [14]. In agreement with the literature, we found mutations in
1112 MMR genes in the majority (13/20, 65%) of tumors with sequencing results, most of them
1113 (45%) with somatic mutations in *MSH6* (**Figure 2**). Except for case 055-S, only monoallelic
1114 mutations were identified. Three of those cases (033-S, 034-S, and 064-S) had germline VUS
1115 coupled with somatic inactivating mutations in *MSH6*. Loss of MSH6 protein was also detected
1116 in those tumors based on MMR IHC, suggesting that the germline missense mutations in *MSH6*
1117 could be the first hit. The segregation analysis of those germline variants in their families, as
1118 well as functional assays to determine the impact of those variants in the repair activity of the
1119 MMR pathway, would be helpful to support this hypothesis. In concordance with our findings,
1120 other studies have also found a single somatic mutation in MMR genes in a substantial portion
1121 of LLS tumors [14,39], indicating that, if required, the second hit in the MMR gene is not
1122 always identified by sequencing techniques.
1123 We identified somatic mutations in the exonuclease domain of *POLE* in 4/20 (20%) of
1124 cases. Those tumors were characterized by high TMB, an overrepresentation of C>T, C>A, and
1125 T>C base substitutions, most of them being associated with COSMIC signatures SBS10a and
1126 SBS10b, and a replicative strand bias, with mutations occurring more frequently in the leading
1127 strand of DNA. The POLE-exo mutations identified (P286R, V411L, and S459F) are well-
1128 characterized hotspot mutations commonly found in hypermutated endometrial carcinomas
1129 with mutational features similar to those tumors identified in our cohort [40]. Interestingly, one
1130 of those cases also has a germline loss-of-function variant in the exonuclease domain of *POLE*
1131 (067-S, **Figure 1**). In a dedicated analysis, we have shown that this case had twice the number
1132 of somatic mutations in comparison with endometrial tumors with the same POLE-exo
1133 mutation (S459F) without other variants in *POLE* [17], which was further confirmed by another
1134 case in our cohort (020-S, **Figure 2**, **Supplementary Table S1**). The four tumors with POLE-
1135 exo* mutations were also mutated in the *MSH6*, all of them being characterized by G>T
1136 substitutions that resulted in glutamic acid-to-stop codon mutations. In addition, the *MSH6*
1137 variants had VAF values similar or slightly inferior to the ones found in the POLE-exo*
1138 mutations (**Supplementary Figure S1**). The *MSH6* mutations might have been raised due to

1139 the mutator phenotype triggered by the POLE-exo* mutations, as previously observed [39,40],
1140 and could explain the MSI and MMR IHC pattern observed in those tumors.

1141 Our study has some limitations. Since the mutation analyses were based on a targeted
1142 sequencing approach, the mutational landscape described is restricted to the genomic regions
1143 covered by the panel. Therefore, some features, including TMB, mutational signatures, and
1144 replicative strand bias, are estimates and might differ from their values at the whole genome
1145 level. Additionally, even though the existence of three tumors with high levels of MSI without
1146 any mutation in MMR genes in our cohort suggests the involvement of alternative drivers of
1147 MMR deficiency in endometrial tumors, our sequencing approach could not assess some rare
1148 factors already described as alternative causes of MMR-D phenotype in tumors, including
1149 *SETD2* mutations, overexpression of microRNAs that downregulate *MLH1*, *MSH2*, and *MSH6*,
1150 and deep intronic and structural variants with the potential to inactivate some MMR genes [25].

1151 Altogether, our results confirm the somatic inactivation of MMR genes as the leading
1152 cause of LLS, associating the majority of cancers with a sporadic origin. However, a small
1153 portion of cases might be hereditary due to germline mutations in cancer-predisposing genes,
1154 which are unrelated to the MMR-D observed in their tumors. Our study provides a
1155 comprehensive characterization of LLS in endometrial cancer and supports the analysis of both
1156 germline and somatic mutations to address better management for LLS patients and their
1157 relatives based on tumor etiology.
1158

1159 **Ethics approval and consent to participate**

1160

1161 This study was approved by the Ethics and Research Committee of the Clinics Hospital
1162 of the Ribeirão Preto Medical School (protocol number 1.578.206/2016). Informed written
1163 consent was obtained from all patients included in the study.

1164

1165 **Acknowledgments**

1166

1167 The authors would like to thank the patients for their kind collaboration and contribution
1168 to this study.

1169

1170 **Author contributions**

VF, WJ, and RR conceived and planned the study design. RR and GM performed Molecular Biology experiments. FC and MB provided the clinicopathological data and reviewed histologic slides. RR and AY led the writing of the manuscript. RR, SN, and AY supported data acquisition and data analysis. VF, SN, PK, and WJ provided a critical review of the manuscript. All authors reviewed the final version of the manuscript and gave full consent for publication.

Role of the funding source

Costs of the study design and molecular analyses were funded by the São Paulo Research Foundation (FAPESP grant 2017/04283-8) and National Council for Scientific and Technological Development (CNPq grant 423007/2016-5).

Declaration of competing interest

The authors declared no potential conflicts of interest regarding the research, authorship, and/or publication of this article.

Submission of declaration and verification

This manuscript has not been published previously, is not under consideration for publication elsewhere, and this publication is approved by all authors and the responsible authorities where the work has been carried out. This manuscript will not be published elsewhere in the same form.

Availability of data

The raw data from the targeted sequencing will be made available by the corresponding author under request.

References

[1] D. Kanopiene, J. Vidugiriene, K.P. Valuckas, G. Smailyte, S. Uleckiene, J. Bacher, Endometrial cancer and microsatellite instability status, Open Med (Wars). 10 (2014) 70–76. https://doi.org/10.1515/med-2015-0005.

[2] INCA - Instituto Nacional de Câncer. (2019). https://www.inca.gov.br/estimativa (accessed June 14, 2022).

[3] K. Banno, I. Kisu, M. Yanokura, K. Umene, Y. Nogami, K. Tsuji, K. Masuda, A. Ueki, Y. Kobayashi, W. Yamagami, N. Susumu, D. Aoki, Hereditary Endometrial Cancer: Lynch Syndrome, Curr Obstet Gynecol Rep. 2 (2013) 11–18. https://doi.org/10.1007/s13669-012-0029-0.

[4] N.A.J. Ryan, M.A. Glaire, D. Blake, M. Cabrera-Dandy, D.G. Evans, E.J. Crosbie, The proportion of endometrial cancers associated with Lynch syndrome: a systematic review of the literature and meta-analysis, Genetics in Medicine. 21 (2019) 2167–2180. https://doi.org/10.1038/s41436-019-0536-8.

[5] L.A. Meyer, R.R. Broaddus, K.H. Lu, Endometrial Cancer and Lynch Syndrome: Clinical and Pathologic Considerations, Cancer Control. 16 (2009) 14–22.

[6] H. Hampel, Genetic counseling and cascade genetic testing in Lynch syndrome, Familial Cancer. 15 (2016) 423–427. https://doi.org/10.1007/s10689-016-9893-5.

[7] H.T. Lynch, C.L. Snyder, T.G. Shaw, C.D. Heinen, M.P. Hitchins, Milestones of Lynch syndrome: 1895–2015, Nat Rev Cancer. 15 (2015) 181–194. https://doi.org/10.1038/nrc3878.

[8] N. Pećina-Šlaus, A. Kafka, I. Salamon, A. Bukovac, Mismatch Repair Pathway, Genome Stability and Cancer, Frontiers in Molecular Biosciences. 7 (2020). https://www.frontiersin.org/article/10.3389/fmolb.2020.00122 (accessed June 14, 2022).

1232

1233 [9] V.L. Cox, A.A. Saeed Bamashmos, W.C. Foo, S. Gupta, S. Yedururi, N. Garg, H.C. Kang,
1234 Lynch Syndrome: Genomics Update and Imaging Review, RadioGraphics. 38 (2018)
1235 483–499. https://doi.org/10.1148/rg.2018170075.
1236

1237 [10] A. Wang, J. McCracken, Y. Li, L. Xu, The practice of universal screening for Lynch
1238 syndrome in newly diagnosed endometrial carcinoma, Health Science Reports. 1 (2018)
1239 e43. https://doi.org/10.1002/hsr2.43.
1240

1241 [11] K. Tanakaya, Current clinical topics of Lynch syndrome, Int J Clin Oncol. 24 (2019)
1242 1013–1019. https://doi.org/10.1007/s10147-018-1282-7.
1243

1244 [12] J.M. Carethers, E.M. Stoffel, Lynch syndrome and Lynch syndrome mimics: The growing
1245 complex landscape of hereditary colon cancer, World Journal of Gastroenterology. 21
1246 (2015) 9253–9261. https://doi.org/10.3748/wjg.v21.i31.9253.
1247

1248 [13] D.D. Buchanan, C. Rosty, M. Clendenning, A.B. Spurdle, A.K. Win, Clinical problems
1249 of colorectal cancer and endometrial cancer cases with unknown cause of tumor mismatch
1250 repair deficiency (suspected Lynch syndrome), Appl Clin Genet. 7 (2014) 183–193.
1251 https://doi.org/10.2147/TACG.S48625.
1252

1253 [14] S. Haraldsdottir, H. Hampel, J. Tomsic, W.L. Frankel, R. Pearlman, A. de la Chapelle,
1254 C.C. Pritchard, Colon and Endometrial Cancers With Mismatch Repair Deficiency Can
1255 Arise From Somatic, Rather Than Germline, Mutations, Gastroenterology. 147 (2014)
1256 1308-1316.e1. https://doi.org/10.1053/j.gastro.2014.08.041.
1257

1258 [15] J.M. Carethers, Differentiating Lynch-like from Lynch Syndrome, Gastroenterology. 146
1259 (2014) 602–604. https://doi.org/10.1053/j.gastro.2014.01.041.
1260

1261 [16] R.C.A. Rosa, J.O. Santis, L.A. Teixeira, G.A. Molfetta, J.T.T. dos Santos, V. dos S.
1262 Ribeiro, F. Chahud, A. Ribeiro-Silva, M.O. Brunaldi, W.A. Silva Jr, V.E. de F. Ferraz,
1263 Lynch syndrome identification in a Brazilian cohort of endometrial cancer screened by a

1264 universal approach, Gynecologic Oncology. 159 (2020) 229–238.
1265 https://doi.org/10.1016/j.ygyno.2020.07.013.
1266
1267 [17] First description of ultramutated endometrial cancer caused by germline loss-of-function
1268 and somatic exonuclease domain mutations in POLE gene - PubMed, (n.d.).
1269 https://pubmed.ncbi.nlm.nih.gov/33001133/ (accessed June 17, 2022).
1270
1271 [18] T.J. Pugh, S.S. Amr, M.J. Bowser, S. Gowrisankar, E. Hynes, L.M. Mahanta, H.L. Rehm,
1272 B. Funke, M.S. Lebo, VisCap: inference and visualization of germ-line copy-number
1273 variants from targeted clinical sequencing data, Genet Med. 18 (2016) 712–719.
1274 https://doi.org/10.1038/gim.2015.156.
1275
1276 [19] R. Shen, V.E. Seshan, FACETS: allele-specific copy number and clonal heterogeneity
1277 analysis tool for high-throughput DNA sequencing, Nucleic Acids Res. 44 (2016) e131.
1278 https://doi.org/10.1093/nar/gkw520.
1279
1280 [20] S. Richards, N. Aziz, S. Bale, D. Bick, S. Das, J. Gastier-Foster, W.W. Grody, M. Hegde,
1281 E. Lyon, E. Spector, K. Voelkerding, H.L. Rehm, ACMG Laboratory Quality Assurance
1282 Committee, Standards and guidelines for the interpretation of sequence variants: a joint
1283 consensus recommendation of the American College of Medical Genetics and Genomics
1284 and the Association for Molecular Pathology, Genet Med. 17 (2015) 405–424.
1285 https://doi.org/10.1038/gim.2015.30.
1286
1287 [21] D. Chakravarty, J. Gao, S. Phillips, R. Kundra, H. Zhang, J. Wang, J.E. Rudolph, R.
1288 Yaeger, T. Soumerai, M.H. Nissan, M.T. Chang, S. Chandarlapaty, T.A. Traina, P.K.
1289 Paik, A.L. Ho, F.M. Hantash, A. Grupe, S.S. Baxi, M.K. Callahan, A. Snyder, P. Chi,
1290 D.C. Danila, M. Gounder, J.J. Harding, M.D. Hellmann, G. Iyer, Y.Y. Janjigian, T. Kaley,
1291 D.A. Levine, M. Lowery, A. Omuro, M.A. Postow, D. Rathkopf, A.N. Shoushtari, N.
1292 Shukla, M.H. Voss, E. Paraiso, A. Zehir, M.F. Berger, B.S. Taylor, L.B. Saltz, G.J. Riely,
1293 M. Ladanyi, D.M. Hyman, J. Baselga, P. Sabbatini, D.B. Solit, N. Schultz, OncoKB: A
1294 Precision Oncology Knowledge Base, JCO Precision Oncology. (2017).
1295 https://doi.org/10.1200/PO.17.00011.

1296

1297 [22] F. Blokzijl, R. Janssen, R. van Boxtel, E. Cuppen, MutationalPatterns: comprehensive
1298 genome-wide analysis of mutational processes, Genome Medicine. 10 (2018) 33.
1299 https://doi.org/10.1186/s13073-018-0539-0.

1300

1301 [23] P. Jia, X. Yang, L. Guo, B. Liu, J. Lin, H. Liang, J. Sun, C. Zhang, K. Ye, MSIsensor-pro:
1302 Fast, Accurate, and Matched-normal-sample-free Detection of Microsatellite Instability,
1303 Genomics, Proteomics & Bioinformatics. 18 (2020) 65–71.
1304 https://doi.org/10.1016/j.gpb.2020.02.001.

1305

1306 [24] D.A. Levine, Integrated genomic characterization of endometrial carcinoma, Nature. 497
1307 (2013) 67–73. https://doi.org/10.1038/nature12113.

1308

1309 [25] A. Martínez-Roca, M. Giner-Calabuig, O. Murcia, A. Castillejo, J.L. Soto, A. García-
1310 Heredia, R. Jover, Lynch-like Syndrome: Potential Mechanisms and Management,
1311 Cancers. 14 (2022) 1115. https://doi.org/10.3390/cancers14051115.

1312

1313 [26] M. Morak, B. Heidenreich, G. Keller, H. Hampel, A. Laner, A. de la Chapelle, E.
1314 Holinski-Feder, Biallelic MUTYH mutations can mimic Lynch syndrome, Eur J Hum
1315 Genet. 22 (2014) 1334–1337. https://doi.org/10.1038/ejhg.2014.15.

1316

1317 [27] Prevalence of germline MUTYH mutations among Lynch-like syndrome patients |
1318 Elsevier Enhanced Reader, (n.d.). https://doi.org/10.1016/j.ejca.2014.05.022.

1319

1320 [28] J. Suchy, C. Cybulski, D. Wokołorczyk, O. Oszurek, B. Górski, T. Dębniak, A.
1321 Jakubowska, J. Gronwald, T. Huzarski, T. Byrski, I. Dziuba, M. Gogacz, R. Wiśniowski,
1322 P. Wandzel, Z. Banaszkiewicz, G. Kurzawski, J. Kładny, S.A. Narod, J. Lubiński, CHEK2
1323 mutations and HNPCC-related colorectal cancer, International Journal of Cancer. 126
1324 (2010) 3005–3009. https://doi.org/10.1002/ijc.25003.

1325

1326 [29] M. Dominguez-Valentin, S. Nakken, H. Tubeuf, D. Vodak, P.O. Ekstrøm, A.M. Nissen,
1327 M. Morak, E. Holinski-Feder, A. Martins, P. Møller, E. Hovig, Identification of genetic

1328 variants for clinical management of familial colorectal tumors, BMC Med Genet. 19
1329 (2018) 26. https://doi.org/10.1186/s12881-018-0533-9.
1330
1331 [30] N. Seguí, L.B. Mina, C. Lázaro, R. Sanz-Pamplona, T. Pons, M. Navarro, F. Bellido, A.
1332 López-Doriga, R. Valdés-Mas, M. Pineda, E. Guinó, A. Vidal, J.L. Soto, T. Caldés, M.
1333 Durán, M. Urioste, D. Rueda, J. Brunet, M. Balbín, P. Blay, S. Iglesias, P. Garré, E. Lastra,
1334 A.B. Sánchez-Heras, A. Valencia, V. Moreno, M.Á. Pujana, A. Villanueva, I. Blanco, G.
1335 Capellá, J. Surrallés, X.S. Puente, L. Valle, Germline Mutations in FAN1 Cause
1336 Hereditary Colorectal Cancer by Impairing DNA Repair, Gastroenterology. 149 (2015)
1337 563–566. https://doi.org/10.1053/j.gastro.2015.05.056.
1338
1339 [31] F.A. de O. Garcia, E.S. de Andrade, H. de Campos Reis Galvão, C. da Silva Sábato, N.
1340 Campacci, A.E. de Paula, A.F. Evangelista, I.V.V. Santana, M.E. Melendez, R.M. Reis,
1341 E.I. Palmero, New insights on familial colorectal cancer type X syndrome, Sci Rep. 12
1342 (2022) 2846. https://doi.org/10.1038/s41598-022-06782-8.
1343
1344 [32] S. Sushmitha, R. Murugesan, S. Saraswathi, K. Rathiusha, G. Madhumala, S. Jose, B.
1345 Laura, B. Antara, M. Francesco, P. Surajit, A review on role of ATM gene in hereditary
1346 transfer of colorectal cancer, Acta Biomed. 89 (2018) 463–469.
1347 https://doi.org/10.23750/abm.v89i4.6095.
1348
1349 [33] M.B. Yurgelun, B. Allen, R.R. Kaldate, K.R. Bowles, T. Judkins, P. Kaushik, B.B. Roa,
1350 R.J. Wenstrup, A.-R. Hartman, S. Syngal, Identification of a Variety of Mutations in
1351 Cancer Predisposition Genes in Patients With Suspected Lynch Syndrome,
1352 Gastroenterology. 149 (2015) 604-613.e20. https://doi.org/10.1053/j.gastro.2015.05.006.
1353
1354 [34] K.L. Ring, A.S. Bruegl, B.A. Allen, E.P. Elkin, N. Singh, A.-R. Hartman, M.S. Daniels,
1355 R.R. Broaddus, Germline multi-gene hereditary cancer panel testing in an unselected
1356 endometrial cancer cohort, Mod Pathol. 29 (2016) 1381–1389.
1357 https://doi.org/10.1038/modpathol.2016.135.
1358

1359 [35] J. Chernoff, The two-hit theory hits 50, Mol Biol Cell. 32 (2021) rt1.
1360 https://doi.org/10.1091/mbc.E21-08-0407.
1361
1362 [36] K. Huang, R.J. Mashl, Y. Wu, D.I. Ritter, J. Wang, C. Oh, M. Paczkowska, S. Reynolds,
1363 M.A. Wyczalkowski, N. Oak, A.D. Scott, M. Krassowski, A.D. Cherniack, K.E.
1364 Houlahan, R. Jayasinghe, L.-B. Wang, D.C. Zhou, D. Liu, S. Cao, Y.W. Kim, A. Koire,
1365 J.F. McMichael, V. Hucthagowder, T.-B. Kim, A. Hahn, C. Wang, M.D. McLellan, F. Al-
1366 Mulla, K.J. Johnson, O. Lichtarge, P.C. Boutros, B. Raphael, A.J. Lazar, W. Zhang, M.C.
1367 Wendl, R. Govindan, S. Jain, D. Wheeler, S. Kulkarni, J.F. Dipersio, J. Reimand, F.
1368 Meric-Bernstam, K. Chen, I. Shmulevich, S.E. Plon, F. Chen, L. Ding, Pathogenic
1369 germline variants in 10,389 adult cancers, Cell. 173 (2018) 355-370.e14.
1370 https://doi.org/10.1016/j.cell.2018.03.039.
1371
1372 [37] J.V. Martín-López, Y. Barrios, V. Medina-Arana, M. Andújar, S. Lee, L. Gu, G.-M. Li, J.
1373 Rüschoff, E. Salido, R. Fishel, The hMSH2(M688R) Lynch syndrome mutation may
1374 function as a dominant negative, Carcinogenesis. 33 (2012) 1647–1654.
1375 https://doi.org/10.1093/carcin/bgs199.
1376
1377 [38] G.X. Reyes, B. Zhao, T.T. Schmidt, K. Gries, M. Kloor, H. Hombauer, Identification of
1378 MLH2/hPMS1 dominant mutations that prevent DNA mismatch repair function, Commun
1379 Biol. 3 (2020) 751. https://doi.org/10.1038/s42003-020-01481-4.
1380
1381 [39] A.M. Jansen, T. van Wezel, B.E. van den Akker, M. Ventayol Garcia, D. Ruano, C.M.
1382 Tops, A. Wagner, T.G. Letteboer, E.B. Gómez-García, P. Devilee, J.T. Wijnen, F.J. Hes,
1383 H. Morreau, Combined mismatch repair and POLE/POLD1 defects explain unresolved
1384 suspected Lynch syndrome cancers, Eur J Hum Genet. 24 (2016) 1089–1092.
1385 https://doi.org/10.1038/ejhg.2015.252.
1386
1387 [40] A. León-Castillo, H. Britton, M.K. McConechy, J.N. McAlpine, R. Nout, S. Kommoss,
1388 S.Y. Brucker, J.W. Carlson, E. Epstein, T.T. Rau, T. Bosse, D.N. Church, C.B. Gilks,
1389 Interpretation of somatic POLE mutations in endometrial carcinoma, The Journal of
1390 Pathology. 250 (2020) 323–335. https://doi.org/10.1002/path.5372.

CHAPTER III: Uncovering the role of WDHD1 in the DNA mismatch repair pathway

We are performing further experiments to be included to the final version before the submission of this manuscript for publication.

1391 **Uncovering the role of WDHD1 in the DNA Mismatch repair pathway**

1392

1393 Reginaldo Cruz Alves Rosa[a,b], Andrey Yurchenko[c], Caroline Pouvelle[b], Sergey Nikolaev[c],
1394 Victor Evangelista de Faria Ferraz[a,d], Patricia Kannouche[b*],

1395

1396 [a] Department of Genetics, Ribeirão Preto Medical School, University of São Paulo, Ribeirão
1397 Preto, Brazil
1398 [b] Université Paris-Saclay, CNRS UMR9019, Gustave Roussy Cancer Campus, Villejuif, France
1399 [c] Université Paris-Saclay, Inserm U981, Gustave Roussy Cancer Campus, Villejuif, France
1400 [d] Center for Medical Genomics at Clinical Hospital of the Ribeirão Preto Medical School,
1401 University of São Paulo, Brazil

1402

1403

1404 * Corresponding author:

1405

1406 Name: Dr. Patricia Kannouche. Address: CNRS UMR9019, Institut Gustave Roussy. 114, Rue
1407 Edouard Vaillant - 94805 Villejuif Cedex, France.
1408 E-mail address: patricia.kannouche@gustaveroussy.fr.
1409 Telephone number: +33142114030 – Fax number: +33142115008

Abstract

Background: Mismatch repair (MMR) deficiency is observed in a substantial fraction of tumors, especially endometrial and colorectal cancer. Germline and somatic inactivation of *MLH1*, *MSH2*, *MSH6*, and *PMS2*, genes that encode for the main components of the MMR pathway, is the leading cause of MMR deficiency. However, a portion of MMR-deficient tumors does not harbor any mutation in MMR genes, suggesting that alteration in other genes could also drive the MMR-deficient phenotype in cancer. It has been reported that the WDHD1 protein, an essential component of eukaryote replisome, interacts with MSH2. However, the relevance of WDHD1-MSH2 interaction on the MMR pathway remains to be elucidated.

