UNIVERSITÀ DEGLI STUDI DEL PIEMONTE ORIENTALE

School of Medicine

Master Degree in Medical Biotechnologies - LM-09

Experimental thesis

Utility of a Next Generation Sequencing gene panel in the diagnosis of Lynch Syndrome

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SUMMARY

Rational of the study

This study focuses on the genetic of Lynch Syndrome (LS), a hereditary condition leading to a higher risk of various cancers, most notably colorectal cancer. The identification of mutations correlated to Lynch Syndrome is important for the follow-up of the patients. The present study aims to evaluate the usefulness of an NGS panel in detecting LS mutations and the introduction of this panel in the diagnostic workflow to enhance diagnostic efficiency and treatment approaches, ultimately leading to better outcomes for affected individuals and families.

Planning of the study

The planning phase of the study involved several key steps:

1. Patients' Enrollment: Selecting individuals who met specific criteria suggestive of Lynch Syndrome for genetic testing and analysis.
2. Collecting and preparing samples for detailed genetic examination to identify mutations associated with LS.
3. Targeted Sequencing and Data Analysis to identify mutations in patients.

Results

In the study, pathogenic variants were found in 39.1% of patients across various genes, with the panel including genes not traditionally associated with Lynch Syndrome (LS). A notable discovery was a mutation in the BRCA1 gene, typically linked to breast and ovarian cancer, in a patient, raising questions about its role in colorectal cancer. The analysis categorized mutations into five genes: MLH1, MSH2, PMS2, EPCAM, and BRCA1. MLH1 and MSH2 showed the highest mutation rates among the patients, suggesting a significant link to Lynch Syndrome. This information helps in understanding the genetic foundations of the cohort and emphasizes the prevalence of MLH1 and MSH2 mutations.

Conclusions

We analyzed 18 colorectal cancer (CRC) patients, The majority of the genetic alterations were deletions, especially in the genes MSH2, MLH1, PMS2, and EPCAM, followed by stop gain mutations and splicing variants mainly in MLH1. Multigene panel testing identified high-penetrance mutations in cancer predisposition genes, and one of the patients had an unexpected result. patient #1243-21 showed a stop-gain mutation in BRCA1: c.3001 G>T, which is more Lynch-like than HBOC. Approximately 60% of the patients were analyzed as Negative however this result can be because of NGS limitations, mainly introns and regulatory regions impact.

1. INTRODUCTION

1.1 LYNCH SYNDROME

Lynch syndrome (LS) is an autosomal dominant inherited syndrome characterized by an increased risk of cancer and is defined by the presence of pathogenic variants in mismatch repair genes. It is the most common inherited cause of colorectal (lifetime risk up to 70%) and endometrial cancer. It is characterized by the development of cancer at younger ages, multiple cancer diagnoses (synchronous and metachronous), and accelerated carcinogenesis. In addition, it is associated with an increased risk of predominantly epithelial tumors in other locations: ovary, stomach, small intestine, pancreas, ureter, and renal pelvis, as well as skin cancer (e.g. sebaceous neoplasia, in the variant known as Muir-Torre) and tumors of the central nervous system.[1]

The historical background of Lynch Syndrome has been shaped by key developments over the years. It was first recognized due to familial patterns of colorectal cancer, a discovery traced back to the early 20th century. Dr. Aldred Scott Warthin's initial observations and Dr. Henry T. Lynch's subsequent research in the late 20th century established the hereditary nature of the syndrome. This pioneering work led to the identification of the genetic basis of Lynch Syndrome, particularly the role of mismatch repair (MMR) genes, in the 1990s. [2] These discoveries laid the groundwork for subsequent genetic research, leading to the identification of MMR genes in the 1990s.[3]

The importance of studying Lynch Syndrome lies in its implications for cancer prevention, diagnosis, and treatment. Understanding the genetic basis and clinical manifestations of Lynch Syndrome can lead to more effective screening strategies, early detection, and personalized treatment options for affected individuals. This not only improves survival rates but also provides insights into the broader mechanisms of carcinogenesis. Additionally, studying Lynch Syndrome has significant implications for family members of affected individuals, as they may also carry genetic mutations and are at an increased risk of developing cancers. [4] We acknowledge that there is an opinion that LS can only be diagnosed in such individuals once cancer has been diagnosed, this is contrary to the hereditary polyposis, which is characterized by the macroscopic syndromic feature of multiple pre-malignant tumors [9]. Allied with this, a major purpose of diagnosis of a cancer-predisposing condition is to identify those who would benefit from surveillance and prophylactic surgery to prevent cancer.[5]

1.1.1 Epidemiology of Lynch Syndrome

Epidemiological studies of Lynch Syndrome provide essential insights into its prevalence, distribution, and inheritance patterns, which are crucial for developing effective screening and prevention strategies.