Objective: To investigate the contribution of WDHD1-MSH2 interaction to the repair of replication errors and the cellular response to 6-TG mediated by the MMR system.

Methodology: Mutation and methylation data were downloaded from The Cancer Genome Atlas Pan-Cancer Project, and the fraction of tumors with microsatellite instability (MSI) and their corresponding etiologies were determined. Unexplained MSI tumors were interrogated for the occurrence of *WDHD1* mutations. The interaction between WDHD1 and MSH2 was validated by immunoprecipitation and site-directed mutagenesis in HEK293T cells. CT26 cells lacking the domain required for the interaction between MSH2 and WDHD1 proteins were generated by CRISPR/Cas9 targeting the last exon of the *Wdhd1* gene. *Msh2*-KO and *Msh6*-KO cells were also generated. Spontaneous mutations and MSI were investigated by whole-exome sequencing of cells cultured for several passages. Wild-type, *Wdhd1*-mutant, and MMR-deficient cell lines were treated with increasing doses of 6-TG, and their surviving percentages were measured by clonogenic assay.

Results: A total of 369/10,192 (3.6%) tumors had MSI, and 11.4% of them did not have alterations in MMR genes. *WDHD1* mutations were identified in three Unexplained MSI tumors. We validated the interaction between WDHD1 and MSH2 and confirmed the C-terminal domain of WDHD1 as the critical region for interaction with MSH2. *Wdhd1*-mutant cells did not accumulate spontaneous mutations and did not have an MSI phenotype after 25 weeks of passage. On the other hand, *Wdhd1*-mutant cells showed mild resistance to 6-TG in comparison to wild-type cells.

1443 **Conclusion:** Altogether, our results indicate that the interaction between WDHD1 and MSH2
1444 is not required for the repair of spontaneous mutations by the MMR pathway, and the disruption
1445 of this interaction does not lead to the MSI phenotype in vitro. However, despite not being
1446 essential, the WDHD1-MSH2 interaction might participate in the cellular response to 6-TG.

1447

1448 **Keywords:** Chemoresistance, CRISPR/Cas9, DNA repair, Whole-exome sequencing

1450 **Introduction**

1451

1452 Mismatch repair (MMR) is a pathway conserved from bacteria to higher eukaryotes and
1453 is critical to maintaining genome integrity by repairing base-base and insertion-deletion loops
1454 generated during DNA replication [1]. MMR is commonly defective in cancer, with frequencies
1455 ranging from 8 to 30% of cases, depending on the tumor type [2]. MMR-deficient (MMR-D)
1456 tumors usually present with a high number of spontaneous mutations, mainly in microsatellite
1457 regions, and are resistant to different chemotherapeutic agents, such as 6-thioguanine (6-TG)
1458 [3,4].

1459 Germline or somatic inactivation of *MLH1*, *MSH2*, *MSH6*, or *PMS2*, the main MMR
1460 genes, are well-described drivers of MMR deficiency. More than 50% of endometrial and
1461 colorectal tumors with MMR deficiency are caused by epigenetic silencing of the *MLH1* gene
1462 or by somatic biallelic mutations in MMR genes [5–8]. In addition, heterozygous germline
1463 mutations in *MLH1*, *MSH2*, *MSH6*, *PMS2*, and *EPCAM* genes, which lead to Lynch syndrome,
1464 are found in around 30% of patients with MMR-D tumors [9,10]. However, about 11% of
1465 MMR-D tumors do not have any somatic or germline alteration in MMR genes; therefore, their
1466 etiology is unclear [10].

1467 Defining the genetic etiology of unexplained MMR-D tumors is critical for the clinical
1468 management and surveillance of those patients [11]. Additionally, identifying alternative routes
1469 driving the MMR-D phenotype in cancer could be therapeutically explored once individuals
1470 with MMR-D tumors are eligible for some promising anti-cancer therapies, such as immune
1471 checkpoint inhibitors [12,13]. Defects in non-MMR genes, including *SETD2* [14], *ARID1* [15],
1472 *MUTYH* [16], *POLD1,* and *POLE* [17], have been found in unexplained MMR-D tumors,
1473 suggesting that alterations in genes not related to the MMR pathway could be an alternative
1474 etiology for the MMR-D phenotype. In addition to those genes, *WDHD1*, which encodes an
1475 essential component of the eukaryotic replisome [18], was identified as potentially being
1476 involved with the MMR pathway by interacting with MSH2 [19]. However, the relevance of
1477 this interaction on the MMR functions remains to be elucidated.

1478 In this study, we characterized the role of WDHD1 in the MMR pathway by
1479 investigating the prevalence of *WDHD1* mutations in tumors with unexplained MMR-D
1480 phenotype in a large pan-cancer cohort. Additionally, we constructed a cellular model lacking
1481 interaction between WDHD1 and MSH2 proteins and accessed the MMR deficiency by means
1482 of spontaneous mutation rate, microsatellite instability, and resistance to 6-TG.

1483

1484 **Materials and Methods**

1485 *TCGA data analysis*

1486

1487 Open-access clinical, genomic, and epigenomic data from the Cancer Genome Atlas
1488 Pan-Cancer Analysis Project (TCGA Pan-Cancer) [20] were downloaded using the Genomic
1489 Data Commons platform [21]. Microsatellite instability was assessed by the MSIsensor tool
1490 [22]. Tumors with ≥ 4 MSIsensor scores were classified as having microsatellite instability
1491 [23]. *MLH1*-methylation was measured from the HumanMethylation27 (HM27) or
1492 HumanMethylation450 (HM450) Illumina Infinitum DNA methylation array data (using the
1493 cg00893636 probe). Tumors with a beta value ≥ 0.1 were considered to have *MLH1*-
1494 hypermethylation [24]. Publicly available whole-exome sequencing data were used to identify
1495 the germline [25] and somatic [26] inactivating variants in the MMR genes. MSI tumors
1496 without inactivating variants in MMR genes and without hypermethylation in the promoter
1497 region of *MLH1* were classified as Unexplained MSI tumors.

1498

1499 *Cell lines and culture conditions*

1500

1501 CT26 cells (murine colorectal cancer cell line) were cultured in RPMI-640 medium
1502 supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin, 100μg/ml
1503 streptomycin (Gibco). HEK293T cells (human embryonic kidney cell line) were cultured in
1504 Dulbecco's Modified Eagle medium (D-MEM) containing sodium pyruvate,
1505 penicillin/streptomycin, and 10% FBS. CT26 and HEK293T cells were cultured at 37°C in an
1506 atmosphere containing 5% CO_2 and were periodically tested for mycoplasma contamination
1507 using the MycoAlert Mycoplasma Detection Kit (LT07-418, Lonza). CT26 cells knocked out
1508 for the *Msh2* gene (*Msh2*-KO) were generated as described elsewhere [27].

1509

1510 *Plasmids*

1511

1512 The human *WDHD1* full-length cDNA (CCDS 9721.1) was obtained by PCR
1513 amplification using the primers 5'-
1514 TAAGCACTCGAGCCTGCCACACGGAAGCCAATGAGATAT-3' and 5'-
1515 TGCTTAGGATCCTTACTCCTGCTTAAATGCAAAAGCTGA-3'. The PCR product was
1516 digested with XhoI and BamHI restriction enzymes and cloned into the XhoI-BamHI digested

1517 pEGFP-C3 plasmid (Clontech) vector. The GFP-MSH2 plasmid was kindly provided by Dr.
1518 Akira Yasui [28].
1519 Plasmids expressing Cas9, GFP, and guide RNAs were constructed by annealing,
1520 phosphorylation, and cloning of the primers 5'-CACCGAGCAAAGAAGCGAAAGCGTG-3'
1521 and 5'-AAACCACGCTTTCGCTTCTTTGCTC-3' (targeting the exon 25 of the murine *Wdhd1*
1522 gene - ENSMUST00000187531.8), and 5'-CACCGGGCACTCGGCGACACCAAGA-3' and
1523 5'- AAACTCTTGGTGTCGCCGAGTGCCC-3' (targeting the exon 1 of the mouse *Msh6* gene
1524 - ENSMUST00000005503.5), into the BbsI-digested px458 plasmid (Addgene #48138).
1525
1526 *Site-directed mutagenesis*
1527
1528 The c.G3274T (p.1092*) nonsense mutation was introduced in the C-terminus of the
1529 WDHD1 sequence present in the GFP-WDHD1 plasmid by PCR using the primers 5'-
1530 TTTCTTCCTGGTTTTCTGTTTAATCACTTTCATCAACCACACG-3' and 5'-
1531 CGTGTQGTTGATGAAAGTGATTAAACAGAAAACCAGGAAGAAA-3' and the
1532 QuikChange Lightning site-directed mutagenesis kit (Agilent) according to the manufacturer's
1533 instructions.
1534
1535 *Immunoprecipitation*
1536
1537 For immunoprecipitation of GFP-tagged proteins (GFP-trap), HEK293T cells were
1538 transfected with GFP-expressing plasmids were lysed in NET-N buffer (50 mM Tris–HCl pH
1539 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, protease inhibitor cocktail, and anti-
1540 phosphatases) for 30 min on ice and sonicated twice at 30% amplitude for 10 seconds. Samples
1541 were cleared by centrifugation at 12,000 g for 5 minutes at 4°C, and the supernatant was
1542 incubated with agarose beads conjugated with GFP nanobody (Chromotek) for 2 hours at room
1543 temperature on a wheel. Beads were washed twice in NET-N buffer, followed by a final wash
1544 in 500 mM NaCl NET-N for 10 minutes at 4°C, and were denaturated in 2x Laemmli buffer at
1545 90°C for 10 minutes.
1546
1547 *Western blotting*
1548
1549 Proteins were separated on 8% acrylamide SDS–polyacrylamide gels and transferred to
1550 PVDF membranes (Millipore). Membranes were blotted with antibodies directed to the

1551 following proteins: WDHD1 (rabbit, #HPA001122, Sigma, 1/2000); MSH2 (rabbit, #ab70270,
1552 Abcam, 1/5000); MSH6 (goat, #A300-022A, Bethyl, 1/2000); GFP (rabbit, #ab290, Abcam,
1553 1/2000), and β-actin (mouse, #A5441, Sigma Aldrich, 1/10,000).
1554
1555 *CRISPR/Cas9*
1556
1557 Five μg of the px458 plasmid were transfected into wild-type CT26 cells using the
1558 JetPEI kit (Polyplus), according to the manufacturer's instructions. After 48 hours, GFP-
1559 positive cells were single-cell sorted by fluorescence-activated cell sorting (FACS) into
1560 individual wells in 96-well plates. Single-cell clones were further expanded in culture.
1561 For validation of CRISPR-mediated gene editing, clones were lysed in lysis buffer (50
1562 mM Tris pH 7.5, 20 mM NaCl, 10 mM MgCl2, 0.1% SDS, and anti-proteases cOmplete
1563 cocktail from Roche) supplemented with 20 U/mL benzonase (Millipore) for 10 minutes at
1564 room temperature and protein expression was evaluated by western blotting. Additionally,
1565 CRISPR-introduced mutations were characterized by Sanger sequencing.
1566
1567 *Clonogenic assay*
1568
1569 The sensitivity of wild-type, *Wdhd1*-mutant, *Msh6*-KO, *Msh6/Wdhd1*-mutant, and
1570 *Msh2*-KO cells to 6-thioguanine (6-TG) was determined by seeding 1000 cells in 100 mm
1571 dishes. Three dishes were plated for each treatment, including untreated cells, DMSO, and
1572 increasing doses of 6-TG (1μM and 3μM). 16-24 hours post-seeding, media was removed and
1573 replaced by 10 mL of RMPI media containing the different treatments, which was removed
1574 after 24 hours and replaced with fresh media. After 8-10 days of culture, dishes were gently
1575 washed with PBS, and colonies were stained with violet crystal solution. Colonies with ≥ 50
1576 cells were counted, and the surviving fractions were calculated using the plating efficiency
1577 method, as described elsewhere [29].
1578
1579 *Cell cycle analysis*
1580
1581 To evaluate the impact of 6-TG on the cell cycle of CT26 cells with different genotypes,
1582 500,000 cells were seeded in 100 mm dishes and were allowed to grow for 24 hours. Following
1583 the seeding step, media was replaced with media containing 1μM of 6-TG. In addition, cells

1584 were also treated with DMSO. 48 hours post-treatment, cells were pelleted by centrifugation at
1585 300 g for 5 minutes at 4°C. Cell pellets were resuspended in 500 μL of PBS, fixed in 80% cold
1586 ethanol, and immediately stored at -20 °C for at least 24 hours. Ethanol was removed by
1587 centrifugation of fixed cells at 300 g for 5 minutes at 4°C, and cell pellets were resuspended in
1588 500 μL of 25 μg/mL propidium iodide (PI) solution, supplemented with 50 μg/mL RNase and
1589 were incubated for 30 minutes at room temperature. PI-stained cells were submitted to cell
1590 cycle analysis by flow cytometry (CytoFlex, Beckman Coulter). Flow cytometry data were
1591 analyzed using FlowJo v.10 (BD Biosciences).
1592
1593 *Whole-exome sequencing*
1594
1595 *Wdhd1*-mutant, *Msh2*-KO, and wild-type CT26 cells were serially passaged twice a
1596 week. Single-cell subclones were obtained from each genotype at 6 and 25 weeks of passage
1597 to monitor mutation burden and microsatellite instability accumulated over time. Genomic
1598 DNA was extracted using the NucleoSpin Tissue kit (Macherey-Nagel). Whole-exome
1599 sequencing (WES) was performed according to the manufacturer protocols (BGI Tech
1600 solutions, Hong Kong) with a BGISEQ-500 sequencer in a paired-end mode.
1601 WES data were analyzed as described elsewhere [27]. Briefly, adaptor-free reads were
1602 mapped to the mm9 mouse reference genome using BWA-MEM. Removal of duplicate reads,
1603 base quality recalibration, and calling of somatic variants were performed according to the
1604 GATK best practice pipeline [30]. Somatic single-nucleotide variants and indels were called
1605 using Mutect2 in the normal-tumor mode, using parental wild-type CT26 cells as the normal
1606 reference. Somatic variants were filtered using Mutect2, FilterMutectCalls, and
1607 FilterByOrientationBias from the GATK v. 4.2.1.0 toolkit, and their functional effect on the
1608 gene function and further annotations were performed using ANNOVAR [31]. Somatic
1609 variants with a Mutect2 "PASS" flag supported by at least one read from each DNA strand, and
1610 at least three reads in total were considered for further analyses. Microsatellite instability
1611 analysis was performed using MSIsensor-pro [22] in the normal-tumor mode.
1612
1613 *Statistical analyses*
1614
1615 Tests used for each analysis are indicated in the legends. *P*-values of <0.05 were
1616 considered significant unless stated differently in the legends. Statistical analyses were
1617 performed using R (version 4.2.1).

1618 **Results**

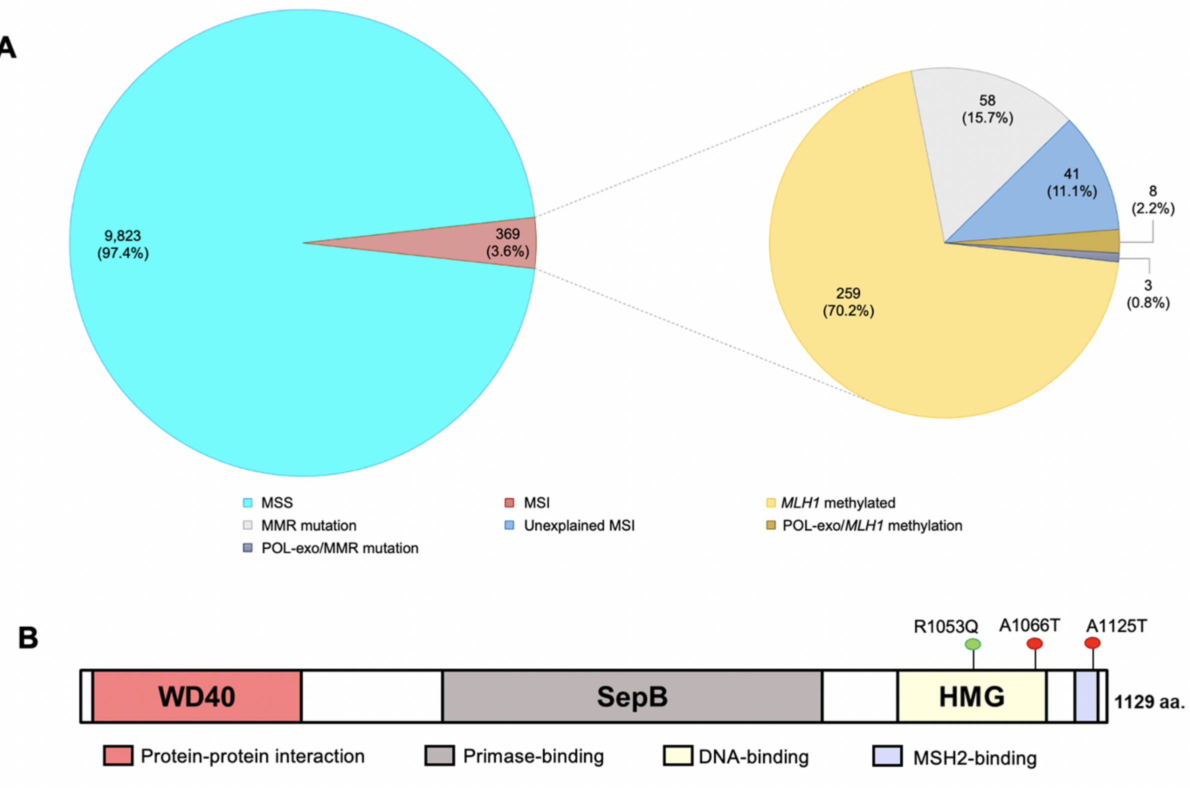
1619

1620 *Unexplained MSI tumors are found in different tumors types and have mutations in the C-*
1621 *terminus of the WDHD1 gene*

1622

1623 A total of 10,192 tumors from the TCGA project had sufficient molecular data available
1624 to identify MMR-deficient tumors and their respective etiologies (**Figure 1A**). 369/10,192
1625 (3.6%) tumors were found to have MSI and were classified as MMR-deficient tumors. Most
1626 MMR-deficient tumors had *MLH1* promoter methylation (259/369, 70.2%). Additionally,
1627 15.7% (58/369) of MSI tumors were found to have somatic or germline-inactivating mutations
1628 in MMR genes. A small fraction of MSI tumors had mutations in the exonuclease domain of
1629 the *POLE* gene (POLE-exo) in association with mutations in MMR genes (8/369, 2.2%) or co-
1630 occurring with *MLH1* methylation (3/369, 0.8%). Notably, 41/369 (11.1%) MSI tumors did not
1631 have any inactivating alteration in MMR genes and were classified as "Unexplained MSI"
1632 tumors.

1633



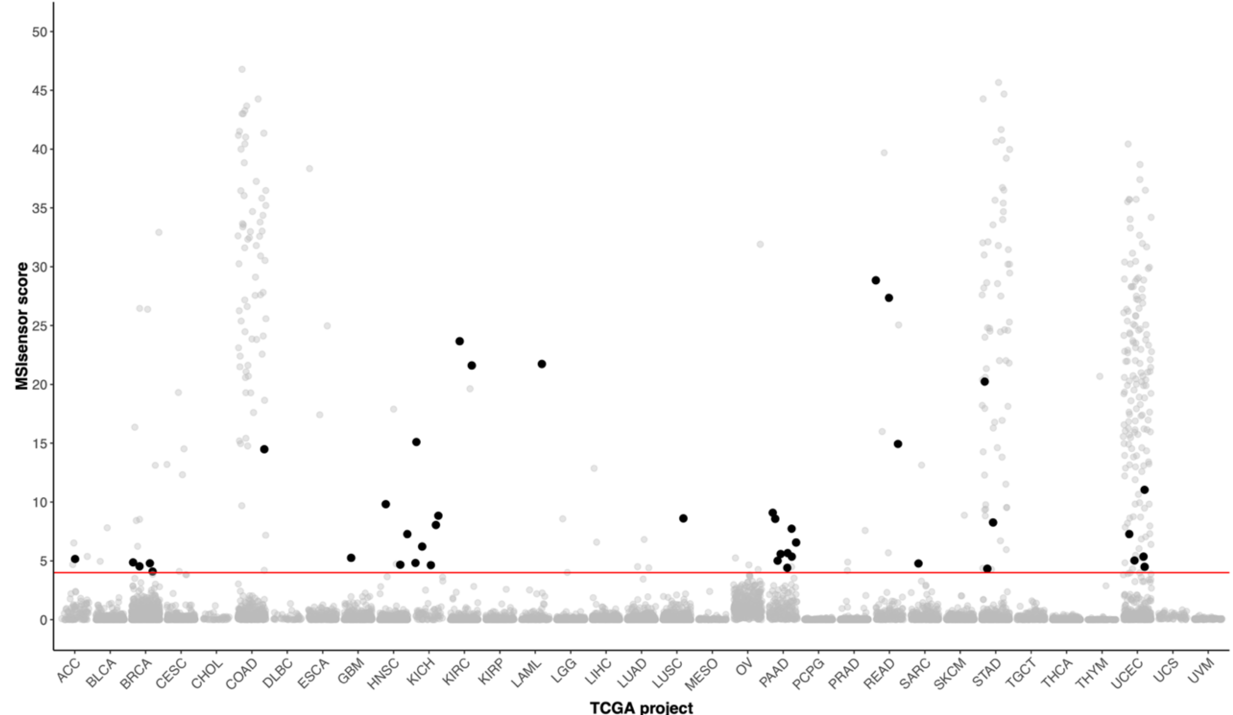
1634

1635

1636 **Figure 1 – Identification of *WDHD1* mutations in Unexplained MSI tumors from the TCGA dataset.** (A)
1637 Frequency of MSI tumors and their respective molecular etiologies. Tumors with MSIsensor scores ≥ 4 were
1638 considered as having MSI/MMR deficiency. Refer to the Materials and Methods for details about the parameters

used to assess the etiology of MSI tumors. (B) Scheme of the human WDHD1 protein with somatic mutations identified in Unexplained MSI tumors. Red circles refer to *WDHD1* mutations identified in uterine carcinomas (UCEC), while the green circle informs the detection of a *WDHD1* mutation in stomach cancer (STAD). MSS: microsatellite stable. MMR: Mismatch repair. MSI: microsatellite unstable. POL-exo: mutations affecting the exonuclease domain of the DNA polymerase epsilon.

Unexplained MSI tumors were found in 14/33 tumor types present in the TCGA dataset (**Supplementary Figure S1**). Around 20% (9/41) of Unexplained MSI tumors were observed in pancreatic adenocarcinomas (PAAD). Additionally, 6/41(14.6%) unexplained cases were found in kidney chromophobe tumors (KICH), 5/41 (12.2%) in uterine carcinomas (UCEC), and 4/41 (9.7%) in invasive breast carcinomas (BRCA). The remaining Unexplained MSI cases were distributed among cancer types frequently associated with MSI, such as colon (COAD), rectal (READ), and stomach (STAD) carcinomas, as well as in tumor types not classically related to the MSI phenotype, including adrenocortical carcinomas (ACC), glioblastoma multiforme (GBM), head and neck squamous cell carcinoma (HNSC), acute myeloid leukemia (LAML), and sarcomas (SARC). We identified somatic mutations affecting the C-terminus of the *WDHD1* gene in three Unexplained MSI cases, including two uterine (UCEC) and one stomach (STAD) carcinomas (**Figure 1B**).