1.1.2 Prevalence Globally and in Specific Populations

Lynch Syndrome is one of the most common hereditary cancer syndromes, accounting for about 3-5% of all colorectal cancers. Globally, its prevalence varies, with estimates suggesting that approximately 1 in 279 to 1 in 440 individuals carry a mutation associated with Lynch Syndrome. [6] The prevalence is notably higher in certain populations due to founder mutations. For example, in Finland, certain MLH1 and MSH2 mutations are more common, reflecting a specific population history. [7] Moreover, studies have indicated variations in gene mutation frequency among different ethnic groups, which can influence disease patterns and risk assessment strategies. [8]

1.2 OVERVIEW OF GENETICS IN CANCER

Cancer is fundamentally a genetic disease caused by the accumulation of mutations in genes that regulate cell growth and division. These mutations can be inherited (germline) or acquired (somatic). The genes typically involved in cancer development can be categorized into two main groups: oncogenes and tumor suppressor genes. Oncogenes are mutated forms of normal genes (proto-oncogenes) that when altered, promote cancerous growth. Tumor suppressor genes, on the other hand, are responsible for controlling cell division and repairing DNA errors. When these genes are inactivated or lost, uncontrolled cell growth can occur, leading to cancer. [9]

Advancements in genomic technologies, particularly next-generation sequencing, have enabled the detailed mapping of cancer genomes, revealing the complexity and heterogeneity of genetic changes in various cancers. This has led to the identification of numerous cancer susceptibility genes and the understanding of their roles in DNA repair, cell cycle control, and apoptosis. [10]

1.2.1 Germline Mutations vs Somatic Mutations

Germline mutations are genetic alterations that occur in the sperm or egg cells and are passed from parents to offspring. These mutations are present in every cell of the body from conception and can be inherited from one generation to the next. Germline mutations are responsible for about 5%–10% of all cancers and are significant in hereditary cancer syndromes like Lynch Syndrome. They are identified through genetic tests using blood samples or buccal cells from saliva samples and affect reproductive cells; hence they can pass from generation to generation, leading to inherited cancer syndromes.

On the other hand, somatic mutations occur in individual cells during a person's lifetime and are not inherited from parents or passed on to children. These mutations can result from environmental factors, such as tobacco use, ultraviolet light or radiation, and aging, and they lead to sporadic cancers. Unlike germline mutations, somatic mutations are present only in specific cells, not throughout the body, and they occur after conception, typically in non-reproductive cells. These mutations are identified by analyzing tumor tissues or liquid biopsies containing circulating tumor cells. (Fig. 1)

The distinction between germline and somatic mutations is crucial for cancer diagnosis and treatment. Understanding whether a mutation is germline or somatic helps determine the hereditary risk, appropriate screening strategies, and targeted therapies for patients and their families. For instance, individuals with germline mutations may require more intensive surveillance and may benefit from preventive measures or treatments aimed at reducing the risk of cancer development.

Recent studies have compared somatic and germline mutation rates, revealing that somatic mutation rates are significantly higher than germline rates, underscoring the different roles these mutations play in the body and their impact on cancer development. This distinction highlights the importance of accurate genetic testing and counseling in identifying and managing hereditary cancer syndromes like Lynch Syndrome.[11]