Supplementary Figure S1 – Microsatellite unstable tumors among 33 tumor types from the TCGA dataset. The horizontal red line indicates the MSIsensor threshold to classify a tumor as having MSI using the MSIsensor tool. Dots above the red line are MSI tumors. Black dots indicate Unexplained MSI cases. ACC: Adrenocortical

1664 carcinoma; BLCA: Bladder Urothelial Carcinoma; BRCA: Breast invasive carcinoma; CESC: Cervical squamous
1665 cell carcinoma and endocervical adenocarcinoma; CHOL: Cholangiocarcinoma; COAD: Colorectal
1666 adenocarcinoma; DLBC: Diffuse Large B-cell Lymphoma; ESCA: Esophageal carcinoma; GBM: Glioblastoma
1667 Multiforme; HNSC: Head and Neck squamous cell carcinoma; KICH: Kidney Chromophobe; KIRC: Kidney renal
1668 clear cell carcinoma; KIRP: Kidney renal papillary cell carcinoma; LAML: Acute Myeloid Leukemia; LGG: Brain
1669 Lower Grade Glioma; LIHC: Liver Hepatocellular Carcinoma; LUAD: Lung Adenocarcinoma; LUSC: Lung
1670 Squamous Cell Carcinoma; MESO: Mesothelioma; OV: Ovarian serous cystadenocarcinoma; PAAD: Pancreatic
1671 adenocarcinoma; PCPG: Pheochromocytoma and Paraganglioma; PRAD: Prostate Adenocarcinoma; READ:
1672 Rectum Adenocarcinoma; SARC: Sarcoma; SKCM: Skin Cutaneous Melanoma; STAD: Stomach
1673 adenocarcinoma; TGCT: Testicular Germ Cell Tumors; THCA: Thyroid carcinoma; THYM: Thymoma; UCEC:
1674 Uterine Corpus Endometrial Carcinoma; UCS: Uterine Carcinosarcoma; UVM: Uveal Melanoma.
1675

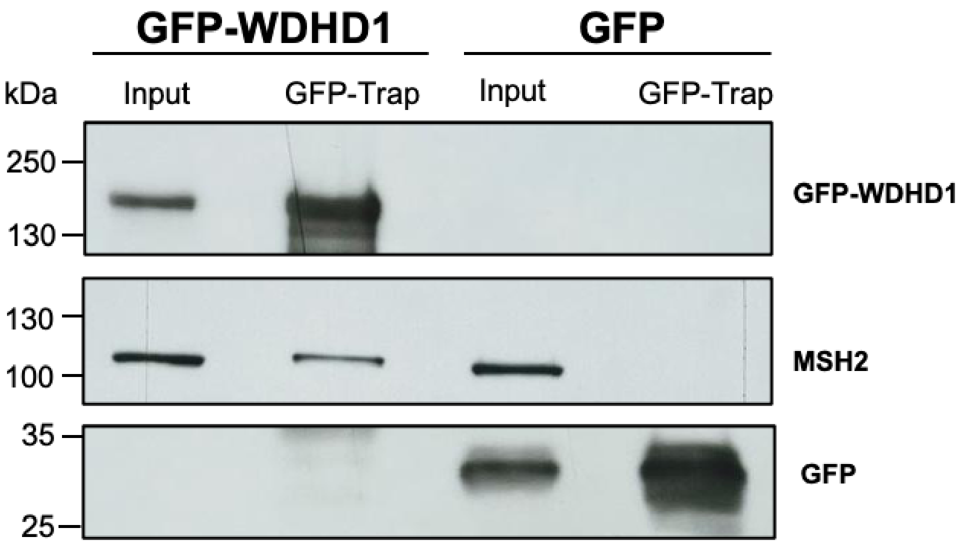
1676

1677 *WDHD1 interacts with MSH2 protein at the C-terminal of WDHD1 protein*

1678

1679 Chen *et al*. have previously characterized the interactome of the MMR pathway by using
1680 tandem-affinity purification with mass spectrometry and identified the interaction between
1681 WDHD1 and MSH2 proteins [19]. We used immunoprecipitation of GFP-tagged WDHD1
1682 protein to validate this interaction and found that MSH2 co-immunoprecipitated with GFP-
1683 WDHD1 (**Figure 2**). Similarly, the WDHD1 protein also co-immunoprecipitated with GFP-
1684 MSH2 (**Supplementary Figure S2**).

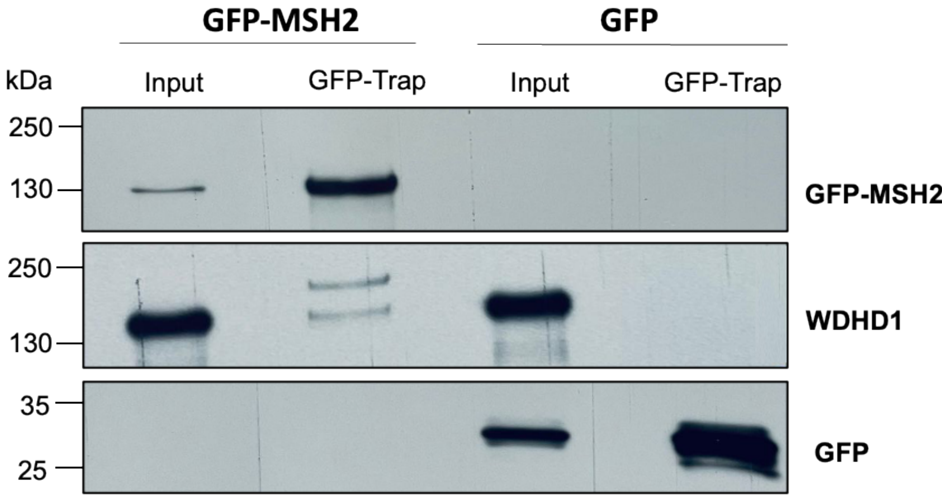
1685



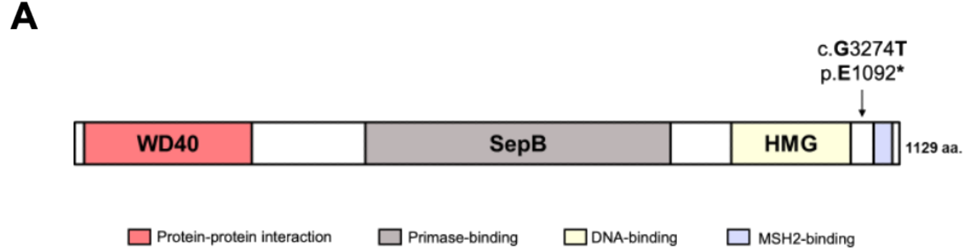
1686

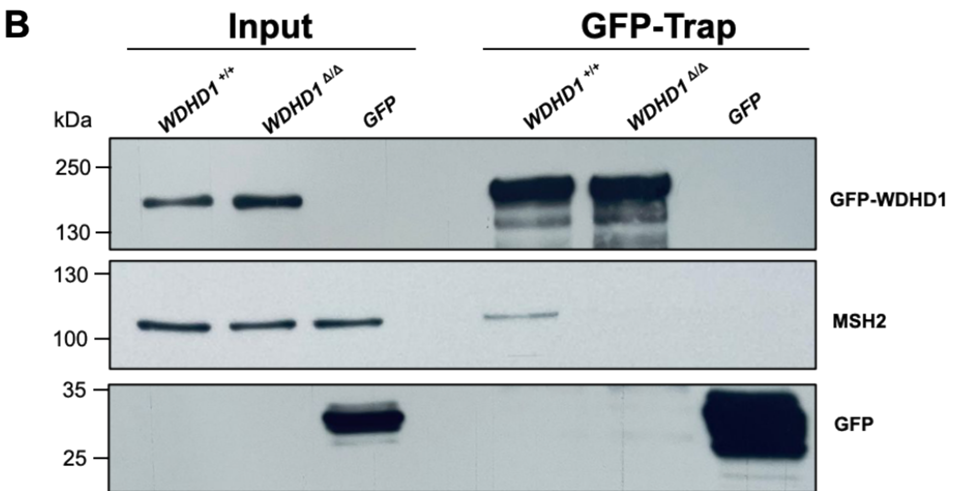
1687

1688 **Figure 2 – Validation of WDHD1-MSH2 interaction.** HEK293T cells were transfected with either the GFP-
1689 WDHD1 or GFP plasmids. MSH2 protein was detected in input and immunoprecipitation (identified as GFP-Trap
1690 on the top of the figure) fractions of cells transfected with GFP-WDHD1 and in the input but not in the
1691 immunoprecipitation fraction of cells transfected with GFP plasmid, validating the interaction between WDHD1
1692 and MSH2 proteins.
1693



1694
1695
1696 **Supplementary Figure S2 – Validation of WDHD1-MSH2 interaction by immunoprecipitation of the GFP-**
1697 **MSH2 protein.** HEK293T cells were transfected with either the GFP-MSH2 or GFP plasmids. WDHD1 protein
1698 was detected in input and immunoprecipitation (identified as GFP-Trap on the top of the figure) fractions of cells
1699 transfected with GFP-MSH2 and in the input but not in the immunoprecipitation fraction of cells transfected with
1700 GFP plasmid, validating the interaction between WDHD1 and MSH2 proteins. The presence of two bands
1701 identified by the anti-WDHD1 antibody in the GFP-Trap fraction of cells transfected with the GFP-MSH2 plasmid
1702 might indicate the presence of post-translational modifications in the WDHD1 protein.
1703
1704
1705 A series of truncating mutations has been previously used to identify the minimal
1706 MSH2-binding region in the C terminus of WDHD1 (residues 1122-1226) [19]. We introduced
1707 a truncating mutation (c.G3274T, p.E1092*) in the WDHD1 sequence (**Figure 3A**) and
1708 confirmed the C-terminus of WDHD1 protein as its MSH2-binding domain once the deletion
1709 of this region abolished interaction with MSH2 (**Figure 3B**).
1710





1711

1712

1713 **Figure 3 – Validation of the minimal MSH2-binding site in WDHD1 protein**. (A) Schematic representation of
1714 the human WDHD1 protein. A truncating mutation (c.G3274T, p.E1092*) was introduced upstream of the MSH2-
1715 binding domain in the C-terminus of WDHD1 by site-directed mutagenesis. Refer to Materials and Methods for
1716 more details. (B) Co-immunoprecipitation of MSH2 and the wild-type (+/+) or truncated (Δ/Δ) WDHD1 protein.
1717 MSH2 protein was detected in the input fraction of HEK293T cells transfected with plasmids expressing GFP-
1718 WDHD1+/+, GFP-WDHD1$^{\Delta/\Delta}$, or GFP proteins and in the immunoprecipitation (GFP-Trap) fraction of cells
1719 expressing the wild-type GFP-WDHD1 protein, but not in the immunoprecipitation fraction of cells transfected
1720 with the mutated GFP-WDHD1 or GFP plasmids, validating the C-terminus (residuals 1122-1226) of WDHD1
1721 protein as the binding site for MSH2.

1722

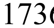
1723 *Cells lacking interaction between MSH2 and WDHD1 are microsatellite stable and do not*
1724 *accumulate spontaneous mutations in vitro*

1725

1726 Once the interaction between MSH2 and WDHD1 proteins has been validated, and the
1727 C-terminus of WDHD1 has been confirmed as required for this interaction, we created a cellular
1728 model with truncating mutations in the last exon of the *Wdhd1* gene to abolish the WDHD1-
1729 MSH2 interaction, and interrogated the impact of this disrupted interaction on the repair of
1730 spontaneous mutations mediated by the MMR pathway.

1731 We used the CRISPR/Cas9 system to generate CT26 cells harboring homozygous and
1732 compound heterozygous frameshift mutations in the *Wdhd1* gene (**Supplementary Figure**

S3A) by targeting the exon 25. We confirmed the expression of truncated WDHD1 protein in both homozygous and compound heterozygous clones by western blot (**Supplementary Figure S3B**).

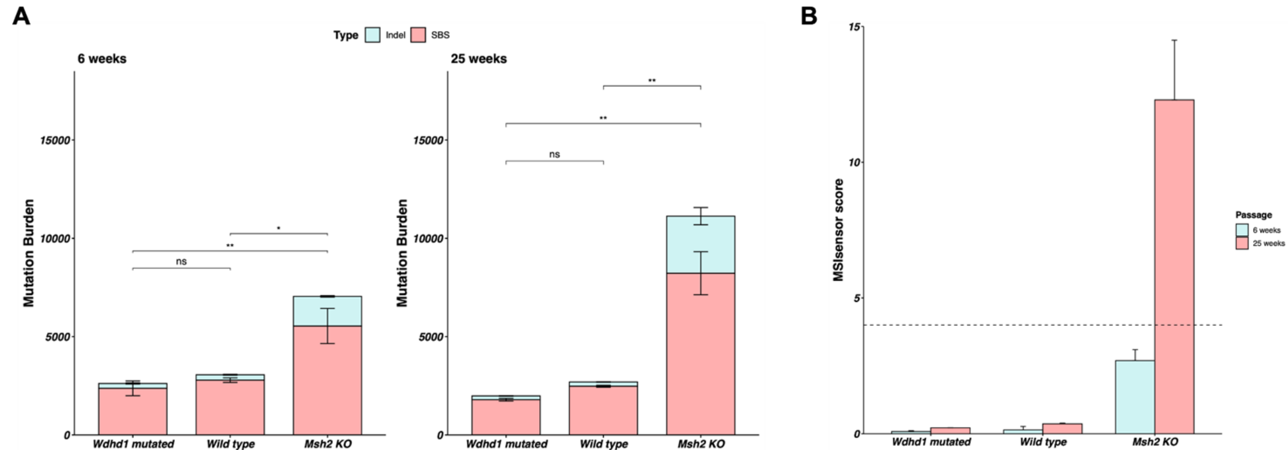


Supplementary Figure S3 – Characterization of *Wdh1*-mutant clones. Single-cell clones harboring homozygous or heterozygous frameshift mutations in the *Wdhd1* gene were obtained by transfecting the px458 plasmid expressing the CRISPR/Cas9 components, including a guide RNA targeting the exon 25 of *Wdhd1* in wild-type CT26 cells. The identification of a single 2-nucleotide deletion with a contribution of 100% indicates the generation of a homozygous clone, while the presence of two distinct frameshift mutations (a deletion of two nucleotides and an insertion of one nucleotide), each of them with a contribution of around 50%, indicates the generation of a compound heterozygous clone. (B) Expression of WDHD1 protein in wild-type and *Wdhd1*-mutant (WDHD1$^{\Delta/\Delta}$) CT26 cells. Wild-type cells express a single band corresponding to the full-length WDHD1 protein, while homozygous and compound heterozygous Wdhd1-mutant clones stably express only truncated WDHD1 protein as a result of the frameshift mutations introduced by the CRISPR/Cas9 system.

The accumulation of spontaneous mutations was assessed by whole-exome sequencing of wild-type, *Wdhd1*-mutant, and *Msh2*-KO cells, after 6 and 25 weeks of cell culture (**Figure 4A**). *Msh2*-KO cells showed a 2-fold increase in the mean mutation burden compared to wild-type cells (5,513 vs. 2,763 mutations, p = 0.00970) and a 2.6-fold increase in contrast with *Wdhd1*-mutant cells (5,513 vs. 2,124 mutations, p = 0.00613) cultured for six weeks. After 25 weeks of culture, *Msh2*-KO cells had a 4-fold increase in the total number of mutations compared with wild-type cells (11,128 vs. 2,696 mutations, p = 0.00507) and around a 5.6-fold increase in comparison with *Wdhd1*-mutated cells (11,128 vs. 1,983 mutations, p = 0.00401). No difference in the total number of mutations was found between the wild-type and *Wdhd1*-mutant cells cultured for 5 weeks (2,763 vs. 2,124, p = 0.48100) and 25 weeks (2,696 vs. 1,983, p = 0.72700). Additionally, *Msh2*-KO duplicated the number of mutations from 5 to 25 weeks of culture (5,513 vs. 11,128 mutations), while wild-type and *Wdhd1*-mutant cells did not accumulate a substantial number of mutations after 25 weeks of growth.

Microsatellite instability was evaluated using the MSIsensor tool in whole-exome sequencing data from *Msh2*-KO, wild-type, and *Wdhd1*-mutant cells cultured for 5 and 25 weeks (**Figure 4B**). *Msh2*-KO cells had MSIsensor scores superior to 4 after 25 weeks of culture, characterizing them as having MSI. On the other hand, wild-type and *Wdhd1*-mutant

1768 cells did not achieve the threshold for microsatellite instability calling. Together, those findings
1769 indicate that the interaction between MSH2 and WDHD1 is not required for the MMR-
1770 mediated repair of spontaneous mutations generated during DNA replication. Additionally,
1771 disruption of MSH2-WDHD1 interaction does not lead to an MSI phenotype *in vitro*.
1772



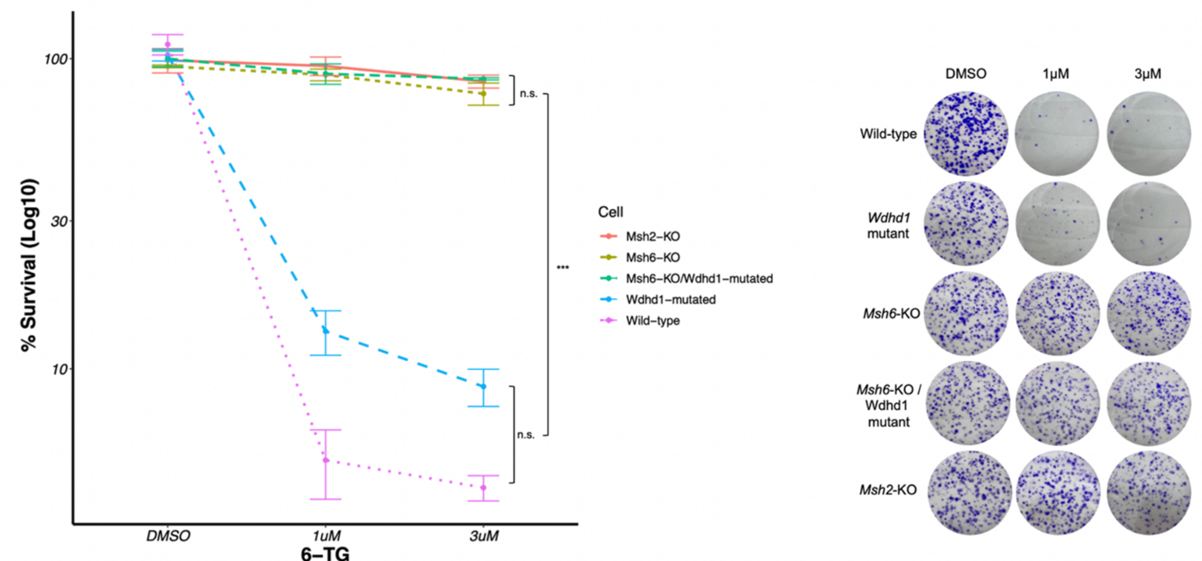
1773
1774
1775 **Figure 4 – Accumulation of spontaneous mutations and microsatellite instability in cells lacking WDHD1-**
1776 **MSH2 protein interaction.** (A) The mean number of mutations accumulated in wild-type, *Wdhd1*-mutant, and
1777 *Msh2*-KO cells were compared after 5 and 25 weeks in culture. The mean number of indels and single-base
1778 substitutions (SBS) were obtained from whole-exome sequencing of two single-cell clones per cell line at 6 and
1779 25 weeks. Mean values were compared using Tukey's test. * ($p < 0.01$), ** ($p<0.001$). (B) Microsatellite scores
1780 were calculated using the MSIsensor tool. The horizontal line represents the threshold of 4 used for microsatellite
1781 instability detection.
1782

1783 *WDHD1 mutant cells have mild resistance to 6-Thioguanine*

1784

1785 In addition to repairing replication mismatches, the MMR pathway also participates in
1786 the cellular response to 6-TG, which comprises the recognition of 6-TG-mediated DNA adducts
1787 by MutSα proteins and consequent cell cycle arrest and apoptosis in a pathway involving ATM
1788 and CHEK2 [4]. The inactivation of MSH2 and other MMR components leads to resistance to
1789 6-TG [32]. Therefore, we interrogated whether the interaction between WDHD1 and MSH2 is
1790 required for the MMR-mediated cellular response to 6-TG by conducting a clonogenic assay
1791 on cells treated with increasing doses of 6-TG (1μM and 3μM)
1792 *Msh2-KO* and *Msh6-KO* cells are highly resistant to 6-TG in comparison with wild-type
1793 cells (p-value < 0.05, Tukey's test) (**Figure 5**). There were no statistical differences in surviving
1794 fractions among *Msh2*-KO, *Msh6*-KO, and *Msh6*-KO/*Wdhd1*-mutant cells. However, *Msh6*-
1795 KO/*Wdhd1*-mutant were slightly more resistant to 6-TG at the 3μM dose than *Msh6*-KO cells
1796 (86.13% ±0.73% vs 77.12% ± 6.31%, p-value = 0.639 – Tukey's test). Interestingly, *Wdhd1*-

1797 mutant cells were twice more resistant to 6-TG than wild-type cells in both 1µM (13.22% ±
1798 2.16% vs. 5.08% ± 1.28%, p-value = 0.787 – Tukey's test), and 3µM (8.77% ± 1.20% vs. 4.14%
1799 ± 0.39%, p-value = 0.999 – Tukey's test) doses. Those results indicate that the disruption of the
1800 WDHD1-MSH2 interaction leads to a mild resistance to 6-TG treatment, and the disruption of
1801 MSH2-WDHD1 interaction seems to amplify the resistance to 6-TG in cells lacking MSH6
1802 protein.

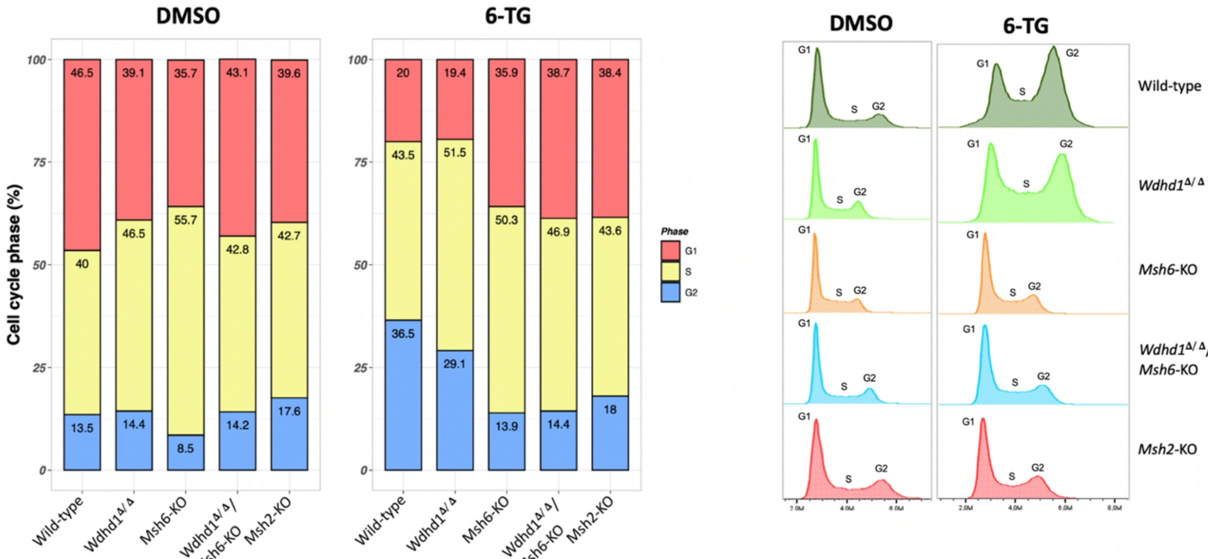


1803
1804

1805 **Figure 5 – Effect of 6-thioguanine on the surviving fraction of *Wdhd1*-mutant cells.** The survival fraction of
1806 cells to DMSO (6-TG solvent) and different doses of 6-TG assessed by the clonogenic assay are shown on the left
1807 panel. Representative results of one out of three independent experiments are displayed as means and standard
1808 deviation values. The surviving fraction of different cell lines in the three treatments were compared by using the
1809 two-way ANOVA and the post-hoc Tukey's honestly significant difference test. Representative images of crystal
1810 violet-stained colonies are shown in the right panel. Refer to the Materials and Methods section for more details.
1811 n.s. (no statistical significance was achieved). *** (p-value < 0.001).
1812

1813 We also investigated the impact of 6-TG on the cell cycle of *Wdhd1*-mutant cells in
1814 comparison with wild-type and MMR-deficient cell lines by flow cytometry (**Figure 6**). About
1815 80% of the MMR-proficient (wild-type) and *Wdhd1*-mutant cell populations exposed to 1µM
1816 of 6-TG for 48 hours were represented by cells in the S or G2 phases, corresponding to a 2-fold
1817 S/G2 increase in comparison with cells treated with DMSO. In contrast, we did not notice an
1818 increase in S/G2 fractions in *Msh6*-KO, double mutant cells (*Wdhd1*-mutant/*Msh6*-KO), and
1819 *Msh2*-KO cells after treatment with 6-TG in comparison with their counterparts treated with
1820 DMSO. Therefore, the disruption of the MSH2-WDHD1 interaction does not modify the S/G2
1821 cell cycle arrest mediated by 6-TG.
1822

1823



1824

1825 1826 1827 1828 1829 1830 1831 **Figure 6 – Cell cycle analysis of cells treated with 6-thioguanine.** *Wdhd1*-mutant, wild-type, and MMR-mutated cells were treated with 1μM of 6-TG for 48 hours and were fixed and stained with propidium iodide for cell cycle analysis by flow cytometry. The G1, S, and G2 fractions present in the left panel were calculated from viable, single cells using the cell cycle module from the FlowJo software. More details are found in the Materials and Methods section. The right panel shows a representative histogram with G1, S, and G2 cells treated with DMSO and 1μM of 6-TG for 48 hours. The x-axis shows the PI intensity values recovered from the PE filter. Two independent experiments were performed. WDHD1$^{\Delta/\Delta}$: *Wdhd1*-mutant cells.

1832

1833 **Discussion**

1834

1835 MMR deficiency is caused mainly by genetic or epigenetic silencing of MMR genes
1836 [33]. However, a fraction of MMR-deficient tumors does not have alterations in the MMR
1837 components. In this study, we conducted a comprehensive analysis of more than 10,000 tumors
1838 from the TCGA dataset and detected microsatellite instability in 3.6% of the TCGA cohort
1839 (**Figure 1A**), consistent with previous studies [23,34]. By combining methylation with somatic
1840 and germline mutation data, we identified the molecular etiologies of MSI tumors and
1841 confirmed the methylation of the *MLH1* gene as the main cause of MSI in cancer. Interestingly,
1842 11.4% of tumors did not have an etiology explained by inactivating events in MMR genes.
1843 Similar results have recently been reported in a meta-analysis conducted on more than 50,000
1844 colorectal carcinomas, where the authors found tumors with an unexplained MMR-deficient
1845 phenotype in around 10% of MMR-deficient tumors [10]. An integrative analysis of
1846 unexplained MSI tumors identified in the TCGA cohort, comprising the available mutation,
1847 methylation, transcriptomics, and protein expression data, will be of utmost utility for exploring
1848 novel molecular drivers of MSI, with the potential to generate new knowledge about the MMR
1849 pathway.

1850 The WDHD1 protein is essential for DNA replication in eukaryotic cells, acting as a
1851 hub connecting the replication machinery to several factors required for chromosome
1852 duplication [18]. WDHD1 also plays a role in genome integrity by preventing fork resection-
1853 mediated double-strand breaks by promoting fork protection [35]. In addition, WDHD1 was
1854 found to interact with the MSH2 protein through a small domain comprising the residuals 1122-
1855 1126 in the C-terminus of the WDHD1 protein [19]. The role of this interaction in the
1856 functionality of the MMR pathway has not been investigated so far. After validating the
1857 WDHD1-MSH2 interaction in HEK293T cells by combining site-directed mutagenesis and
1858 immunoprecipitation of GFP-tagged WDHD1 protein (**Figure 2; Figure 3**), we constructed a
1859 cellular model lacking the MSH2-binding domain by introducing truncating mutations in the
1860 C-terminus of the *Wdhd1* gene in CT26 cells (**Supplementary Figure S3**) to investigate the
1861 impact of the WDHD1-MSH2 interaction on the repair of replication errors mediated by the
1862 MMR pathway.
1863 CT26 cells were serially passaged for weeks to allow for the accumulation of replication
1864 errors, as previously demonstrated by our group [27]. We did not observe an increase of
1865 spontaneous mutations in the *Wdhd*1-mutant and wild-type cells after 25 weeks of culture
1866 (**Figure 4A**). Similarly, only *Msh2*-KO cells cultured for 25 weeks had an MSI phenotype
1867 (**Figure 4B**). The time cells were maintained in culture was based on studies where key MMR
1868 genes, such as *Mlh1* [36] and *Msh2,* were inactivated [37]. We can speculate that the disruption
1869 of the WDHD1-MSH2 interaction does not have the same impact on the repair of replication
1870 errors by the MMR pathway as presented by those key MMR components, suggesting that those
1871 cells would require more time to accumulate mutations than cells with inactivation in MMR
1872 genes. However, we did not detect any tendency towards an accumulation of mutations in the
1873 *Wdhd1*-mutant cells in comparison with the wild-type counterpart after 25 weeks of culture.
1874 In addition to the repair of spontaneous mutations generated during DNA replication,
1875 MMR proteins are also involved in the cellular response to DNA adducts generated by several
1876 mutagenic agents, including 6-thioguanine, a base analog commonly used in anti-cancer
1877 chemotherapies [32,38]. Similar to the repair of replicative mismatches, MutSα and MutLα
1878 participate in the recognition of 6-TG adducts and activates the DNA damage signaling to
1879 trigger cell cycle arrest and apoptosis in an ATM/CHEK2 manner [39,40]. The inactivation of
1880 MutS or MutL components renders cells resistant to 6-TG and is a known mechanism of
1881 resistance to 6-TG-based chemotherapy in some cancers [32,39]. Therefore, evaluating the
1882 sensitivity to 6-TG treatment is an alternative approach to detecting MMR deficiency in cellular
1883 models. Our results showed a mild resistant phenotype in *Wdhd1*-mutant cells treated with

1884 different doses of 6-TG in comparison with wild-type cells (**Figure 5**). Additionally, the
1885 inactivation of *Msh6* in *Wdhd1*-mutant cells resulted in a discrete but reproducible increase in
1886 the surviving fraction of cells treated with 3μM of 6-TG compared to *Msh6*-KO cells with wild-
1887 type *Wdhd1* gene. Differently from our results, by knocking down the *WDHD1* gene in HeLa
1888 cells, Chen et al. [19] reported around 100% of survival in cells treated with 1μM, 3μM, or
1889 8μM of 6-TG, similar to their percentages obtained with the knock-down of *MSH2*. This
1890 discordance may be explained, at least partially, by the different cell lines used in both studies.
1891 Additionally, we investigated the sensitivity to 6-TG in a cellular model expressing a truncating
1892 WDHD1 protein, lacking exclusively the C-terminus domain required for interaction with
1893 MSH2, while Chen *et al*. obtained high resistance to 6-TG in cells with reduced expression of
1894 the WDHD1 protein. Thus, it is not possible to address the strong resistant phenotype obtained
1895 by Chen *et al*. to the disputation of WDHD1-MSH2 interaction rather than to a pleiotropic
1896 effect created by the reduction of WDHD1 expression.

1897 In summary, we have identified a fraction of MSI tumors in a large pan-cancer cohort
1898 and addressed the molecular etiologies of the MSI phenotype detected among 33 different
1899 tumor types. About 11% of MSI tumors did not have genetic or epigenetic inactivating events
1900 in MMR genes, but mutations in the C-terminus of WDHD1 were found in a fraction of them.
1901 We have validated the previously reported interaction between WDHD1 and MSH2 proteins
1902 and engineered a cellular model lacking this interaction. After 25 weeks in cell culture, our
1903 *Wdhd1*-mutant model did not accumulate replication errors and did not have an MSI phenotype.
1904 However, the abolishment of the WDHD1-MSH2 interaction conferred mild resistance to 6-
1905 TG. Taken together, our results indicate that the interaction between WDHD1 and MSH2 is
1906 not required for the repair of replication errors by the MMR pathway, and the disruption of this
1907 interaction does not lead to the MSI phenotype in vitro. However, despite not being essential,
1908 the WDHD1-MSH2 interaction might participate in the cellular response to 6-TG. The role of
1909 WDHD1 in the resistance to 6-TG-based chemotherapy should be further investigated.

1910

1911 **Author contributions**

1912

1913 PK and RR conceived and planned the study design. RR performed Molecular Biology
1914 experiments. RR and AY led the writing of the manuscript. RR, SN, and AY supported data
1915 acquisition and data analysis. PK, VF, and SN provided a critical review of the manuscript. All
1916 authors reviewed the final version of the manuscript and gave full consent for publication.

Funding source

This work was supported by the SIRIC SOCRATE, Gustave Roussy (grant number INCa-DGOS-Inserm 12551). R.C.A.R. was supported by the Eiffel Scholarship Program of Excellence (Campus France) and by SIRIC SOCRATE, Gustave Roussy (grant number INCa-DGOS-Inserm 12551).

Declaration of competing interest

The authors declared no potential conflicts of interest regarding the research, authorship, and/or publication of this article.

Submission of declaration and verification

This manuscript has not been published previously, is not under consideration for publication elsewhere, and this publication is approved by all authors and the responsible authorities where the work has been carried out. This manuscript will not be published elsewhere in the same form.

Availability of data

The raw data from the targeted sequencing will be made available by the corresponding author under request.

1940 **References**

1941

1942 [1] D. Liu, G. Keijzers, L.J. Rasmussen, DNA mismatch repair and its many roles in
1943 eukaryotic cells, Mutat Res Rev Mutat Res. 773 (2017) 174–187.
1944 https://doi.org/10.1016/j.mrrev.2017.07.001.

1945 [2] E. Vilar, S.B. Gruber, Microsatellite instability in colorectal cancer—the stable
1946 evidence, Nat Rev Clin Oncol. 7 (2010) 153–162. https://doi.org/10.1038/nrclinonc.2009.237.

1947 [3] G. Poulogiannis, I.M. Frayling, M.J. Arends, DNA mismatch repair deficiency in
1948 sporadic colorectal cancer and Lynch syndrome, Histopathology. 56 (2010) 167–179.
1949 https://doi.org/10.1111/j.1365-2559.2009.03392.x.

1950 [4] L.M. Martin, B. Marples, M. Coffey, M. Lawler, T.H. Lynch, D. Hollywood, L.
1951 Marignol, DNA mismatch repair and the DNA damage response to ionizing radiation: Making
1952 sense of apparently conflicting data, Cancer Treatment Reviews. 36 (2010) 518–527.
1953 https://doi.org/10.1016/j.ctrv.2010.03.008.

1954 [5] X. Li, X. Yao, Y. Wang, F. Hu, F. Wang, L. Jiang, Y. Liu, D. Wang, G. Sun, Y. Zhao,
1955 MLH1 promoter methylation frequency in colorectal cancer patients and related
1956 clinicopathological and molecular features, PLoS One. 8 (2013) e59064.
1957 https://doi.org/10.1371/journal.pone.0059064.

1958 [6] A.S. Bruegl, B. Djordjevic, D.L. Urbauer, S.N. Westin, P.T. Soliman, K.H. Lu, R.
1959 Luthra, R.R. Broaddus, Utility of MLH1 methylation analysis in the clinical evaluation of
1960 Lynch Syndrome in women with endometrial cancer, Curr Pharm Des. 20 (2014) 1655–1663.
1961 https://doi.org/10.2174/13816128113199990538.

1962 [7] S. Haraldsdottir, H. Hampel, J. Tomsic, W.L. Frankel, R. Pearlman, A. de la Chapelle,
1963 C.C. Pritchard, Colon and Endometrial Cancers With Mismatch Repair Deficiency Can Arise
1964 From Somatic, Rather Than Germline, Mutations, Gastroenterology. 147 (2014) 1308-1316.e1.
1965 https://doi.org/10.1053/j.gastro.2014.08.041.

1966 [8] W.R.R. Geurts-Giele, C.H.M. Leenen, H.J. Dubbink, I.C. Meijssen, E. Post, H.F.B.M.
1967 Sleddens, E.J. Kuipers, A. Goverde, A.M.W. van den Ouweland, M.G.F. van Lier, E.W.
1968 Steyerberg, M.E. van Leerdam, A. Wagner, W.N.M. Dinjens, Somatic aberrations of mismatch

1969 repair genes as a cause of microsatellite-unstable cancers, J Pathol. 234 (2014) 548–559.
1970 https://doi.org/10.1002/path.4419.

1971 [9] N.A.J. Ryan, M.A. Glaire, D. Blake, M. Cabrera-Dandy, D.G. Evans, E.J. Crosbie, The
1972 proportion of endometrial cancers associated with Lynch syndrome: a systematic review of the
1973 literature and meta-analysis, Genetics in Medicine. 21 (2019) 2167–2180.
1974 https://doi.org/10.1038/s41436-019-0536-8.

1975 [10] E.L. Eikenboom, A.-S. van der Werf-'t Lam, M. Rodríguez-Girondo, C.J. Van Asperen,
1976 W.N.M. Dinjens, R.M.W. Hofstra, M.E. Van Leerdam, H. Morreau, M.C.W. Spaander, A.
1977 Wagner, M. Nielsen, Universal Immunohistochemistry for Lynch Syndrome: A Systematic
1978 Review and Meta-analysis of 58,580 Colorectal Carcinomas, Clin Gastroenterol Hepatol. 20
1979 (2022) e496–e507. https://doi.org/10.1016/j.cgh.2021.04.021.

1980 [11] L.E. Oldfield, T. Li, A. Tone, M. Aronson, M. Edwards, S. Holter, R. Quevedo, E. Van
1981 de Laar, J. Lerner-Ellis, A. Pollett, B. Clarke, U. Tabori, S. Gallinger, S.E. Ferguson, T.J. Pugh,
1982 An Integrative DNA Sequencing and Methylation Panel to Assess Mismatch Repair
1983 Deficiency, J Mol Diagn. 23 (2021) 242–252. https://doi.org/10.1016/j.jmoldx.2020.11.006.

1984 [12] A. Cercek, M. Lumish, J. Sinopoli, J. Weiss, J. Shia, M. Lamendola-Essel, I.H. El Dika,
1985 N. Segal, M. Shcherba, R. Sugarman, Z. Stadler, R. Yaeger, J.J. Smith, B. Rousseau, G. Argiles,
1986 M. Patel, A. Desai, L.B. Saltz, M. Widmar, K. Iyer, J. Zhang, N. Gianino, C. Crane, P.B.
1987 Romesser, E.P. Pappou, P. Paty, J. Garcia-Aguilar, M. Gonen, M. Gollub, M.R. Weiser, K.A.
1988 Schalper, L.A. Diaz, PD-1 Blockade in Mismatch Repair-Deficient, Locally Advanced Rectal
1989 Cancer, N Engl J Med. 386 (2022) 2363–2376. https://doi.org/10.1056/NEJMoa2201445.

1990 [13] D.T. Le, J.N. Uram, H. Wang, B.R. Bartlett, H. Kemberling, A.D. Eyring, A.D. Skora,
1991 B.S. Luber, N.S. Azad, D. Laheru, B. Biedrzycki, R.C. Donehower, A. Zaheer, G.A. Fisher,
1992 T.S. Crocenzi, J.J. Lee, S.M. Duffy, R.M. Goldberg, A. de la Chapelle, M. Koshiji, F. Bhaijee,
1993 T. Huebner, R.H. Hruban, L.D. Wood, N. Cuka, D.M. Pardoll, N. Papadopoulos, K.W. Kinzler,
1994 S. Zhou, T.C. Cornish, J.M. Taube, R.A. Anders, J.R. Eshleman, B. Vogelstein, L.A. Diaz, PD-
1995 1 Blockade in Tumors with Mismatch-Repair Deficiency, N Engl J Med. 372 (2015) 2509–
1996 2520. https://doi.org/10.1056/NEJMoa1500596.

1997 [14] M. Lu, B. Zhao, M. Liu, L. Wu, Y. Li, Y. Zhai, X. Shen, Pan-cancer analysis of SETD2
1998 mutation and its association with the efficacy of immunotherapy, NPJ Precis Oncol. 5 (2021)
1999 51. https://doi.org/10.1038/s41698-021-00193-0.

2000 [15] J. Shen, Z. Ju, W. Zhao, L. Wang, Y. Peng, Z. Ge, Z.D. Nagel, J. Zou, C. Wang, P.
2001 Kapoor, X. Ma, D. Ma, J. Liang, S. Song, J. Liu, L.D. Samson, J.A. Ajani, G.-M. Li, H. Liang,
2002 X. Shen, G.B. Mills, G. Peng, ARID1A deficiency promotes mutability and potentiates
2003 therapeutic antitumor immunity unleashed by immune checkpoint blockade, Nat Med. 24
2004 (2018) 556–562. https://doi.org/10.1038/s41591-018-0012-z.

2005 [16] A. Castillejo, G. Vargas, M.I. Castillejo, M. Navarro, V.M. Barberá, S. González, E.
2006 Hernández-Illán, J. Brunet, T. Ramón y Cajal, J. Balmaña, S. Oltra, S. Iglesias, A. Velasco, A.
2007 Solanes, O. Campos, A.B. Sánchez Heras, J. Gallego, E. Carrasco, D. González Juan, A.
2008 Segura, I. Chirivella, M.J. Juan, I. Tena, C. Lázaro, I. Blanco, M. Pineda, G. Capellá, J.L. Soto,
2009 Prevalence of germline MUTYH mutations among Lynch-like syndrome patients, Eur J
2010 Cancer. 50 (2014) 2241–2250. https://doi.org/10.1016/j.ejca.2014.05.022.

2011 [17] P.A. Konstantinopoulos, U.A. Matulonis, POLE mutations as an alternative pathway
2012 for microsatellite instability in endometrial cancer: implications for Lynch syndrome testing,
2013 Cancer. 121 (2015) 331–334. https://doi.org/10.1002/cncr.29057.

2014 [18] F. Villa, A.C. Simon, M.A. Ortiz Bazan, M.L. Kilkenny, D. Wirthensohn, M.
2015 Wightman, D. Matak-Vinkovíc, L. Pellegrini, K. Labib, Ctf4 Is a Hub in the Eukaryotic
2016 Replisome that Links Multiple CIP-Box Proteins to the CMG Helicase, Mol Cell. 63 (2016)
2017 385–396. https://doi.org/10.1016/j.molcel.2016.06.009.

2018 [19] Z. Chen, M. Tran, M. Tang, W. Wang, Z. Gong, J. Chen, Proteomic Analysis Reveals
2019 a Novel Mutator S (MutS) Partner Involved in Mismatch Repair Pathway, Mol Cell Proteomics.
2020 15 (2016) 1299–1308. https://doi.org/10.1074/mcp.M115.056093.

2021 [20] Cancer Genome Atlas Research Network, J.N. Weinstein, E.A. Collisson, G.B. Mills,
2022 K.R.M. Shaw, B.A. Ozenberger, K. Ellrott, I. Shmulevich, C. Sander, J.M. Stuart, The Cancer
2023 Genome Atlas Pan-Cancer analysis project, Nat Genet. 45 (2013) 1113–1120.
2024 https://doi.org/10.1038/ng.2764.

2025 [21] R.L. Grossman, A.P. Heath, V. Ferretti, H.E. Varmus, D.R. Lowy, W.A. Kibbe, L.M.
2026 Staudt, Toward a Shared Vision for Cancer Genomic Data, N Engl J Med. 375 (2016) 1109–
2027 1112. https://doi.org/10.1056/NEJMp1607591.

2028 [22] P. Jia, X. Yang, L. Guo, B. Liu, J. Lin, H. Liang, J. Sun, C. Zhang, K. Ye, MSIsensor-
2029 pro: Fast, Accurate, and Matched-normal-sample-free Detection of Microsatellite Instability,
2030 Genomics, Proteomics & Bioinformatics. 18 (2020) 65–71.
2031 https://doi.org/10.1016/j.gpb.2020.02.001.

2032 [23] M.H. Bailey, C. Tokheim, E. Porta-Pardo, S. Sengupta, D. Bertrand, A. Weerasinghe,
2033 A. Colaprico, M.C. Wendl, J. Kim, B. Reardon, P.K.-S. Ng, K.J. Jeong, S. Cao, Z. Wang, J.
2034 Gao, Q. Gao, F. Wang, E.M. Liu, L. Mularoni, C. Rubio-Perez, N. Nagarajan, I. Cortés-Ciriano,
2035 D.C. Zhou, W.-W. Liang, J.M. Hess, V.D. Yellapantula, D. Tamborero, A. Gonzalez-Perez, C.
2036 Suphavilai, J.Y. Ko, E. Khurana, P.J. Park, E.M. Van Allen, H. Liang, MC3 Working Group,
2037 Cancer Genome Atlas Research Network, M.S. Lawrence, A. Godzik, N. Lopez-Bigas, J.
2038 Stuart, D. Wheeler, G. Getz, K. Chen, A.J. Lazar, G.B. Mills, R. Karchin, L. Ding,
2039 Comprehensive Characterization of Cancer Driver Genes and Mutations, Cell. 173 (2018) 371-
2040 385.e18. https://doi.org/10.1016/j.cell.2018.02.060.

2041 [24] A.C. Berger, A. Korkut, R.S. Kanchi, A.M. Hegde, W. Lenoir, W. Liu, Y. Liu, H. Fan,
2042 H. Shen, V. Ravikumar, A. Rao, A. Schultz, X. Li, P. Sumazin, C. Williams, P. Mestdagh, P.H.
2043 Gunaratne, C. Yau, R. Bowlby, A.G. Robertson, D.G. Tiezzi, C. Wang, A.D. Cherniack, A.K.
2044 Godwin, N.M. Kuderer, J.S. Rader, R.E. Zuna, A.K. Sood, A.J. Lazar, A.I. Ojesina, C.
2045 Adebamowo, S.N. Adebamowo, K.A. Baggerly, T.-W. Chen, H.-S. Chiu, S. Lefever, L. Liu,
2046 K. MacKenzie, S. Orsulic, J. Roszik, C.S. Shelley, Q. Song, C.P. Vellano, N. Wentzensen,
2047 Cancer Genome Atlas Research Network, J.N. Weinstein, G.B. Mills, D.A. Levine, R. Akbani,
2048 A Comprehensive Pan-Cancer Molecular Study of Gynecologic and Breast Cancers, Cancer
2049 Cell. 33 (2018) 690-705.e9. https://doi.org/10.1016/j.ccell.2018.03.014.