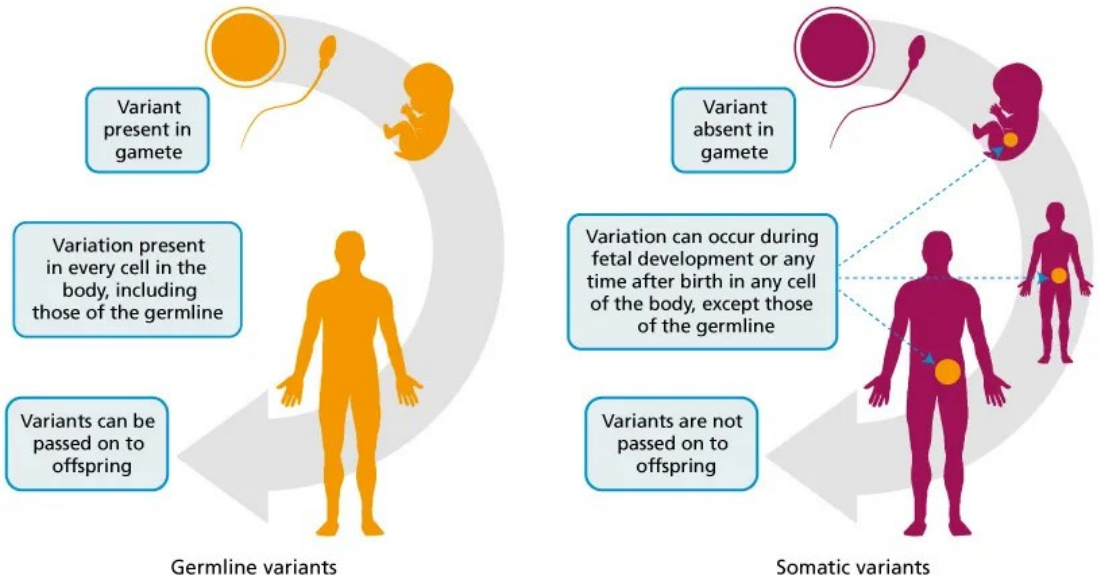

Figure 1 Differences between Germline variants and Somatic Variants

1.2.2 Genes Involved in Lynch Syndrome

Lynch Syndrome is caused by mutations in several genes that are responsible for DNA mismatch repair (MMR). The primary genes implicated in this condition are MLH1, MSH2, MSH6, PMS2, and EPCAM. These genes play crucial roles in correcting DNA replication errors, thus maintaining genomic stability. (Fig. 2)