2050 [25] K. Huang, R.J. Mashl, Y. Wu, D.I. Ritter, J. Wang, C. Oh, M. Paczkowska, S. Reynolds,
2051 M.A. Wyczalkowski, N. Oak, A.D. Scott, M. Krassowski, A.D. Cherniack, K.E. Houlahan, R.
2052 Jayasinghe, L.-B. Wang, D.C. Zhou, D. Liu, S. Cao, Y.W. Kim, A. Koire, J.F. McMichael, V.
2053 Hucthagowder, T.-B. Kim, A. Hahn, C. Wang, M.D. McLellan, F. Al-Mulla, K.J. Johnson, O.
2054 Lichtarge, P.C. Boutros, B. Raphael, A.J. Lazar, W. Zhang, M.C. Wendl, R. Govindan, S. Jain,
2055 D. Wheeler, S. Kulkarni, J.F. Dipersio, J. Reimand, F. Meric-Bernstam, K. Chen, I.

2056 Shmulevich, S.E. Plon, F. Chen, L. Ding, S.J. Caesar-Johnson, J.A. Demchok, I. Felau, M.
2057 Kasapi, M.L. Ferguson, C.M. Hutter, H.J. Sofia, R. Tarnuzzer, Z. Wang, L. Yang, J.C.
2058 Zenklusen, J. (Julia) Zhang, S. Chudamani, J. Liu, L. Lolla, R. Naresh, T. Pihl, Q. Sun, Y. Wan,
2059 Y. Wu, J. Cho, T. DeFreitas, S. Frazer, N. Gehlenborg, G. Getz, D.I. Heiman, J. Kim, M.S.
2060 Lawrence, P. Lin, S. Meier, M.S. Noble, G. Saksena, D. Voet, H. Zhang, B. Bernard, N.
2061 Chambwe, V. Dhankani, T. Knijnenburg, R. Kramer, K. Leinonen, Y. Liu, M. Miller, S.
2062 Reynolds, I. Shmulevich, V. Thorsson, W. Zhang, R. Akbani, B.M. Broom, A.M. Hegde, Z.
2063 Ju, R.S. Kanchi, A. Korkut, J. Li, H. Liang, S. Ling, W. Liu, Y. Lu, G.B. Mills, K.-S. Ng, A.
2064 Rao, M. Ryan, J. Wang, J.N. Weinstein, J. Zhang, A. Abeshouse, J. Armenia, D. Chakravarty,
2065 W.K. Chatila, I. de Bruijn, J. Gao, B.E. Gross, Z.J. Heins, R. Kundra, K. La, M. Ladanyi, A.
2066 Luna, M.G. Nissan, A. Ochoa, S.M. Phillips, E. Reznik, F. Sanchez-Vega, C. Sander, N.
2067 Schultz, R. Sheridan, S.O. Sumer, Y. Sun, B.S. Taylor, J. Wang, H. Zhang, P. Anur, M. Peto,
2068 P. Spellman, C. Benz, J.M. Stuart, C.K. Wong, C. Yau, D.N. Hayes, J.S. Parker, M.D.
2069 Wilkerson, A. Ally, M. Balasundaram, R. Bowlby, D. Brooks, R. Carlsen, E. Chuah, N. Dhalla,
2070 R. Holt, S.J.M. Jones, K. Kasaian, D. Lee, Y. Ma, M.A. Marra, M. Mayo, R.A. Moore, A.J.
2071 Mungall, K. Mungall, A.G. Robertson, S. Sadeghi, J.E. Schein, P. Sipahimalani, A. Tam, N.
2072 Thiessen, K. Tse, T. Wong, A.C. Berger, R. Beroukhim, A.D. Cherniack, C. Cibulskis, S.B.
2073 Gabriel, G.F. Gao, G. Ha, M. Meyerson, S.E. Schumacher, J. Shih, M.H. Kucherlapati, R.S.
2074 Kucherlapati, S. Baylin, L. Cope, L. Danilova, M.S. Bootwalla, P.H. Lai, D.T. Maglinte, D.J.
2075 Van Den Berg, D.J. Weisenberger, J.T. Auman, S. Balu, T. Bodenheimer, C. Fan, K.A.
2076 Hoadley, A.P. Hoyle, S.R. Jefferys, C.D. Jones, S. Meng, P.A. Mieczkowski, L.E. Mose, A.H.
2077 Perou, C.M. Perou, J. Roach, Y. Shi, J.V. Simons, T. Skelly, M.G. Soloway, D. Tan, U.
2078 Veluvolu, H. Fan, T. Hinoue, P.W. Laird, H. Shen, W. Zhou, M. Bellair, K. Chang, K.
2079 Covington, C.J. Creighton, H. Dinh, H. Doddapaneni, L.A. Donehower, J. Drummond, R.A.
2080 Gibbs, R. Glenn, W. Hale, Y. Han, J. Hu, V. Korchina, S. Lee, L. Lewis, W. Li, X. Liu, M.
2081 Morgan, D. Morton, D. Muzny, J. Santibanez, M. Sheth, E. Shinbrot, L. Wang, M. Wang, D.A.
2082 Wheeler, L. Xi, F. Zhao, J. Hess, E.L. Appelbaum, M. Bailey, M.G. Cordes, L. Ding, C.C.
2083 Fronick, L.A. Fulton, R.S. Fulton, C. Kandoth, E.R. Mardis, M.D. McLellan, C.A. Miller, H.K.
2084 Schmidt, R.K. Wilson, D. Crain, E. Curley, J. Gardner, K. Lau, D. Mallery, S. Morris, J.
2085 Paulauskis, R. Penny, C. Shelton, T. Shelton, M. Sherman, E. Thompson, P. Yena, J. Bowen,
2086 J.M. Gastier-Foster, M. Gerken, K.M. Leraas, T.M. Lichtenberg, N.C. Ramirez, L. Wise, E.
2087 Zmuda, N. Corcoran, T. Costello, C. Hovens, A.L. Carvalho, A.C. de Carvalho, J.H. Fregnani,
2088 A. Longatto-Filho, R.M. Reis, C. Scapulatempo-Neto, H.C.S. Silveira, D.O. Vidal, A. Burnette,
2089 J. Eschbacher, B. Hermes, A. Noss, R. Singh, M.L. Anderson, P.D. Castro, M. Ittmann, D.

2090 Huntsman, B. Kohl, X. Le, R. Thorp, C. Andry, E.R. Duffy, V. Lyadov, O. Paklina, G.
2091 Setdikova, A. Shabunin, M. Tavobilov, C. McPherson, R. Warnick, R. Berkowitz, D. Cramer,
2092 C. Feltmate, N. Horowitz, A. Kibel, M. Muto, C.P. Raut, A. Malykh, J.S. Barnholtz-Sloan, W.
2093 Barrett, K. Devine, J. Fulop, Q.T. Ostrom, K. Shimmel, Y. Wolinsky, A.E. Sloan, A. De Rose,
2094 F. Giuliante, M. Goodman, B.Y. Karlan, C.H. Hagedorn, J. Eckman, J. Harr, J. Myers, K.
2095 Tucker, L.A. Zach, B. Deyarmin, H. Hu, L. Kvecher, C. Larson, R.J. Mural, S. Somiari, A.
2096 Vicha, T. Zelinka, J. Bennett, M. Iacocca, B. Rabeno, P. Swanson, M. Latour, L. Lacombe, B.
2097 Têtu, A. Bergeron, M. McGraw, S.M. Staugaitis, J. Chabot, H. Hibshoosh, A. Sepulveda, T.
2098 Su, T. Wang, O. Potapova, O. Voronina, L. Desjardins, O. Mariani, S. Roman-Roman, X.
2099 Sastre, M.-H. Stern, F. Cheng, S. Signoretti, A. Berchuck, D. Bigner, E. Lipp, J. Marks, S.
2100 McCall, R. McLendon, A. Secord, A. Sharp, M. Behera, D.J. Brat, A. Chen, K. Delman, S.
2101 Force, F. Khuri, K. Magliocca, S. Maithel, J.J. Olson, T. Owonikoko, A. Pickens, S.
2102 Ramalingam, D.M. Shin, G. Sica, E.G. Van Meir, H. Zhang, W. Eijckenboom, A. Gillis, E.
2103 Korpershoek, L. Looijenga, W. Oosterhuis, H. Stoop, K.E. van Kessel, E.C. Zwarthoff, C.
2104 Calatozzolo, L. Cuppini, S. Cuzzubbo, F. DiMeco, G. Finocchiaro, L. Mattei, A. Perin, B.
2105 Pollo, C. Chen, J. Houck, P. Lohavanichbutr, A. Hartmann, C. Stoehr, R. Stoehr, H. Taubert,
2106 S. Wach, B. Wullich, W. Kycler, D. Murawa, M. Wiznerowicz, K. Chung, W.J. Edenfield, J.
2107 Martin, E. Baudin, G. Bubley, R. Bueno, A. De Rienzo, W.G. Richards, S. Kalkanis, T.
2108 Mikkelsen, H. Noushmehr, L. Scarpace, N. Girard, M. Aymerich, E. Campo, E. Giné, A.L.
2109 Guillermo, N. Van Bang, P.T. Hanh, B.D. Phu, Y. Tang, H. Colman, K. Evason, P.R. Dottino,
2110 J.A. Martignetti, H. Gabra, H. Juhl, T. Akeredolu, S. Stepa, D. Hoon, K. Ahn, K.J. Kang, F.
2111 Beuschlein, A. Breggia, M. Birrer, D. Bell, M. Borad, A.H. Bryce, E. Castle, V. Chandan, J.
2112 Cheville, J.A. Copland, M. Farnell, T. Flotte, N. Giama, T. Ho, M. Kendrick, J.-P. Kocher, K.
2113 Kopp, C. Moser, D. Nagorney, D. O'Brien, B.P. O'Neill, T. Patel, G. Petersen, F. Que, M.
2114 Rivera, L. Roberts, R. Smallridge, T. Smyrk, M. Stanton, R.H. Thompson, M. Torbenson, J.D.
2115 Yang, L. Zhang, F. Brimo, J.A. Ajani, A.M.A. Gonzalez, C. Behrens, J. Bondaruk, R.
2116 Broaddus, B. Czerniak, B. Esmaeli, J. Fujimoto, J. Gershenwald, C. Guo, A.J. Lazar, C.
2117 Logothetis, F. Meric-Bernstam, C. Moran, L. Ramondetta, D. Rice, A. Sood, P. Tamboli, T.
2118 Thompson, P. Troncoso, A. Tsao, I. Wistuba, C. Carter, L. Haydu, P. Hersey, V. Jakrot, H.
2119 Kakavand, R. Kefford, K. Lee, G. Long, G. Mann, M. Quinn, R. Saw, R. Scolyer, K. Shannon,
2120 A. Spillane, J. Stretch, M. Synott, J. Thompson, J. Wilmott, H. Al-Ahmadie, T.A. Chan, R.
2121 Ghossein, A. Gopalan, D.A. Levine, V. Reuter, S. Singer, B. Singh, N.V. Tien, T. Broudy, C.
2122 Mirsaidi, P. Nair, P. Drwiega, J. Miller, J. Smith, H. Zaren, J.-W. Park, N.P. Hung, E. Kebebew,
2123 W.M. Linehan, A.R. Metwalli, K. Pacak, P.A. Pinto, M. Schiffman, L.S. Schmidt, C.D. Vocke,

2124 N. Wentzensen, R. Worrell, H. Yang, M. Moncrieff, C. Goparaju, J. Melamed, H. Pass, N.
2125 Botnariuc, I. Caraman, M. Cernat, I. Chemencedji, A. Clipca, S. Doruc, G. Gorincioi, S. Mura,
2126 M. Pirtac, I. Stancul, D. Tcaciuc, M. Albert, I. Alexopoulou, A. Arnaout, J. Bartlett, J. Engel,
2127 S. Gilbert, J. Parfitt, H. Sekhon, G. Thomas, D.M. Rassl, R.C. Rintoul, C. Bifulco, R.
2128 Tamakawa, W. Urba, N. Hayward, H. Timmers, A. Antenucci, F. Facciolo, G. Grazi, M.
2129 Marino, R. Merola, R. de Krijger, A.-P. Gimenez-Roqueplo, A. Piché, S. Chevalier, G.
2130 McKercher, K. Birsoy, G. Barnett, C. Brewer, C. Farver, T. Naska, N.A. Pennell, D. Raymond,
2131 C. Schilero, K. Smolenski, F. Williams, C. Morrison, J.A. Borgia, M.J. Liptay, M. Pool, C.W.
2132 Seder, K. Junker, L. Omberg, M. Dinkin, G. Manikhas, D. Alvaro, M.C. Bragazzi, V. Cardinale,
2133 G. Carpino, E. Gaudio, D. Chesla, S. Cottingham, M. Dubina, F. Moiseenko, R. Dhanasekaran,
2134 K.-F. Becker, K.-P. Janssen, J. Slotta-Huspenina, M.H. Abdel-Rahman, D. Aziz, S. Bell, C.M.
2135 Cebulla, A. Davis, R. Duell, J.B. Elder, J. Hilty, B. Kumar, J. Lang, N.L. Lehman, R. Mandt,
2136 P. Nguyen, R. Pilarski, K. Rai, L. Schoenfield, K. Senecal, P. Wakely, P. Hansen, R. Lechan,
2137 J. Powers, A. Tischler, W.E. Grizzle, K.C. Sexton, A. Kastl, J. Henderson, S. Porten, J.
2138 Waldmann, M. Fassnacht, S.L. Asa, D. Schadendorf, M. Couce, M. Graefen, H. Huland, G.
2139 Sauter, T. Schlomm, R. Simon, P. Tennstedt, O. Olabode, M. Nelson, O. Bathe, P.R. Carroll,
2140 J.M. Chan, P. Disaia, P. Glenn, R.K. Kelley, C.N. Landen, J. Phillips, M. Prados, J. Simko, K.
2141 Smith-McCune, S. VandenBerg, K. Roggin, A. Fehrenbach, A. Kendler, S. Sifri, R. Steele, A.
2142 Jimeno, F. Carey, I. Forgie, M. Mannelli, M. Carney, B. Hernandez, B. Campos, C. Herold-
2143 Mende, C. Jungk, A. Unterberg, A. von Deimling, A. Bossler, J. Galbraith, L. Jacobus, M.
2144 Knudson, T. Knutson, D. Ma, M. Milhem, R. Sigmund, A.K. Godwin, R. Madan, H.G.
2145 Rosenthal, C. Adebamowo, S.N. Adebamowo, A. Boussioutas, D. Beer, T. Giordano, A.-M.
2146 Mes-Masson, F. Saad, T. Bocklage, L. Landrum, R. Mannel, K. Moore, K. Moxley, R. Postier,
2147 J. Walker, R. Zuna, M. Feldman, F. Valdivieso, R. Dhir, J. Luketich, E.M.M. Pinero, M.
2148 Quintero-Aguilo, C.G. Carlotti, J.S. Dos Santos, R. Kemp, A. Sankarankuty, D. Tirapelli, J.
2149 Catto, K. Agnew, E. Swisher, J. Creaney, B. Robinson, C.S. Shelley, E.M. Godwin, S. Kendall,
2150 C. Shipman, C. Bradford, T. Carey, A. Haddad, J. Moyer, L. Peterson, M. Prince, L. Rozek, G.
2151 Wolf, R. Bowman, K.M. Fong, I. Yang, R. Korst, W.K. Rathmell, J.L. Fantacone-Campbell,
2152 J.A. Hooke, A.J. Kovatich, C.D. Shriver, J. DiPersio, B. Drake, R. Govindan, S. Heath, T. Ley,
2153 B. Van Tine, P. Westervelt, M.A. Rubin, J.I. Lee, N.D. Aredes, A. Mariamidze, Pathogenic
2154 Germline Variants in 10,389 Adult Cancers, Cell. 173 (2018) 355-370.e14.
2155 https://doi.org/10.1016/j.cell.2018.03.039.

2156 [26] K. Ellrott, M.H. Bailey, G. Saksena, K.R. Covington, C. Kandoth, C. Stewart, J. Hess,
2157 S. Ma, K.E. Chiotti, M. McLellan, H.J. Sofia, C. Hutter, G. Getz, D. Wheeler, L. Ding, MC3
2158 Working Group, Cancer Genome Atlas Research Network, Scalable Open Science Approach
2159 for Mutation Calling of Tumor Exomes Using Multiple Genomic Pipelines, Cell Syst. 6 (2018)
2160 271-281.e7. https://doi.org/10.1016/j.cels.2018.03.002.

2161 [27] L. Nebot-Bral, A. Hollebecque, A.A. Yurchenko, L. de Forceville, M. Danjou, J.-M.
2162 Jouniaux, R.C.A. Rosa, C. Pouvelle, S. Aoufouchi, P. Vuagnat, C. Smolenschi, E. Colomba,
2163 A. Leary, A. Marabelle, J.-Y. Scoazec, L. Cassard, S. Nikolaev, N. Chaput, P. Kannouche,
2164 Overcoming resistance to αPD-1 of MMR-deficient tumors with high tumor-induced
2165 neutrophils levels by combination of αCTLA-4 and αPD-1 blockers, J Immunother Cancer. 10
2166 (2022) e005059. https://doi.org/10.1136/jitc-2022-005059.

2167 [28] Z. Hong, J. Jiang, K. Hashiguchi, M. Hoshi, L. Lan, A. Yasui, Recruitment of mismatch
2168 repair proteins to the site of DNA damage in human cells, J Cell Sci. 121 (2008) 3146–3154.
2169 https://doi.org/10.1242/jcs.026393.

2170 [29] N.A.P. Franken, H.M. Rodermond, J. Stap, J. Haveman, C. van Bree, Clonogenic assay
2171 of cells in vitro, Nat Protoc. 1 (2006) 2315–2319. https://doi.org/10.1038/nprot.2006.339.

2172 [30] G.A. Van der Auwera, M.O. Carneiro, C. Hartl, R. Poplin, G. del Angel, A. Levy-
2173 Moonshine, T. Jordan, K. Shakir, D. Roazen, J. Thibault, E. Banks, K.V. Garimella, D.
2174 Altshuler, S. Gabriel, M.A. DePristo, From FastQ Data to High-Confidence Variant Calls: The
2175 Genome Analysis Toolkit Best Practices Pipeline, Current Protocols in Bioinformatics. 43
2176 (2013) 11.10.1-11.10.33. https://doi.org/10.1002/0471250953.bi1110s43.

2177 [31] K. Wang, M. Li, H. Hakonarson, ANNOVAR: functional annotation of genetic variants
2178 from high-throughput sequencing data, Nucleic Acids Research. 38 (2010) e164.
2179 https://doi.org/10.1093/nar/gkq603.

2180 [32] Z. Li, A.H. Pearlman, P. Hsieh, DNA mismatch repair and the DNA damage response,
2181 DNA Repair (Amst). 38 (2016) 94–101. https://doi.org/10.1016/j.dnarep.2015.11.019.

2182 [33] A. Martínez-Roca, M. Giner-Calabuig, O. Murcia, A. Castillejo, J.L. Soto, A. García-
2183 Heredia, R. Jover, Lynch-like Syndrome: Potential Mechanisms and Management, Cancers. 14
2184 (2022) 1115. https://doi.org/10.3390/cancers14051115.

2185 [34] R. Bonneville, M.A. Krook, E.A. Kautto, J. Miya, M.R. Wing, H.-Z. Chen, J.W. Reeser,
2186 L. Yu, S. Roychowdhury, Landscape of Microsatellite Instability Across 39 Cancer Types, JCO
2187 Precis Oncol. 2017 (2017). https://doi.org/10.1200/PO.17.00073.

2188 [35] T. Abe, R. Kawasumi, M. Giannattasio, S. Dusi, Y. Yoshimoto, K. Miyata, K.
2189 Umemura, K. Hirota, D. Branzei, AND-1 fork protection function prevents fork resection and
2190 is essential for proliferation, Nat Commun. 9 (2018) 3091. https://doi.org/10.1038/s41467-018-
2191 05586-7.

2192 [36] G. Germano, S. Lamba, G. Rospo, L. Barault, A. Magrì, F. Maione, M. Russo, G.
2193 Crisafulli, A. Bartolini, G. Lerda, G. Siravegna, B. Mussolin, R. Frapolli, M. Montone, F.
2194 Morano, F. de Braud, N. Amirouchene-Angelozzi, S. Marsoni, M. D'Incalci, A. Orlandi, E.
2195 Giraudo, A. Sartore-Bianchi, S. Siena, F. Pietrantonio, F. Di Nicolantonio, A. Bardelli,
2196 Inactivation of DNA repair triggers neoantigen generation and impairs tumour growth, Nature.
2197 552 (2017) 116–120. https://doi.org/10.1038/nature24673.

2198 [37] R. Mandal, R.M. Samstein, K.-W. Lee, J.J. Havel, H. Wang, C. Krishna, E.Y. Sabio, V.
2199 Makarov, F. Kuo, P. Blecua, A.T. Ramaswamy, J.N. Durham, B. Bartlett, X. Ma, R. Srivastava,
2200 S. Middha, A. Zehir, J.F. Hechtman, L.G. Morris, N. Weinhold, N. Riaz, D.T. Le, L.A. Diaz,
2201 T.A. Chan, Genetic diversity of tumors with mismatch repair deficiency influences anti–PD-1
2202 immunotherapy response, Science. 364 (2019) 485–491.
2203 https://doi.org/10.1126/science.aau0447.

2204 [38] L. Chen, H.-X. Yan, X.-W. Liu, W.-X. Chen, Clinical efficacy and safety of 6-
2205 thioguanine in the treatment of childhood acute lymphoblastic leukemia: A protocol for
2206 systematic review and meta-analysis, Medicine (Baltimore). 99 (2020) e20082.
2207 https://doi.org/10.1097/MD.0000000000020082.

2208 [39] D. Gupta, C.D. Heinen, The mismatch repair-dependent DNA damage response:
2209 Mechanisms and implications, DNA Repair (Amst). 78 (2019) 60–69.
2210 https://doi.org/10.1016/j.dnarep.2019.03.009.

2211 [40] W. Xu, G.-M. Li, DNA repair | DNA Mismatch Repair and the DNA Damage Response,
2212 in: J. Jez (Ed.), Encyclopedia of Biological Chemistry III (Third Edition), Elsevier, Oxford,
2213 2021: pp. 232–235. https://doi.org/10.1016/B978-0-12-819460-7.00238-3.

CONCLUSION

In Chapter I, we adopted a universal tumor screening approach to select cases for genetic testing and found a 4.1% prevalence of Lynch syndrome in a Brazilian cohort of 242 primary endometrial carcinomas. Most MMR-deficient endometrial tumors, without *MLH1* methylation, did not have germline variants in MMR genes and were classified as having Lynch-like syndrome. Those Lynch-like individuals had their cancer diagnosis at intermediate age between hereditary and sporadic cases.

Next, in Chapter II, we explored the genetic etiology of Lynch-like cases identified in Chapter I by combining germline and somatic mutation analysis in 63 genes related to cancer predisposition and DNA repair. Germline variants in cancer-related genes, such as *ATM*, *ATR*, *CHEK2*, *FAN1*, and *MUTYH*, were present in 26% of individuals, which were associated with a higher incidence of cancer in their families than in cases without germline mutations. Tumor sequencing confirmed the inactivation of MMR genes, mainly *MSH6*, as the most frequent etiology for the MMR deficiency phenotype in endometrial cancer. Interestingly, POLE-exonuclease somatic mutations were found to be frequent drivers of MMR deficiency in Lynch-like tumors, probably resulting in the inactivation of MMR genes. Mutational signatures and microsatellite instability scores obtained from tumor sequencing reflected the mutational status of MMR genes.

Finally, in Chapter III, we characterized *WDHD1*, a gene not included in the gene panel used in Chapters I and II, as a potential driver of MMR deficiency based on published biochemistry and genomic data. Around 11% of MSI tumors from the TCGA cohort were not explained by alterations in MMR genes. Mutations in the C-terminus of *WDHD1* were found in a subset of those unexplained MSI tumors. We confirmed the interaction between WDHD1 and MSH2 proteins and validated the C-terminus of WDHD1 as critical for this interaction. The CRISPR/Cas9 system was shown to be an effective methodology to generate cells expressing truncated WDHD1 protein, lacking the MSH2-binding domain. Disruption of WDHD1 and MSH2 did not trigger an MSI phenotype in vitro, excluding the *WDHD1* gene as an alternative driver of MMR deficiency. However, *Wdhd1*-mutant cells had mild resistance to 6-TG, suggesting that the WDHD1-MSH2 interaction might be involved with the cellular response to this drug, mediated by the MMR pathway.

Altogether, our results corroborate the inactivation of MMR genes as the leading cause of MMR deficiency in cancer. Germline mutations in genes from other DNA repair pathways may be found in individuals with MMR-deficient tumors and, even though not causing MMR

deficiency, may confer a familial predisposition to cancer. Mutations in the exonuclease domain of *POLE* are an early event in a portion of endometrial cancers and may trigger MMR deficiency by generating a hypermutator phenotype, making MMR genes prone to be inactivated by truncating mutations. Lastly, the *WDHD1* gene should not be considered an alternative driver of MMR deficiency. However, its role in the resistance to 6-TG should be further investigated.

SCIENTIFIC PRODUCTIONS ACHIEVED DURING THE PH.D.

Articles published in scientific journals

Nebot-Bral, L., Hollebecque, A., Yurchenko, A.A., Forceville, L., Danjou, J.M., Jouniaux, **Rosa, R.C.A.** et al. (July, 2022). Overcoming resistance to αPD-1 of MMR-deficient tumors with high tumor-induced neutrophils levels by a combination of αCTLA-4 and αPD-1 blockers. *Journal of ImmunoTherapy of Cancer*. v.10, doi: 10.1136/jitc-2022-005059

Castro, L.P., Melo, D.B.V., Souza, T.A., Timoteo, A.R.S., Coutinho, J.D.L., Almeida, I.C.P., Henriques, S.R.M., Azevedo, F.M., **Rosa, R.C.A.**, et al. (2022). XPC and POLH/XPV genes mutated in a genetic cluster of xeroderma pigmentosum patients in Northeast Brazil. *Frontiers in Genetics*. v.12, doi: 10.3389/fgene.2021.784963

Barros, I.I., Leao, V., Santis, J.O., **Rosa, R.C.A.**, et al. (2021). Non-Syndromic Intellectual Disability and Its Pathways: A Long Non-coding RNA Perspective. *Non-Coding RNA*, 7(22). doi: 10.3390/ncrna7010022

Rosa, R.C.A., Santis, J.O., Teixeira, L.A., et al. (2020). Lynch syndrome identification in a Brazilian cohort of endometrial cancer screened by a universal approach. *Gynecologic Oncology*, 159(1). doi: https://doi.org/10.1016/j.ygyno.2020.07.013

Rosa, R.C.A., Yurchenko, A.A., Chahud, F., et al. (2020). Ultramutated endometrial cancer with germline loss-of-function and somatic exonuclease domain mutations in *POLE*. *Genetics and Molecular Biology*, 43(4). doi: https://doi.org/10.1590/1678-4685-GMB-2020-0100

Castro, L.P., Sahbatou, M., Kehdy, F.S.G., Farias, A. A., Yurchenko, A.A., De Souza, T.A., **Rosa, R.C.A.,** et al. (2020). The Iberian legacy into a young genetic xeroderma pigmentosum cluster in central Brazil. *Mutat Res Genet Toxicol Environ Mutagen*, 852. doi: https://doi.org/10.1016/j.mrgentox.2020.503164

Carvalho, S.C.S, Cury, N.M., Broto, D.B., De Araújo, L.F., **Rosa, R.C.A.**, et al. (2020). Germline variants in DNA repair genes associated with hereditary breast and ovarian cancer

syndrome: analysis of a 21 gene panel in the Brazilian population. *BMC Medical Genomics*, 13(21). doiI: https://doi.org/10.1186/s12920-019-0652-y

Oral and poster presentation

Oral presentations

Rosa, R.C.A., Yurchenko, A.A., Nikolaev, S. et al. (2022). Uncovering the role of WDHD1 in the DNA mismatch repair pathway. *8th Workshop of the Graduate Program in Genetics of the University of São Paulo.* Ribeirão Preto, SP, Brazil.

Rosa, R.C.A., Chahud, F., Silva, A.R., et al. (2019). Molecular diagnosis of Lynch syndrome in DNA-mismatch repair-deficient endometrial cancer. *31st Brazilian Congress of Medical Genetics*. Salvador, BA, Brazil.

Poster presentations

Rosa, R.C.A., Kannouche, P. (2022). Uncovering the role of WDHD1 in the Mismatch repair pathway. *14th Edition of the French 3R meeting: "Replication, Recombination, Repair".* Giens, France.

Rosa, R.C.A., Chahud, F., Silva, A.R., et al. (2019). Screening of germline mutations in genes related to DNA repair among Lynch-Like patients diagnosed with endometrial cancer. *65th Brazilian Congress of Genetics*. Águas de Lindoia, SP, Brazil.

Rosa, R.C.A., Chahud, F., Silva, A.R., et al. (2018). Molecular characterization of endometrial tumors for the proficiency of the DNA-mismatch repair system: a universal screening for Lynch Syndrome. *6th FARM DNA Congress.* São Paulo, SP, Brazil.

Rosa, R.C.A., Chahud, F., Silva, et al. (2018). Molecular characterization of endometrial tumors for the proficiency of the DNA-mismatch repair system. *Frontiers in Molecular Oncology Congress.* São Paulo, SP, Brazil.

Scholarships

2020: Eiffel Scholarship Program of Excellence **-** French Ministry for Europe and Foreign Affairs.

2020: Institutional Program for Internationalization (PrInt) – CAPES.

2018: Scholarship from The Brazilian National Council for Scientific and Technological Development (CNPq) for Ph.D. studies.

APPENDIX

Case report of germline and somatic POLE-exo mutations in an EC case

Genetics and Molecular Biology 43, 4, e20200100 (2020)
Copyright © 2020, Sociedade Brasileira de Genética.
DOI: https://doi.org/10.1590/1678-4685-GMB-2020-0100

Short Communication
Human and Medical Genetics

First description of ultramutated endometrial cancer caused by germline loss-of-function and somatic exonuclease domain mutations in *POLE* gene

Reginaldo Cruz Alves Rosa[1*], Andrey A. Yurchenko[2*], Fernando Chahud[3], Alfredo Ribeiro-Silva[3], Mariângela Ottoboni Brunaldi[3], Wilson Araújo Silva Jr[1], Patricia L. Kannouche[4], Sergey Nikolaev[2], Victor Evangelista de Faria Ferraz[1,5]

[1]*Universidade de São Paulo, Faculdade de Medicina de Ribeirão Preto, Departamento de Genética, Ribeirão Preto, SP, Brazil.*
[2]*Université Paris Saclay, Inserm U981, Gustave Roussy Cancer Campus, Villejuif, France.*
[3]*Universidade de São Paulo, Faculdade de Medicina de Ribeirão Preto, Departamento de Patologia e Medicina Legal, Ribeirão Preto, SP, Brazil.*
[4]*Paris-Sud University, CNRS-UMR 8200, Equipe labellisée Ligue Contre le Cancer, Gustave Roussy Cancer Campus, Villejuif, France.*
[5]*Universidade de São Paulo, Centro de Genômica Médica, Hospital das Clínicas, Faculdade de Medicina de Ribeirão Preto, Ribeirão Preto, SP, Brazil.*

Abstract

Endometrial cancer (EC) harboring heterozygous *POLE* proofreading inactivating mutations (*POLE*-exo*) is associated with an increased number of somatic mutations that result in a distinctive anti-tumor immune response. However, the consequences of such *POLE* mutations in the context of the missing wild-type allele have not yet been described in endometrial tumors. A 72-year-old woman harboring a germline monoallelic frameshift mutation (p.Pro269fsTer26) in *POLE* was diagnosed with an EC having a somatic heterozygous mutation in the exonuclease domain of *POLE* (S459F). Targeted gene sequencing revealed an ultramutated phenotype (381 mutations/Mb) in the tumor and a 2-fold excess of mutations on the DNA leading strand. Additionally, we observed a mutational signature similar to the COSMIC signature 10, a higher mutation rate in this tumor than in endometrial tumors with heterozygous *POLE*-exo*, and an increased number of T lymphocytes. This is the first report of an ultramutated EC harboring a somatic *POLE*-exo* mutation in association with a germline loss-of-function mutation in this gene. The absence of a wild type *POLE* allele led to a particularly high mutational burden.

Keywords: Endometrial cancer, *POLE* exonuclease mutation, Targeted sequencing, TMB, Ultramutated phenotype.

Received: April 9, 2020; Accepted: July 8, 2020.

Endometrial cancer (EC) is a heterogeneous malignancy characterized by several different histologic subtypes with endometrioid carcinoma being the most common (McConechy *et al.*, 2016). Recently, there have been significant advances in defining the molecular alterations that contribute to tumorigenesis in EC. The Cancer Genome Atlas Research Network (TCGA) divides EC into four categories based on recurrent molecular features: an ultramutated phenotype caused by *POLE* mutations, a hypermutator phenotype caused by the DNA mismatch repair deficiency (MMRD) leading to microsatellite instability (MSI), a copy number low phenotype, and a copy number high phenotype (Levine *et al.*, 2013).

Send correspondence to Victor Evangelista de Faria Ferraz. Universidade de São Paulo, Faculdade de Medicina de Ribeirão Preto, Departamento de Genética, Av. Bandeirantes, 3900, Vila Monte Alegre, 14049-900, Ribeirão Preto, SP, Brazil. E-mail: vferraz@usp.br.
* These authors have contributed equally to this work.

The *POLE* gene encodes the catalytic subunit of DNA polymerase ε (Pol ε), which replicates the leading strand during DNA replication (Burgers *et al.*, 2017). In addition to DNA-binding and polymerase domains, Pol ε has proofreading activity through its exonuclease domain. This capacity is essential for the maintenance of replication fidelity, and this proofreading function may act, not only on newly misincorporated nucleotides, but also on mismatches produced by non-proofreading polymerases (Palles *et al.*, 2013). Up to 12% of all endometrial carcinomas harbor *POLE* mutations that tend to cluster in the exonuclease domain (*POLE*-exo*), especially in the conserved residues 268 to 471 (Billingsley *et al.*, 2016; Bellone *et al.*, 2017; Barbari *et al.*, 2018). Tumors harboring such mutations are associated with an ultramutated phenotype, increased neoantigen load, increased tumor infiltrating lymphocytes, and increased potential for responding to immunotherapy (Imboden *et al.*, 2019).

2214 **First description of ultramutated endometrial cancer caused by germline loss-of-**
2215 **function and somatic exonuclease domain mutations in *POLE* gene**
2216
2217
2218 Reginaldo Cruz Alves Rosa[1†], Andrey A. Yurchenko[2†], Fernando Chahud[3], Alfredo Ribeiro-
2219 Silva[3], Mariângela Ottoboni Brunaldi[3], Wilson Araújo Silva Jr[1], Patricia L. Kannouche[4],
2220 Sergey Nikolaev[2], Victor Evangelista de Faria Ferraz[1,5*]
2221
2222
2223 [1] Department of Genetics, Ribeirão Preto Medical School, University of São Paulo, Ribeirão
2224 Preto, Brazil
2225 [2] Inserm U981, Gustave Roussy Cancer Campus, Université Paris Saclay, Villejuif, France
2226 [3] Department of Pathology and Legal Medicine, Ribeirão Preto Medical School, University of
2227 São Paulo, Ribeirão Preto, Brazil
2228 [4] Paris-Sud University, CNRS-UMR 8200, Equipe labellisée Ligue Contre le Cancer, Gustave
2229 Roussy Cancer Campus, Villejuif, France.
2230 [5] Center for Medical Genomics at Clinical Hospital of the Ribeirão Preto Medical School,
2231 University of São Paulo, Brazil
2232
2233 † These authors have contributed equally to this work.
2234
2235 **Running title:** *POLE* variants in endometrial cancer
2236
2237 *** Correspondence:**
2238 Dr. Victor Evangelista de Faria Ferraz
2239 vferraz@usp.br
2240 ORCID: http://orcid.org/0000-0003-0337-4588

2241 **Abstract**

2242

2243 Endometrial cancer (EC) harboring heterozygous *POLE* proofreading inactivating mutations
2244 (*POLE*-exo*) is associated with an increased number of somatic mutations that results in a
2245 distinctive anti-tumor immune response. However, the consequences of such *POLE* mutations
2246 in the context of the missing wild-type allele have not yet been described in endometrial tumors.
2247 A 72-year-old woman harboring a germline monoallelic frameshift mutation (p.Pro269fsTer26)
2248 in *POLE* was diagnosed with an EC having a somatic heterozygous mutation in the exonuclease
2249 domain of *POLE* (S459F). Targeted gene sequencing revealed an ultramutated phenotype (381
2250 mutations/Mb) in the tumor and a 2-fold excess of mutations on the DNA leading strand.
2251 Additionally, we observed a mutational signature similar to the COSMIC signature 10, a higher
2252 mutation rate in this tumor than in endometrial tumors with heterozygous *POLE*-exo*, and an
2253 increased number of T lymphocytes. This is the first report of an ultramutated EC harboring a
2254 somatic *POLE*-exo* mutation in association with a germline loss-of-function mutation in this
2255 gene. The absence of a wild type *POLE* allele led to a particularly high mutational burden.

2256

2257 **Keywords:** Endometrial cancer, *POLE* exonuclease mutation, Targeted sequencing, TMB,
2258 Ultramutated phenotype

Endometrial cancer (EC) is a heterogeneous malignancy characterized by several different histologic subtypes with endometrioid carcinoma being the most common (McConechy *et al*., 2016). Recently, there have been significant advances in defining the molecular alterations that contribute to tumorigenesis in EC. The Cancer Genome Atlas Research Network (TCGA) divides EC into four categories based on recurrent molecular features: an ultramutated phenotype caused by *POLE* mutations, a hypermutator phenotype caused by the DNA mismatch repair deficiency (MMRD) leading to microsatellite instability (MSI), a copy number low phenotype, and a copy number high phenotype (The Cancer Genome Atlas Network *et al*., 2013).

The *POLE* gene encodes the catalytic subunit of DNA polymerase ε (Pol ε), which replicates the leading strand during DNA replication (Burgers *et al*., 2017). In addition to DNA-binding and polymerase domains, Pol ε has proofreading activity through its exonuclease domain. This capacity is essential for the maintenance of replication fidelity, and this proofreading function may act, not only on newly misincorporated nucleotides but also on mismatches produced by non-proofreading polymerases (Palles *et al*., 2013). Up to 12% of all endometrial carcinomas harbor *POLE* mutations that tend to cluster in the exonuclease domain (*POLE*-exo*), especially in the conserved residues 268 to 471 (Billingsley *et al*., 2016, Bellone *et al*., 2017, Barbari *et al*., 2018). Tumors harboring such mutations are associated with an ultramutated phenotype, increased neoantigen load, increased tumor infiltrating lymphocytes, and increased potential for responding to immunotherapy (Imboden *et al*., 2019).

Germline mutations in the exonuclease domain *POLE* are infrequent; most *POLE*-exo* mutations are somatic and occur in sporadic tumors almost exclusively in a heterozygous state because of their dominant nature (Wong *et al*., 2016, Barbari *et al*., 2017). Additionally, there is no associated *POLE* inactivation by somatic loss of heterozygosity (LOH) when tumors occur in carriers of germline *POLE* mutations (Palles *et al*., 2013). However, a minority of tumors with *POLE*-exo* show LOH or other inactivating mutations that could act as 'second hits' (Heitzer *et al*., 2014). Curiously, loss or inactivation of the second allele has been reported in a few colorectal tumors with mutations disturbing Pol ε proofreading activity and at least one example illustrates that this mutation may have phenotypic consequences for disease presentation (The Cancer Genome Atlas Network, 2012). However, similar findings have not been reported for endometrial tumors (Shinbrot *et al*., 2014).

Here, we report a 72-year-old woman diagnosed with a FIGO Grade 1 and FIGO Stage 1B endometrial endometrioid adenocarcinoma at 63 years old. A total hysterectomy and salpingo-oophorectomy were performed. The patient reported no familial history of cancer.

2293 Immunohistochemistry (IHC) of the MMR proteins and MSI analysis were performed. The
2294 tumor had an intact expression of MLH1, MSH2, MSH6, and PMS2 proteins based on
2295 immunohistochemical analysis and was classified as MSI-low based on the MSI assay. A
2296 germline and somatic mutation screening were performed, and the mutational profile and its
2297 immunologic characterization of the endometrial tumor were accessed (for details of material
2298 and methods, see **Mat-Met S1** in Supplementary Material). The study was approved by the
2299 Scientific and Research Committee of the Clinics Hospital of the Ribeirão Preto Medical
2300 School (1.578.206). Informed written consent was obtained from the patient.

2301 For germline mutation screening, a targeted sequencing assay of the coding, canonical
2302 splice sites, and both 5' and 3' untranslated regions of 63 genes (Table S1), including Lynch
2303 syndrome-associated genes and *POLE,* was performed in DNA extracted from peripheral
2304 blood. Single nucleotide variants (SNVs) and Copy number variation (CNV) were evaluated.
2305 Only the germline frameshift mutation NM_006231:c.806delC (p.Pro269fsTer26) in *POLE*
2306 was identified (**Figure 1A**), with a variant allele frequency (VAF) of 0.50 (total coverage = 729
2307 reads), as expected for a heterozygous germline variant.

2308 Since this germline mutation could not explain the tumor MSI-low phenotype present
2309 in the EC, a further mutational search was performed using the tumor DNA. For somatic
2310 analysis, targeted sequencing using the same gene panel described for germline analysis was
2311 performed on genomic DNA extracted from a representative tumor area (at least 70% of tumor
2312 cells) from Formalin-Fixed Paraffin-Embedded (FFPE) blocks. Both somatic SNVs and CNVs
2313 were called on the matching tumor-blood DNA samples. As expected, the germline frameshift
2314 mutation in *POLE* was also detected in tumor sequencing, with a VAF = 0.51 (total coverage
2315 = 242 reads), supporting its germline origin. Additionally, a somatic mutation in the
2316 exonuclease domain of *POLE*, S459F (NM_006231:c.1376C>T, p.Ser459Phe), was observed
2317 with VAF = 0.298 (**Figure 1B**). As long as this mutation is heterozygous, it is expected to be
2318 present in ~60% of cells in the tumor sample, these estimates are based on a tumor purity of
2319 80% from the histological examination. We did not find any pathogenic mutation neither in the
2320 MMR genes (*MLH1*, *MSH2*, *MSH6*, and *PMS2*) nor in the exonuclease domain of *POLD1*.

2321 The tumor mutational profile was investigated to determine whether the genomic
2322 alterations were consistent with a *POLE* ultramutator phenotype. A total of 190 mutations were
2323 identified in the sequenced region of the 63 gene panel (0.49 Mb). Considering only the coding
2324 region, 0.257 Mb distributed along 937 exons of 63 cancer-related genes, a total of 95 mutations
2325 were identified, resulting in a mutation rate of 381 mutations/Mb. A total of 65 nonsynonymous

mutations were identified in the targeted exons, resulting in an estimated tumor mutation burden (TMB) of 253 nonsynonymous mutations/Mb.

The trinucleotide context of mutations was investigated, and a mutational signature analysis was performed using the database of the known mutational signatures in human cancers from Alexandrov *et al*. (2013). Given the high number of somatic mutations identified, we had sufficient data to derive a mutational signature that was closely related to COSMIC signature 10 (Cosine similarity = 0.97, **Figure 2A**). These findings are indicative of mutations in synthesis associated with errors in the proofreading activity of Pol ε. Most nucleotide substitutions detected in the tumor sample were represented by C>A, C>T, and T>G, with a relative contribution to the total amount of substitution mutations of 0.43, 0.33, and 0.18, respectively (**Figure 2B**).