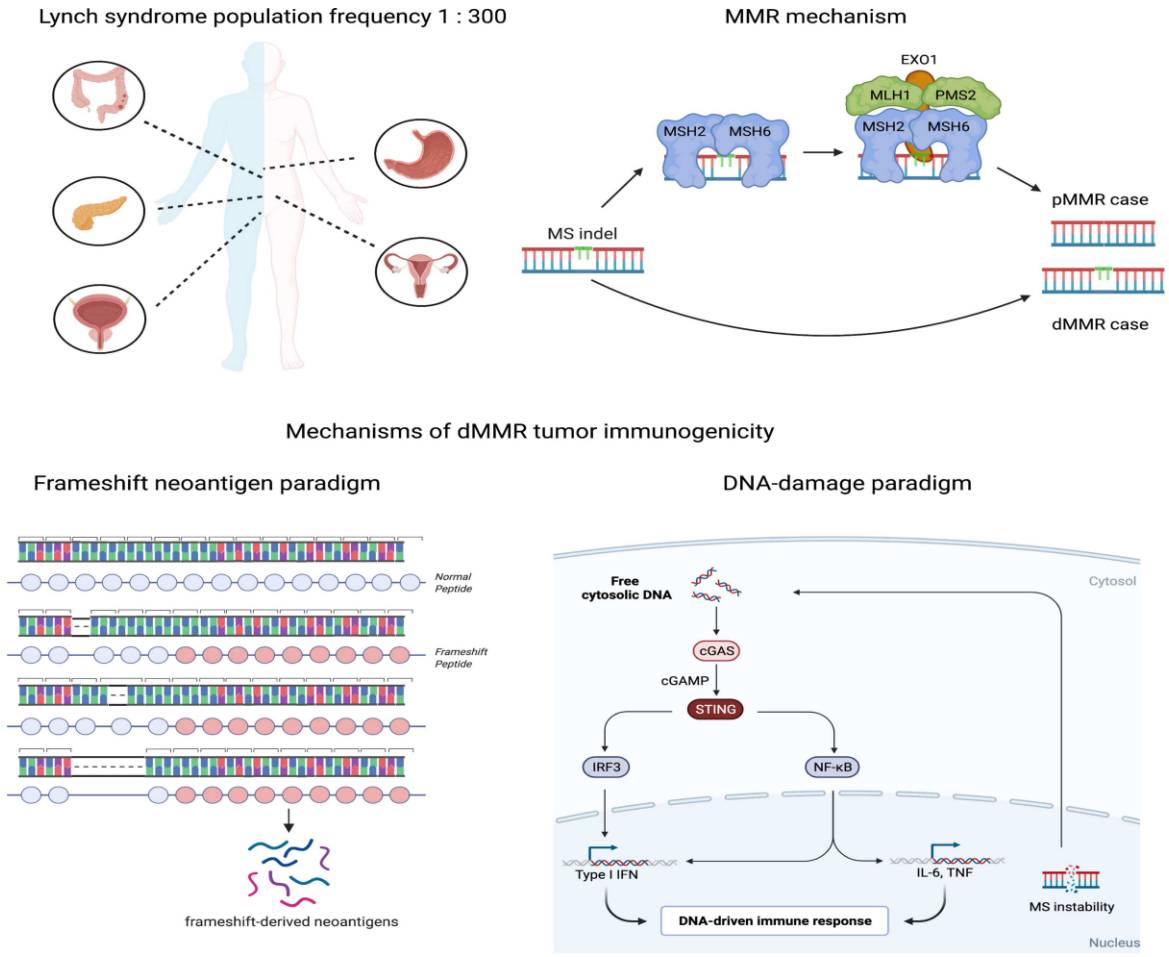

Figure 2 Population, molecular and immunological aspects of mismatch-repair deficient (dMMR) tumors. (A) Predispositions to different cancers conferred by Lynch syndrome condition. Approximately 1 in 300 people in the U.S. has the Lynch Syndrome associated allele Carriers have 80% lifetime risk developing cancer including: colorectal, stomach, pancreas, urinary track and prostate for males and urinary, ovary or uterus tracks for females. In total, Lynch syndrome accounts for 2-3% solid tumor cases. (B) Mismatch repair (MMR) mechanism. MS indels occurring during DNA replication are repaired by MMR system (proficient MMR). MSH2-MSH3 or MSH2-MSH6 complexes, called MutSa or MutSb, detect the error and recruit the MLH1-PMS1, MLH1-PMS2 or MLH1-MLH3 (MutLa/b/g complexes respectively) to bind to the DNA and bring DNA exonuclease with PCNA to the mutation site. The mismatch is then excised and repaired following by DNA resynthesis and re-ligation. These aberrations are left unrepaired in case of MMR deficiency. (C) Two complementary paradigms explaining immune responses in dMMR tumors: neoantigen-driven (left part), and innate immune driven (right part).

MLH1 (MutL protein Homolog 1): Mutations in MLH1 account for approximately 50% of Lynch Syndrome cases. They are associated with a high risk of colorectal and endometrial cancers. (Fig. 3) [12]

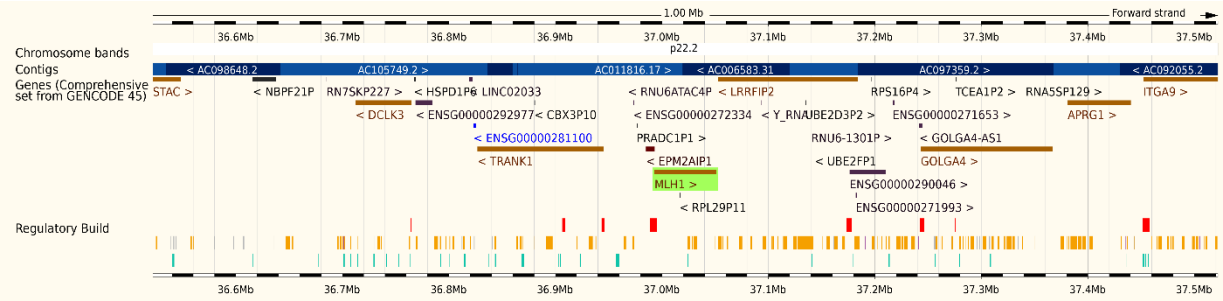

Figure 3 mutL homolog 1 Chromosomal location 3p22.2

MSH2 (MutS Homolog 2): MSH2 mutations are the second most common cause of Lynch Syndrome. Individuals with MSH2 mutations have a similar cancer risk profile to those with MLH1 mutations. (Fig. 4) [13]

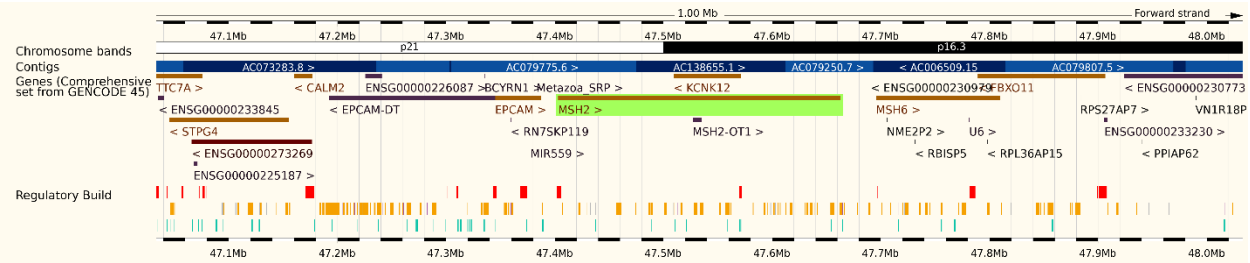

Figure 4 mutS homolog 2 Chromosomal location 2p21-p16.3

MSH6: MSH6 mutations are associated with a slightly lower risk of colorectal cancer but a higher risk of endometrial cancer compared to MLH1 and MSH2 mutations. (Fig. 5) [14]

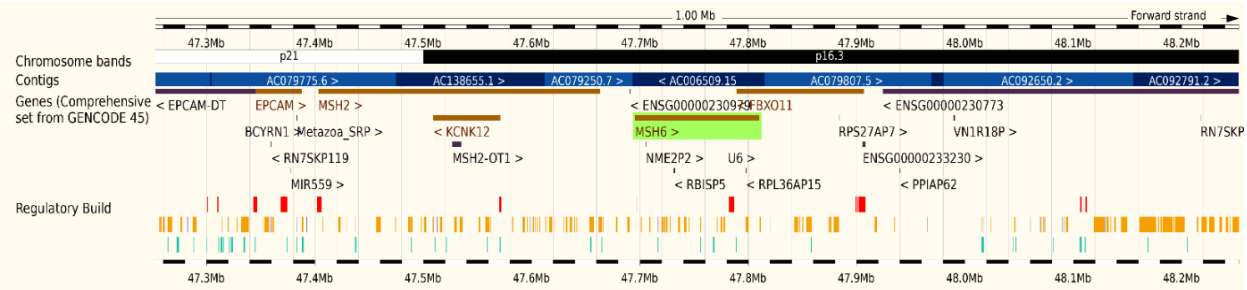

Figure 5 mutS homolog 6 Chromosomal location 2p16.3

PMS2 (Post-Meiotic Segregation Increased 2): PMS2 mutations confer a lower risk of colorectal cancer but are still significant for Lynch Syndrome screening. (Fig. 6) [15]

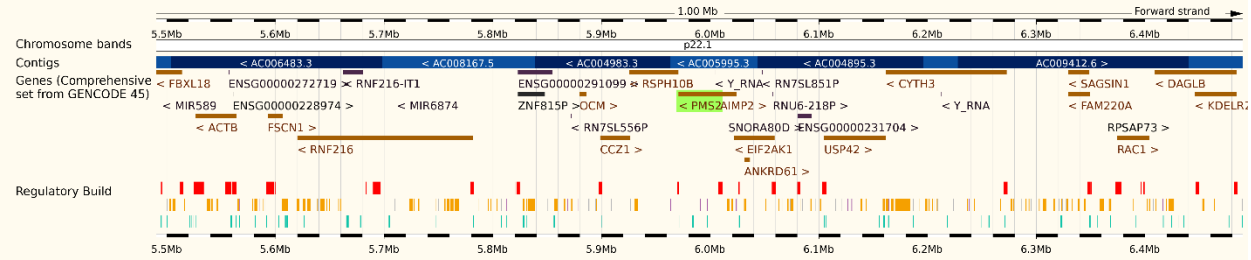

Figure 6 PMS1 homolog 2, mismatch repair system component Chromosomal location 7p22.1