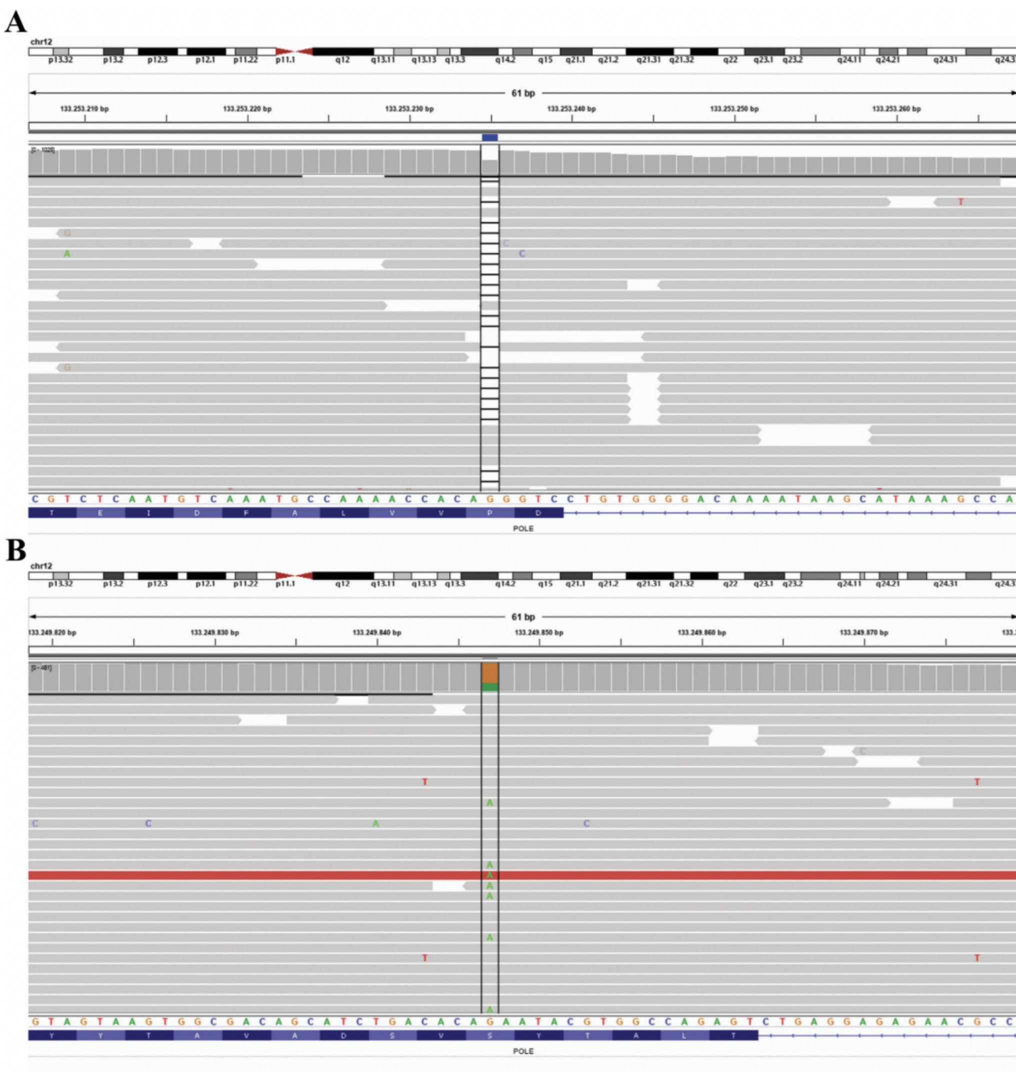
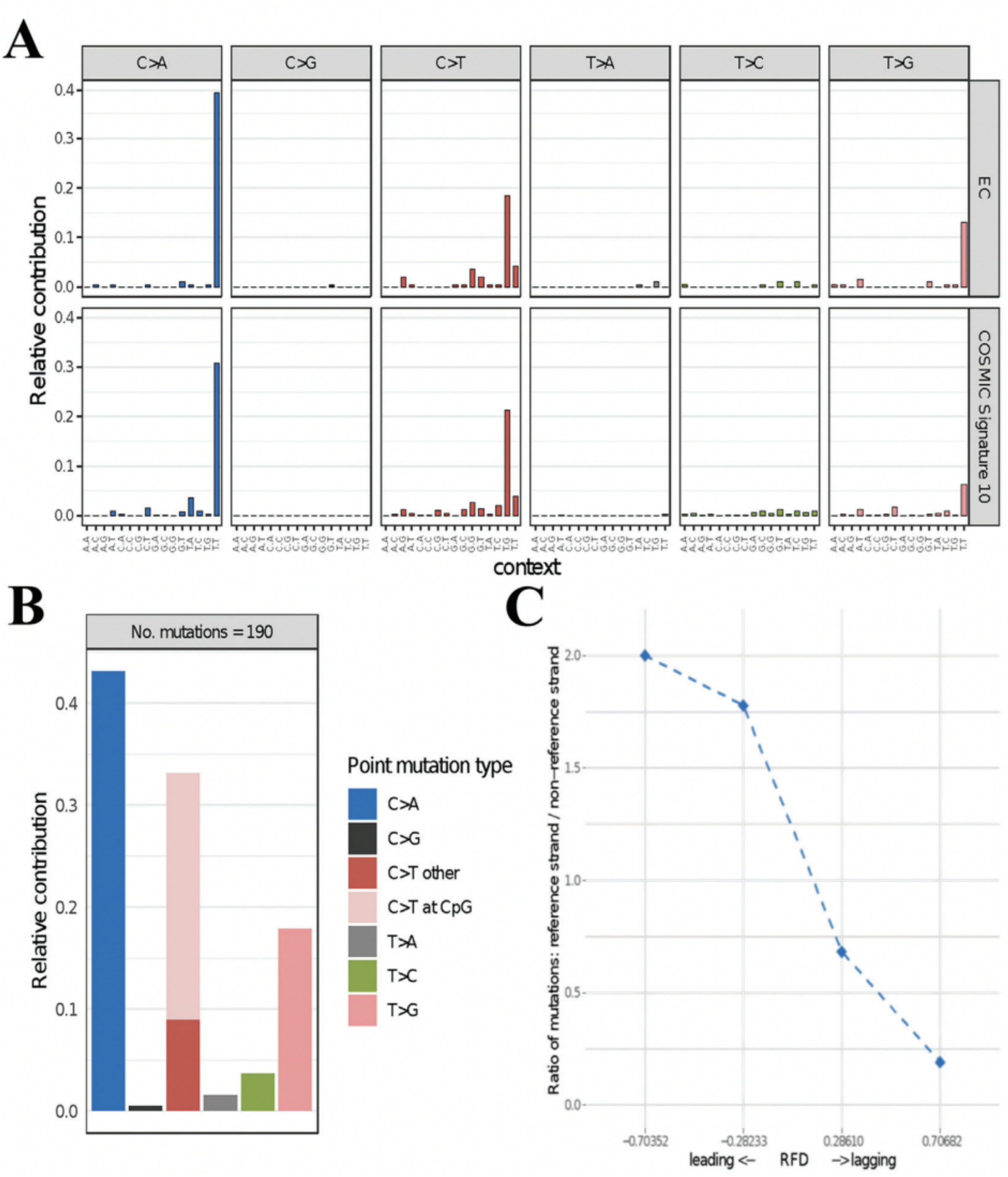


Figure 1 - Integrative Genomics Viewer snapshot of *POLE* mutations with reference POLE nucleotide and amino acid sequences. (A) germline frameshift c.806delC and (B) somatic c.1376C>T (S459F) exonuclease mutation.

2341 In addition to mutational signature analysis, we calculated the mutation strand bias
2342 asymmetry between the leading and lagging DNA strands. There was a 2-fold excess of
2343 mutations on the leading strand of DNA in comparison with the lagging strand (**Figure 2C**).
2344 These molecular findings highlight the strong effect of defects in Pol ε proofreading activity in
2345 this reported EC.



2346

2347 **Figure 2 - Mutational profile based on targeted sequencing data of a 63 cancer-related gene panel.** (A)
2348 mutational signature extracted from the endometrial carcinoma in comparison to the COSMIC signature 10. (B)
2349 relative contribution of each point mutation type to the total amount of somatic mutations. (C) Strand bias analysis
2350 showing a predominance of mutations on the leading strand. EC, endometrial cancer.

2351 In order to compare the mutation rate and total TMB between the studied tumor and
2352 endometrial cancers with heterozygous *POLE*-exo* mutations, we downloaded 25 exomes of
2353 endometrial carcinoma from ICGC portal with *POLE*-exo* somatic mutations and absence of
2354 MSI (Zhang *et al*., 2011). To minimize the influence of different capture kits we estimated
2355 TMB only in the coding regions of our gene panel. The mutation rate observed in the
2356 endometrial carcinoma reported here (381 mutations/Mb) is more than 2-fold higher than the
2357 average rate observed in 25 endometrial carcinomas harboring heterozygous *POLE*-exo*
2358 mutations (153 mutations/Mb, ranging from 47 to 276). One out of 25 endometrial carcinomas
2359 harbored the heterozygous *POLE*-exo* S459F and presented a rate of 167 mutations/Mb (**Table**
2360 **1)**. These data confirm that EC harboring a LoF genetic event in *POLE* in association with a
2361 heterozygous *POLE*-exo* mutation confers an augmented mutator phenotype in comparison
2362 with EC with single *POLE*-exo* alterations.

2363 For evaluation of tumor-associated lymphocytes, the mean number of CD3+, CD4+,
2364 and CD8+ of intraepithelial T lymphocytes, i.e., T lymphocytes located within the tumor
2365 epithelium was calculated. IHC staining for T lymphocyte markers revealed a predominance of
2366 CD8+ lymphocytes in the intra-tumoral area in comparison with CD4+ T cells, with mean
2367 numbers of 29.9 CD8+, and 10.9 CD4+ T-cells. A mild (1+) presence of CD3+, CD4+, and
2368 CD8+ lymphocytes was observed in the peri-tumoral region (**Figure 3**).

2369 This is the first case of an endometrial carcinoma harboring a somatic *POLE*
2370 exonuclease mutation related to an ultra-mutator phenotype acting as a 'second hit' in
2371 association with a germline truncating mutation of the gene. Germline heterozygous missense
2372 mutations affecting the *POLE* exonuclease domain are associated with a syndrome called
2373 Polymerase Proofreading-Associated Polyposis (PPAP) that increases the risk for the
2374 development of multiple colorectal adenomas and colorectal cancer (Briggs *et al*., 2013).
2375 However, a diagnosis of PPAP is not consistent with findings in the patient presented in this
2376 case report since she carries a germline frameshift mutation at the beginning of the *POLE*
2377 exonuclease domain that creates a premature termination codon. Truncating mutations in *POLE*
2378 gene are unlikely to lead to the PPAP phenotype since a successful DNA synthesis must occur
2379 before the proofreading activity of Pol ε (Heitzer *et al*., 2014). However, the co-occurrence of
2380 a germline truncating mutation with a somatic ultra-mutator phenotype-associated variant in
2381 *POLE* suggests a complete loss of Pol ε proofreading activity in the endometrial tumor. Thus,
2382 by itself the germline frameshift mutation does not confer a genetic predisposition to EC and
2383 cannot lead to a mutator phenotype in the tumor, but might contribute to increasing the

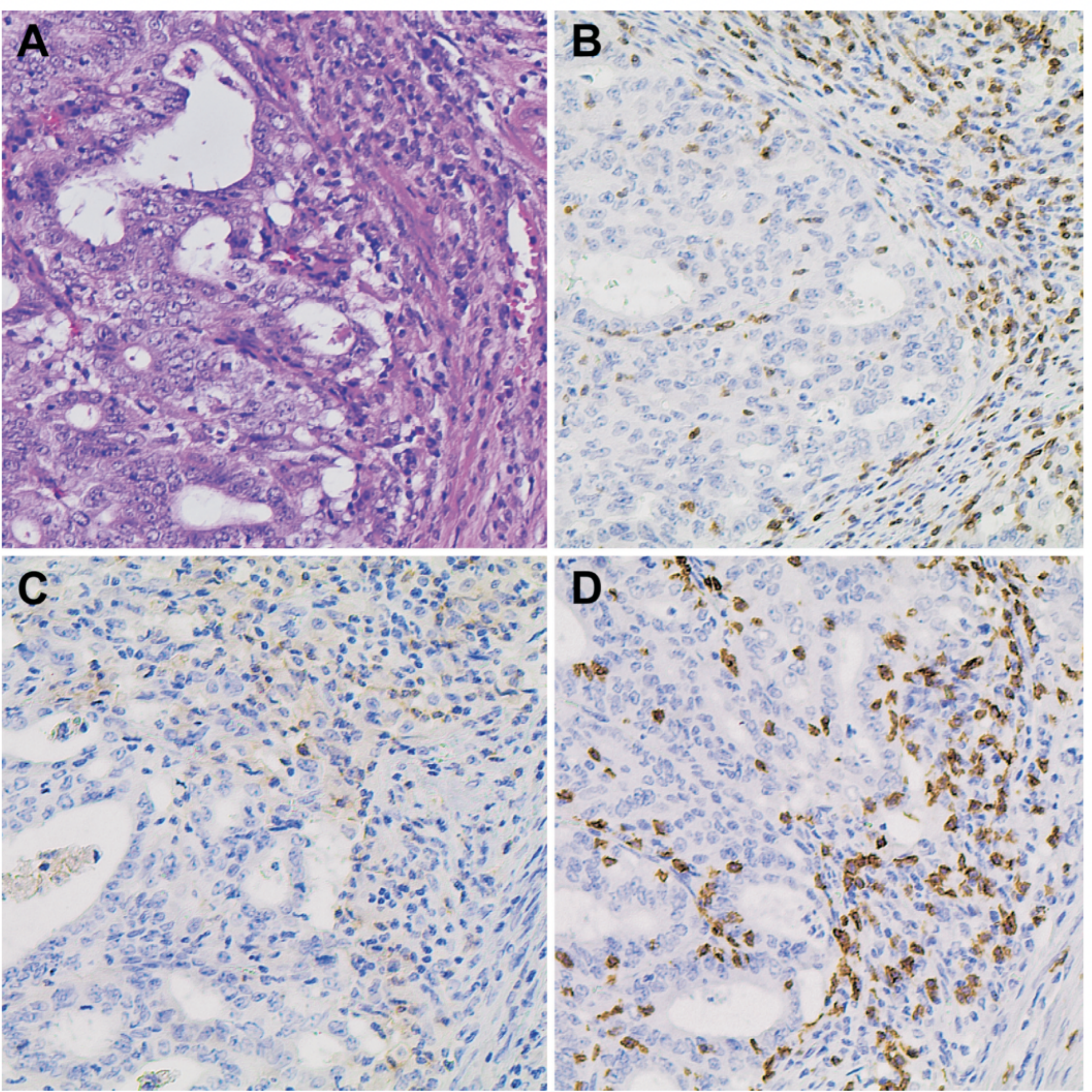
2384 mutational load because only proofreading-deficient Pol-ε will replicate DNA in these tumor
2385 cells.
2386

Sample	*POLE*-exo* mutation	Total amount of mutations [a]	Muta-tions/Mb	Nonsynony-mous mutations	TMB [b]
Report	S459F	95	381	65	253
SA485042	P286R	71	276	53	206
SA475378	P286R	70	272	47	183
SA552345	P286R	69	268	44	171
SA472709	P286R	59	230	42	163
SA466958	P286R	61	237	41	160
SA470974	P286R	64	249	41	160
SA462048	P286R	63	245	39	152
SA467568	P286R	47	183	35	136
SA483959	P286R	48	187	32	125
SA541518	P286R	39	152	29	113
SA472897	S459F	43	167	29	113
SA20267	P286R	38	148	27	105
SA561528	P286R	49	191	24	93
SA469202	P286R	31	121	20	78
SA473549	P286R	26	101	20	78
SA526095	P286R	28	109	18	70
SA541610	P286R	24	93	18	70
SA526120	P286R	26	101	15	58
SA92158	P286R	29	113	15	58
SA482148	P286R	24	93	13	51
SA479614	P286R	19	74	12	47
SA467039	V411L	16	62	10	39
SA476079	V411L	15	58	9	35
SA474561	P286R	12	47	7	27
SA519177	P286R	12	47	6	23

2387

2388 **Table 1 - Mutational profile of endometrial carcinomas harboring heterozygous POLE-exo* mutations in**
2389 **comparison with endometrial cancer reported.** [a] all mutations were identified along the 0.257 Mb of the 63
2390 gene panel, including synonymous and nonsynonymous mutations. [b] expressed as the number of nonsynonymous
2391 mutations/Mb. TMB, tumor mutational burden. Report: endometrial cancer case characterized in the present study.

2392 Some *POLE*-exo* mutations have been described to be associated with an ultra-mutator
2393 phenotype, with varying levels of mutation. Previous functional studies have demonstrated the
2394 exonuclease deficiency effect of the *POLE* S459F mutation *in vitro* (Shinbrot *et al*., 2014) as
2395 well as the moderate mutator effect in yeast (Barbari *et al*., 2018). The EC reported here was
2396 MSI-low. Co-occurrence of MSI and *POLE*-exo* mutations, usually with the P286R mutation,
2397 in endometrial tumors has already been reported (Haradhvala *et al*., 2018). However, all tumors
2398 described to date that harbor the S459F mutation in *POLE* were found to be microsatellite stable
2399 (Shinbrot *et al*., 2014, Andrianova *et al*., 2017, Barbari *et al*., 2018). Our study is the first report
2400 of a tumor harboring the S459F mutation this is associated with an MSI-low phenotype.
2401



2402

2403 **Figure 3 - Immunohistochemical staining for T lymphocyte markers on the peri and intra-tumoral areas of**
2404 **the EC (original magnification x200)**. (A) H&E (hematoxylin-eosin). Brown nuclear staining is indicative of
2405 positive expression of (B) CD3, (C) CD4, and (D) CD8 markers.
2406

Somatic mutations found in cancer genomes are the consequence of the intrinsic infidelity of the DNA replication machinery, exogenous or endogenous mutagen exposures, enzymatic modification of DNA, or defective DNA repair and other processes. Different mutational processes often generate variation in the combinations of mutation types, termed mutational signatures (Alexandrov *et al*., 2013). More than thirty mutational signatures have already been identified across 40 different types of human cancer (Forbes *et al*., 2017). We identified a mutational signature that closely resembles the COSMIC signature 10, which is known to be associated with *POLE*-exo* mutations (Alexandrov *et al*., 2013). The *POLE* mutational signature is characterized by a 100-fold increase in C>A transversions in the context TCT and a 30-fold increase in C>T transitions in the context TCG (Rayner *et al*., 2016). This mutational pattern results in a strong bias for particular amino acid changes, with an overrepresentation of serine to tyrosine or leucine, and arginine to isoleucine or glutamine substitutions, and a substantial increase in glutamic acid to stop codon mutations (Rayner *et al*., 2016). Although mutational signatures are preferably determined by genomic analysis, such as whole genome sequencing (WGS) and whole exome sequencing (WES), we were able to identify a mutational signature related to *POLE*-exo* mutations through targeted sequencing of the coding and regulatory regions of only 63 genes. These findings support that mutational signatures can be extracted from sequencing data derived from a small gene panel in tumors that are highly mutated (Hoeck *et al*., 2019). In addition, we observed a strong strand bias effect with mutations occurring predominantly in the leading strand in comparison with the lagging strand. This phenomenon, in addition to the mutational signature close to COSMIC signature 10, highlights the major effect of *POLE* proofreading inactivation in the EC reported here.

TMB is a quantitative measure of the total number of somatic nonsynonymous mutations per coding area of a tumor genome and is associated with the emergence of neoantigens that trigger anti-tumor immunity (Allgäuer *et al*., 2018, Meléndez *et al*, 2018). We identified a total of 65 nonsynonymous mutations along 0.257 Mb coding regions of the sequenced gene panel, resulting in an estimated TMB of 253 nonsynonymous mutations/Mb. Although a wider genomic analysis is required to achieve the precise TMB (Büttner *et al*., 2019), the absolute amount of somatic nonsynonymous mutations (65 mutations/0.257 Mbp) observed in the EC reported here is superior to the threshold of 20 mutations/Mb commonly used to classify a tumor with high TMB and as an immunotherapy responder (Allgäuer *et al*., 2018, Endris *et al*., 2019). The absolute amount of nonsynonymous mutations as well as the total number of mutations (95 mutations/0.257) identified in our report, is higher than those identified in EC harboring heterozygous *POLE*-exo* mutations. We used the GATK pipeline,

2441 which has high sensitivity and specificity for somatic mutation calling and checked the
2442 occurrence of FFPE-derived artifacts in the sequencing data. Thus, the higher mutational load
2443 identified in the endometrial tumor in comparison with the ICGC/TCGA Pole-exo* tumors is
2444 not supposed to be led by interstudy differences.

2445 The occurrence of two proofreading-inactivating events in *POLE* is extremely rare,
2446 suggesting that *POLE* may not act as a classical tumor suppressor gene (Heitzer *et al*., 2014).
2447 There is a single case of colorectal cancer (CRC) in The Cancer Genome Atlas (TCGA) project
2448 carrying the *POLE* S459F mutation and a nonsense mutation at codon 150 of the *POLE* gene,
2449 which was thought to inactivate the second allele (The Cancer Genome Atlas Network, 2012).
2450 This CRC presented a higher number of somatic mutations (376 mutations/Mb) in comparison
2451 with another TCGA-CRC harboring only the S459F mutation in heterozygosity (81
2452 mutations/Mb) (Shinbrot *et al*., 2014). Both mutations present in the TCGA-CRC with two hits
2453 in *POLE* are somatic. Our findings are novel since we report an endometrial carcinoma
2454 harboring one germline *POLE* LoF mutation and one somatic *POLE*-exo* mutation.

2455 Molecular classification of human cancer represents an important step toward the goal
2456 of precision medicine and helps to identify patients who would benefit from targeted
2457 immunotherapy (Liu *et al*., 2019). We observed the occurrence of a greater number of CD8+ T
2458 lymphocytes in comparison with CD4+ T-cells in the peri and intra-tumoral area in our EC
2459 case. *POLE*-exo* mutations have been associated with increased tumor infiltrating
2460 lymphocytes, especially CD8+ (Howitt *et al*., 2015, Bourdais *et al*., 2017).

2461 The characterization of the mutational pattern, as well as the lymphocyte profile
2462 revealed an accentuated Pol ε proof-reading failure in an EC harboring a germline and a
2463 somatic mutation at the *POLE* exonuclease domain. These findings suggest that the mutations
2464 are in trans, *i.e*. located in different DNA strands. The frameshift mutation affects the beginning
2465 of the exonuclease domain of *POLE* and is expected to result in a truncated, immature, or non-
2466 functional protein. If the *POLE* S459F mutation were located at the same strand as the germline
2467 frameshift, the ultramutator effect would likely be silenced by the frameshift. However, as a
2468 limitation of our study, we could not experimentally prove that the frameshift and missense
2469 *POLE*-exo* mutations are in trans and neither that the frameshift indeed led to the silencing of
2470 one *POLE* allele due to the high fragmentation of DNA and RNA derived from FFPE slides.
2471 Additionally, although we have strong evidence supporting that our EC case has a higher
2472 mutational load identified in comparison with tumors harboring heterozygous *POLE*-exo*
2473 mutations, we are aware that the number of mutations identified might have been affected by
2474 interstudy differences in sample preservation methods, library protocols, and bioinformatic

pipelines. Also, although we observed a higher mutational load in the EC with two genetic events at the proof-reading domains of *POLE* in comparison with EC harboring only a heterozygous *POLE*-exo* mutation, we would need to have more tumors with similar findings in order to make statistically significant conclusions about the mutational burden of these tumors relative to the cancers with heterozygous *POLE*-exo* mutations.

In conclusion, our EC case exhibits molecular and histopathological features typically linked to *POLE* exonuclease mutated tumors. The comparison with other tumors with *POLE*-exo* mutations suggests that the absence of the wild-type *POLE* allele renders particularly higher TMB in such tumors. Consequently, detection of a combination of *POLE*-exo* and LoF *POLE* mutations could be considered a prognostic or therapeutic marker.

Acknowledgments

The authors would like to thank the patient for her kind collaboration and contribution to this publication. Also, we thank Dr. Jeremy Squire for his technical support during the manuscript review. The costs of the design of the study and molecular analyses were supported by FAPESP (grant 2017/04283-8); CNPq (grant 423007/2016-5). Analysis and interpretation of data were supported by Foundation ARC 2017, Foundation Gustave Roussy and Swiss Cancer League (grant KFC-3985-08-2016), and INCa (INCa-PLBio2016-144 and INCa-DGOS-Inserm 12551).

Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicial to the impartiality of the reported research.

Authors Contributions

RR and AY performed the acquisition of data and wrote the draft. VF and WJ designed the study and evaluated the patient's personal and familial history of cancer. AY and SN performed the bioinformatics analysis. PK helped in the data interpretation of experiments. FC, AS and MB provided the clinicopathological data. All authors reviewed the manuscript for submission.

2509 **References**

2510

2511 Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, Bignell GR,
2512 Bolli N, Borg A, Børresen-Dale AL *et al*. (2013) Signatures of mutational processes in
2513 human cancer. Nature 500:415-421.
2514 Allgäuer M, Budczies J, Christopoulos P, Endris V, Lier A, Rempel E, Volckmar AL, Kirchner
2515 M, von Winterfeld M, Leichsenring J *et al*. (2018) Implementing tumor mutational
2516 burden (TMB) analysis in routine diagnostics-a primer for molecular pathologists and
2517 clinicians. Transl Lung Cancer Res 7:703–715.
2518 Andrianova MA, Chetan GK, Sibin MK, Mckee T, Merkler D, Narasinga RK, Ribaux P, Blouin
2519 JL, Makrythanasis P, Seplyarskiy VB *et al*. (2017) Germline PMS2 and somatic POLE
2520 exonuclease mutations cause hypermutability of the leading DNA strand in biallelic
2521 mismatch repair deficiency syndrome brain tumors. J Pathol:243, 331-341.
2522 Barbari SR and Shcherbakova PV (2017) Replicative DNA polymerase defects in human
2523 cancers: Consequences, mechanisms, and implications for therapy.DNA Repair (Amst)
2524 56:16-25.
2525 Barbari SR, Kane DP, Moore EA and Shcherbakova PV (2018) Functional Analysis of Cancer-
2526 Associated DNA Polymerase ε Variants in Saccharomyces cerevisiae. G3 (Bethesda)
2527 8:1019-1029.
2528 Bellone S, Bignotti E, Lonardi S, Ferrari F, Centritto F, Masserdotti A, Pettinella F, Black J,
2529 Menderes G, Altwerger G *et al*. (2017) Polymerase ε (POLE) ultra-mutation in uterine
2530 tumors correlates with T lymphocyte infiltration and increased resistance to platinum-
2531 based chemotherapy in vitro. Gynecol Oncol 144:146–152.
2532 Billingsley CC, Cohn DE, Mutch DG, Hade EM and Goodfellow PJ (2016) Prognostic
2533 significance of POLE exonuclease domain mutations in high grade endometrioid
2534 endometrial cancer on survival and recurrence: a sub-analysis. Int J Gynecol Cancer
2535 2:933–938.
2536 Bourdais R, Rousseau B, Pujals A, Boussion H, Joly C, Guillemin A, Baumgaertner I, Neuzillet
2537 C and Tournigand C (2017) Polymerase proofreading domain mutations: New
2538 opportunities for immunotherapy in hypermutated colorectal cancer beyond MMR
2539 deficiency. Crit Rev Oncol Hematol 113:242-248.
2540 Briggs S and Tomlinson S (2013) Germline and somatic polymerase ε and δ mutations define
2541 a new class of hypermutated colorectal and endometrial cancers. J Pathol 230:148–153.

2542 Burgers PM and Kunkel TA (2017) Eukaryotic DNA replication fork. Annu Rev Biochem.
2543 20:417-438.
2544 Büttner R, Longshore JW, López-Ríos F, Merkelbach-Bruse S, Normanno N, Rouleau E and
2545 Penault-Llorca F (2019) Implementing TMB measurement in clinical practice:
2546 considerations on assay requirements. ESMO Open 4:e000442. doi: 10.1136/esmoopen-
2547 2018-000442
2548 Endris V, Buchhalter I, Allgäuer M, Rempel E, Lier A, Volckmar AL, Kirchner M, von
2549 Winterfeld M, Leichsenring J, Neumann O *et al.* (2019) Measurement of tumor
2550 mutational burden (TMB) in routine molecular diagnostics: in silico and real-life analysis
2551 of three larger gene panels. Int J Cancer 144, 2303-2312.
2552 Forbes SA, Beare D, Boutselakis H, Bamford S, Bindal N, Tate J, Cole CG, Ward S, Dawson
2553 E, Ponting L *et al*. 398 (2017) COSMIC: somatic cancer genetics at high-resolution.
2554 Nucleic Acids Res 45:777–399 783.
2555 Haradhvala NJ, Kim J Maruvka YE, Polak P, Rosebrock D, Livitz D, Hess JM, Leshchiner I,
2556 Kamburov A, Mouw KW *et al*. (2018) Distinct mutational signatures characterize
2557 concurrent loss of polymerase proofreading and mismatch repair. Nat Commun 9:1746.
2558 Heitzer E and Tomlinson I (2014) Replicative DNA polymerase mutations in cancer. Curr Opin
2559 Genet Dev 24:107-113.
2560 Hoeck AV, Tjoonk NH, van Boxtel R and Cuppen E. (2019) Portrait of a cancer: mutational
2561 signature analyses for cancer diagnostics. BMC Cancer 19:457-470.
2562 Howitt BE, Shukla SA, Sholl LM, Ritterhouse LL, Watkins JC, Rodig S, Stover E, Strickland
2563 KC, D'Andrea AD, Wu CJ *et al*. (2015) Association of polymerase e-mutated and
2564 microsatellite-instable 409 endometrial cancers with neoantigen load, number of tumor-
2565 infiltrating lymphocytes, and 410 expression of PD-1 and PD-L1. JAMA Oncol 1:1319-
2566 1323.
2567 Imboden S, Nastic D, Ghaderi M, Rydberg F, Rau TT, Mueller MD, Epstein E and Carlson JW
2568 (2019) Phenotype of POLE-mutated endometrial cancer. PLoS One 14:e0214318.
2569 Liu Y (2019) Immune response characterization of endometrial cancer. Oncotarget 10: 982–
2570 992.
2571 McConechy MK, Talhouk A, Leung S, Chiu D, Yang W, Senz J, Reha-Krantz LJ, Lee CH,
2572 Huntsman DG, Gilks *et al.* CB (2016) Endometrial carcinomas with POLE exonuclease
2573 domain mutations have a favorable prognosis. Clin Cancer Res 22:2865-2873.

2574 Meléndez B, Van Campenhout C, Rorive S, Remmelink M, Salmon I. and D'Haene N. (2018)
2575 Methods of measurement for tumor mutational burden in tumor tissue. Transl Lung
2576 Cancer Res 7: 661-667.
2577 Palles C, Cazier JB, Howarth KM, Domingo E, Jones AM, Broderick P, Kemp Z, Spain SL,
2578 Guarino E, Salguero I *et al.* (2013) Germline mutations in the proof-reading domains of
2579 POLE and POLD1 predispose to colorectal adenomas and carcinomas. Nat Genet
2580 45:136–144.
2581 Rayner E, van Gool IC, Palles C, Kearsey SE, Bosse T, Tomlinson I and Church DN (2016) A
2582 panoply of errors: polymerase proofreading domain mutations in cancer. Nat Rev Cancer
2583 16:71-81.
2584 Shinbrot E, Henninger EE, Weinhold N, Covington KR, Göksenin AY, Schultz N, Chao H,
2585 Doddapaneni H, Muzny DM, Gibbs RA *et al*. (2014) Exonuclease mutations in DNA
2586 polymerase epsilon reveal replication strand specific mutation patterns and human origins
2587 of replication. Genome Res 24:1740-1750.
2588 The Cancer Genome Atlas Network (2012) Comprehensive molecular characterization of
2589 human colon and rectal cancer. Nature 487:330-337.
2590 The Cancer Genome Atlas Research N, Kandoth C, Schultz N, Cherniack AD, Akbani R, Liu
2591 Y, Shen H, Robertson AG, Pashtan I, Shen R *et al*. (2013) Integrated genomic
2592 characterization of endometrial carcinoma. Nature 497:67–73.
2593 Wong A, Kuick CH, Wong, WL, Tham JM, Mansor S, Loh E, Jain S, Vikas NN, Tan SH, Chan
2594 SH *et al*. (2016) Mutation spectrum of POLE and POLD1 mutations in South East Asian
2595 women presenting with grade 3 endometrioid endometrial carcinomas. Gynecol Oncol
2596 141:113-20.
2597 Zhang J, Baran J, Cros A, Guberman JM, Haider S, Hsu J, Liang Y, Rivkin E, Wang J, Whitty
2598 B *et al*. (2011) International Cancer Genome Consortium Data Portal--a one-stop shop
2599 for cancer genomics data. Database (Oxford) 2011:bar026.
2600

HUGO Symbol	RefSeq ID	Coding exon count
AKT1	NM_001014431	13
APC	NM_000038	15
ATM	NM 000051	62
ATR	NM_001184	47
AXIN2	NM_004655	10
BARD1	NM_000465	11
BMPR1A	NM 004329	11
BRCA1	NM 007300	23
BRCA2	NM 000059	26
BRIP1	NM_032043	19
CDH1	NM 004360	16
CDK4	NM 000075	7
CDKN2A	NM 000077	3
CHEK2	NM 001005735	15
EGFR	NM 005228	28
EPCAM	NM 002354	9
EXO1	NM 130398	13
FAN1	NM_014967	13
FANCC	NM_000136	14
GALNT12	NM_024642	10
GREM1	NM_013372	1
IGF1R	NM_000875	21
KLLN	NM_001126049	1
MLH1	NM_000249	19
MLH3	NM_001040108	12
MRE11A	NM_005591	19
MSH2	NM_000251	16
MSH3	NM_002439	24
MSH6	NM_000179	10
MUTYH	NM_001128425	16

2601

HUGO Symbol	RefSeq ID	Coding exon count
NBN	NM 002485	16
NTHL1	NM 002528	6
PALB2	NM 024675	13
PCNA	NM 182649	6
PDGFRA	NM 006206	22
PIK3CA	NM 006218	20
PMS1	NM_000534	12
PMS2	NM_000535	15
POLD1	NM_001256849	26
POLE	NM_006231	49
PTEN	NM_000314	9
RAD50	NM_005732	25
RAD51C	NM_058216	9
RAD51D	NM_002878	10
RFC1	NM_001204747	25
RFC2	NM_181471	11
RFC3	NM_002915	9
RFC4	NM_002916	10
RFC5	NM_007370	11
RINT1	NM_021930	15
RPA1	NM_002945	17
RPA2	NM_002946	9
RPA3	NM_002947	4
RPS20	NM_001146227	5
SDHB	NM 003000	8
SDHC	NM 003001	6
SDHD	NM 003002	4
SEC23B	NM 032986	19
SMAD4	NM 005359	11
STK11	NM 000455	9
TGFA	NM 003236	6
TGFBR2	NM_001024847	8
TP53	NM 001276760	8

2602
2603 **Table S1 – Detailed list of the 63 genes used for targeted sequencing.**

Mat Met S1 - Detailed Material and Methods

Immunohistochemistry (IHC)

IHC staining of MLH1, MSH2, MSH6, PMS2, CD3, CD4, and CD8 was performed on 4 µm sections of formalin-fixed and paraffin-embedded (FFPE) tumoral tissue according to standard procedure. The primary antibodies were anti-MLH1 (dilution 1:100; clone 6168-728; BioSB, Santa Barbara, CA), anti-MSH2 (dilution 1:250; clone 25D12; Leica Biosystems, Buffalo Grove, IL), anti-MSH6 (dilution 1:75; clone 6TBP H-141; Santa Cruz Biotechnology, Santa Cruz, CA), anti-PMS2 (dilution 1: 25; clone MOR46; Leica Biosystems, Buffalo Grove, IL), anti-CD3(dilution 1:200; clone PS1; Neomarkers, Fremont, CA), anti-CD4 (dilution 1:50; clone 4F12; Dako, Santa Clara, CA) and anti-CD8 (dilution 1:200; Novocastra, Buffalo Grove, IL) . The analysis was performed by two independent pathologists (FC and ARS). For MMR protein evaluation, adjacent normal endometrium or lymphocytes in the slides were used as an internal positive control and loss of MMR protein expression was defined as the complete absence of nuclear staining in all tumor cells. For evaluation of tumor-associated lymphocytes, the mean number of CD3+, CD4+, and CD8+ of intraepithelial T lymphocytes, i.e., T lymphocytes located within the tumor epithelium, rather than in the peritumoral stroma, was calculated from photomicrographs (40X objective) of 10 high-power fields (HPFs). Peritumoral T lymphocytes (T lymphocytes in the stroma immediately adjacent to the tumor epithelium) were scored using a semiquantitative method (none (0), mild (1+), moderate (2+), marked (3+)) as described by Howitt *et al*. (2015).

Microsatellite instability (MSI)

The pathologists (A.R.S., F.C., and M.O.B.) manually inspected the H&E slides in order to delimitate tumor and non-tumor areas from each case. Non-neoplastic adjacent uterine areas were used as the normal tissue source. Genomic DNA from tumor and normal tissues were extracted from FFPE sections using the Maxwell Rapid Sample Concentrator System (Promega, Madison – WI). MSI status was performed using multiplexed polymerase chain reaction (PCR) for genotyping the monomorphic repetitive markers: BAT26, BAT25, NR21, NR24, and NR27. Primer sequences and PCR conditions were described elsewhere (Buhard *et al*., 2004). Amplicon detection and analysis were performed using an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) and GeneMarker v.1.85 (SoftGenetics, State College, PA), respectively. A diagnosis of MSI-high (MSI-H) was considered positive when

2638 two or more markers showed an altered pattern; MSI-low (MSI-L) when one marker was altered
2639 and microsatellite stable (MSS) when none of the markers showed alteration.
2640
2641 *Targeted sequencing*
2642
2643 For germline analysis, peripheral blood was extracted using QIAamp DNA Mini Kit
2644 (Qiagen, Germantown, MD) following the manufacturer's instructions, and library preparation
2645 was performed using the SureSelectQXT Kit (Agilent Technologies, Santa Clara, CA). For
2646 somatic analysis, genomic DNA was extracted from a representative tumor area (at least 70%
2647 of tumor cells) from FFPE slides, and the library was prepared using the SureSelectXT Kit
2648 (Agilent Technologies). For both germline and somatic mutation analysis, the coding, canonical
2649 splice sites, and both 5' and 3' untranslated regions (UTRs) of 63 genes (Supplementary Table
2650 S1), including Lynch syndrome-associated genes and *POLE*, were sequenced on an Illumina
2651 NextSeq 500/550 system (Illumina, San Diego, CA) on a 2 x 150 bp paired-end mode.
2652 Bioinformatics Analysis
2653 The raw FASTQ files were aligned to the human reference genome GRCh37 with BWA
2654 (Li and Durbin, 2010), sorted with Samtools (Li *et al*., 2009), and the resulting BAM files were
2655 further processed with GATK v. 4.0.10.1 (McKenna *et al*., 2010) to remove duplicated read
2656 pairs and recalibrate read quality scores according to the GATK Best Practises protocol (Van
2657 der Auwera *et al*., 2013). The germline variants were identified using HaplotypeCaller in the
2658 BAM file generated from the blood DNA sample reads and then annotated using ANNOVAR
2659 software (Wang *et al*., 2010). Somatic variants were called on the matching tumour–blood DNA
2660 samples with the Mutect2 algorithm (Cibulskis *et al*., 2013). After the annotation of somatic
2661 variants with Oncotator (Ramos *et al*., 2015) we left only variants with a "PASS" flag and
2662 Variant Allele Frequency (VAF) higher than 5% to reduce the number of false-positive variants
2663 abundant in FFPE samples (Prentice et al., 2018). The final set of somatic variants was
2664 additionally annotated using OncoKB (Chakravarty *et al*., 2017) database to identify known
2665 cancer-driving events in the studied tumour. Copy-number alterations (CNV) in the tumour
2666 were assessed using FACETS software (Shen *et al*., 2017). VisCap was use for germline CNV
2667 investigation (Pugh *et al*., 2016).
2668 Mutational signature analysis and Cosine similarity calculation were performed in R
2669 package Mutational Patterns (Blokzijl *et al*., 2018) using database of the known mutational
2670 signatures in human cancers from Alexandrov *et al*. (2013).

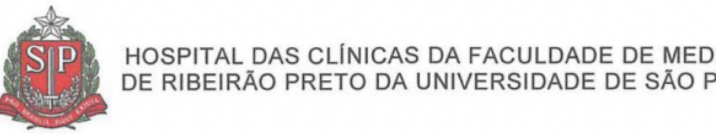
2671 To calculate the strand bias asymmetry between the leading and lagging strands, we
2672 used a map of replication fork direction generated based on the Okazaki fragments sequencing
2673 and subsequent model segmentation from Petryk *et al*. (2016). The probability of replication
2674 fork direction in each 1 kb window was averaged between the HeLa and GM06990 cell lines,
2675 and only regions consistent between them were left for the analysis.
2676 To compare mutation rate and tumor mutational burden (TMB) between the studied
2677 tumor and endometrial cancers with heterozygous *POLE*-exo* mutations, we downloaded VCF
2678 files containing preexisting somatic mutation calls from 25 exomes of endometrial carcinomas
2679 from ICGC portal (https://dcc.icgc.org) with *POLE*-exo* heterozygous somatic mutations,
2680 *POLD1*-exo wild type, and absence of MSI (Zhang *et al*. 2011). We analyzed the same genomic
2681 regions in both the targeted sequencing and the WES data to compare the mutational load
2682 between our EC case and ICGC data.
2683
2684 *Sanger sequencing*
2685
2686 For orthogonal validation, *POLE* variants were confirmed by Sanger sequencing
2687 performed on a 3500xl sequencer using the BigDye 3.1 sequencing kit (Applied Biosystems,
2688 Carlsbad, CA, USA). Primer sequences used were reported previously (Yoshida *et al*., 2011).
2689
2690 **References**
2691
2692 Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, Bignell GR,
2693 Bolli N, Borg A, Børresen-Dale AL *et al*. (2013) Signatures of mutational processes in
2694 human cancer. Nature 500:415-421.
2695 Blokzijl F, Janssen R, van Boxtel R. and Cuppen E (2018) MutationalPatterns: comprehensive
2696 genome-wide analysis of mutational processes. Genome Med. 10:33.
2697 Buhard O, Suraweera N, Lectard A, Duval A and Hamelin R (2004) Quasimonomorphic
2698 mononucleotide repeats for high-level microsatellite instability analysis. Dis Markers.
2699 20:251-257.
2700 Chakravarty D, Gao J, Phillips SM, Kundra R, Zhang H, Wang J, Rudolph JE, Yaeger R,
2701 Soumerai T, Nissan MH *et al.* (2017) OncoKB: a precision oncology knowledge base. JCO
2702 Precis Oncol 1:1-16.

2703 Cibulskis K, Lawrence MS, Carter SL, Sivachenko A, Jaffe D, Sougnez C, Gabriel S, Meyerson
2704 M, Lander ES, Getz G (2013) Sensitive detection of somatic point mutations in impure and
2705 heterogeneous cancer samples. Nat Biotechnol 31:213-219.
2706 Howitt BE, Shukla SA, Sholl LM, Ritterhouse LL, Watkins JC, Rodig S, Stover E, Strickland
2707 KC, D'Andrea AD, Wu CJ *et al*. (2015) Association of polymerase e-mutated and
2708 microsatellite-instable 409 endometrial cancers with neoantigen load, number of tumor-
2709 infiltrating lymphocytes, and 410 expression of PD-1 and PD-L1. JAMA Oncol 1:1319-
2710 1323.
2711 Li, H., and Durbin, R. (2010) Fast and accurate long-read alignment with Burrows–Wheeler
2712 transform. Bioinformatics 26:589-595.
2713 Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth G, Abecasis G,
2714 Durbin R; 1000 Genome Project Data Processing Subgroup (2009) The sequence
2715 alignment/map format and SAMtools. Bioinformatics 25:2078-2079
2716 McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., Garimella
2717 K, Altshuler D, Gabriel S, Daly M *et al*. (2010) The Genome Analysis Toolkit: a
2718 MapReduce framework for analyzing next-generation DNA sequencing data. Genome
2719 Res. 20:1297-1303.
2720 Petryk, N., Kahli, M., d'Aubenton-Carafa, Y., Jaszczyszyn, Y., Shen, Y., Silvain, M., Thermes
2721 C, Chen CL, Hyrien O (2016) Replication landscape of the human genome. Nat Commun
2722 7:10208.
2723
2724 Prentice, L.M., Miller, R.R., Knaggs, J. Mazloomian, A., Hernandez, R.A., Franchini, P., et al.
2725 (2018). Formalin fixation increases deamination mutation signature but should not lead to
2726 false positive mutations in clinical practice. PLoS One13(4):e0196434.
2727 doi:10.1371/journal.pone.0196434
2728 Pugh, T.J, Amr, S.S., Bowser, M.J., Gowrisankar, S., Hynes, E., Mahanta., L.M., Rehm HL,
2729 Funke B, Lebo MS. (2016) VisCap: inference and visualization of germ-line copy-number
2730 variants from targeted clinical sequencing data. Genet Med. 18:712-719.
2731 Ramos, A.H., Lichtenstein, L., Gupta, M., Lawrence, M.S., Pugh, T.J., Saksena, G., Meyerson
2732 M, Getz G (2015) Oncotator: cancer variant annotation tool. Hum Mutat 36:E2423-2429.
2733 Shen R and Seshan VE (2016) FACETS: allele-specific copy number and clonal heterogeneity
2734 analysis tool for high-throughput DNA sequencing. Nucleic Acids Res 44:e131-e131.
2735 Van der Auwera, G.A., Carneiro, M.O., Hartl, C., Poplin, R., Del Angel, G., Levy-Moonshine,
2736 A., Jordan T, Shakir K, Roazen D, Thibault J *et al.* (2013) From FastQ data to

2737 high-confidence variant calls: the genome analysis toolkit best practices pipeline. Curr
2738 Protoc Bioinformatics 43:11-10.
2739 Wang K, Mingyao Li M and Hakon Hakonarson H (2010) ANNOVAR: functional annotation
2740 of genetic variants from high-throughput sequencing data. Nucleic Acids Res 38:e164-
2741 e164.
2742 Zhang J, Baran J, Cros A, Guberman JM, Haider S, Hsu J, Liang Y, Rivkin E, Wang J, Whitty
2743 B *et al*. (2011) International Cancer Genome Consortium Data Portal--a one-stop shop for
2744 cancer genomics data. Database (Oxford) 2011:bar026.

ANNEX

Ethics committee approval



Ribeirão Preto, 08 de junho de 2016.

Ofício nº 1935/2016
CEP/MGV

PROCESSO HCRP nº 5516/2016

Prezados Pesquisadores,

O trabalho intitulado **"DETECÇÃO E DIAGNÓSTICO DE SÍNDROME DE LYNCH E PPAP EM PACIENTES COM CÂNCER DE ENDOMÉTRIO" – Projeto de Pesquisa Versão 2 (modificada em 28/04/2016),** foi analisado pelo Comitê de Ética em Pesquisa, em sua 429ª Reunião Ordinária realizada em 06/06/2016, e enquadrado na categoria: **APROVADO,** bem como o Termo de Consentimento Livre e Esclarecido Versão 2 (modificada em 28/04/2016); o Biorrepositório denominado "Síndromes de Lynch e PPAP" e o Termo de Consentimento para Guarda de Material Biológico.

De acordo com Carta Circular nº 003/2011/CONEP/CNS, datada de 21/03/2011, o sujeito de pesquisa ou seu representante, quando for o caso, deverá rubricar todas as folhas do Termo de Consentimento Livre e Esclarecido – TCLE – apondo sua assinatura na última do referido Termo; o pesquisador responsável deverá da mesma forma, rubricar todas as folhas do Termo de Consentimento Livre e Esclarecido – TCLE – apondo sua assinatura na última página do referido Termo.

Este Comitê segue integralmente a Conferência Internacional de Harmonização de Boas Práticas Clínicas (IGH-GCP), bem como a Resolução nº 466/2012 CNS/MS.

Lembramos que devem ser apresentados a este CEP, o Relatório Parcial e o Relatório Final da pesquisa.

Atenciosamente.

DRª MARCIA GUIMARÃES VILLANOVA
Coordenadora do Comitê de Ética em
Pesquisa do HCRP e da FMRP-USP

Ilustríssimos Senhores
REGINALDO CRUZ ALVES ROSA
PROF. DR. VICTOR EVANGELISTA DE FARIA FERRAZ
Depto. de Genética da FMRP-USP

HOSPITAL DAS CLÍNICAS DA FACULDADE DE MEDICINA DE RIBEIRÃO PRETO DA UNIVERSIDADE DE SÃO PAULO
Campus Universitário – Monte Alegre
14048-900 Ribeirão Preto SP

Comitê de Ética em Pesquisa do HCRP e FMRP-USP
FWA-00002733; IRB-00002186 e
Registro Plataforma Brasil /CONEP nº 5440
(016) 3602-2228
cep@hcrp.usp.br

www.hcrp.usp.br