EPCAM: Deletions in the EPCAM gene lead to epigenetic silencing of the MSH2 gene, contributing to Lynch Syndrome. EPCAM deletions are a rarer cause of the condition. (Fig. 7) [16]

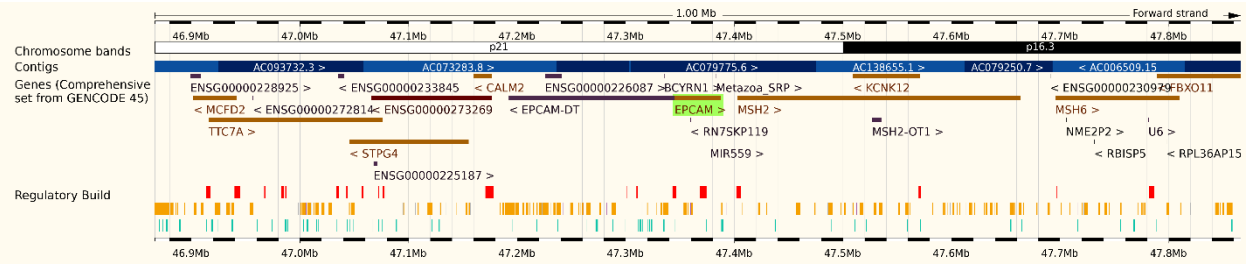

Figure 7 epithelial cell adhesion molecule Chromosomal location 2p21

1.2.3 Mutations and Their Impacts

The mutations in Lynch Syndrome primarily involve loss-of-function alterations in the MMR genes. These mutations lead to microsatellite instability (MSI), a hallmark of Lynch Syndrome-associated cancers. MSI results from the accumulation of uncorrected errors in microsatellite regions of DNA, contributing to tumorigenesis.

The impact of these mutations extends beyond an increased risk of cancer. They also influence the prognosis and response to treatment. For example, Lynch Syndrome-associated colorectal cancers often have better prognosis and different responses to chemotherapy compared to sporadic cases. [17] Furthermore, the identification of these mutations has significant implications for family members, as they can undergo genetic testing and enhanced surveillance for early cancer detection. [18]

1.2.4 Functionality and Role of DNA Mismatch Repair and MMR Genes

Functional DNA mismatch repair (MMR) is vital for basic biology and cancer avoidance. The main function of MMR proteins is to maintain genomic stability by correcting single-base mismatches and insertion/deletion loops (IDL) that may arise during replication. Malfunction of MMR results in a mutator phenotype and microsatellite instability (MSI) characteristic of most tumors from Lynch syndrome (LS) and some 15 % of sporadic tumors MMR proteins also recognize diverse types of endogenous and exogenous damage, such as that induced by oxidation or alkylation, and correct the lesions, or if this is not possible, signal DNA damage to cell cycle arrest or apoptosis.[7] The MMR pathway is bidirectional. The 5' to 3' repair proceeds as follows first, a 5' nick in the newly synthesized strand, which occurs randomly during replication and serves as strand discrimination to the MMR, allows exonuclease 1 (EXO1) to bind. ATP exchange converts the mismatch-bound MutSα into a sliding clamp locked around the DNA. The conformational change releases MutSα from the site of the mismatch and allows it to move along the DNA strand. Moreover, the change permits interaction with MutLα, which subsequently binds the DNA. The MutSα/MutLα complex binds EXO1, after which 5' to 3' movement of the MutSα/MutLα/EXO1 complex allows for excision by EXO1 assisted by the single-stranded DNA binding protein complex replication protein A (RPA), which protects the exposed single-stranded DNA. Then, the DNA polymerase in complex with the proliferating cell nuclear antigen (PCNA) sliding clamp as well as the DNA ligase finishes the repair process (Fig. 8). The 3' and 5' directed repair processes seem to proceed differently, although some details remain to be resolved. [19-21]

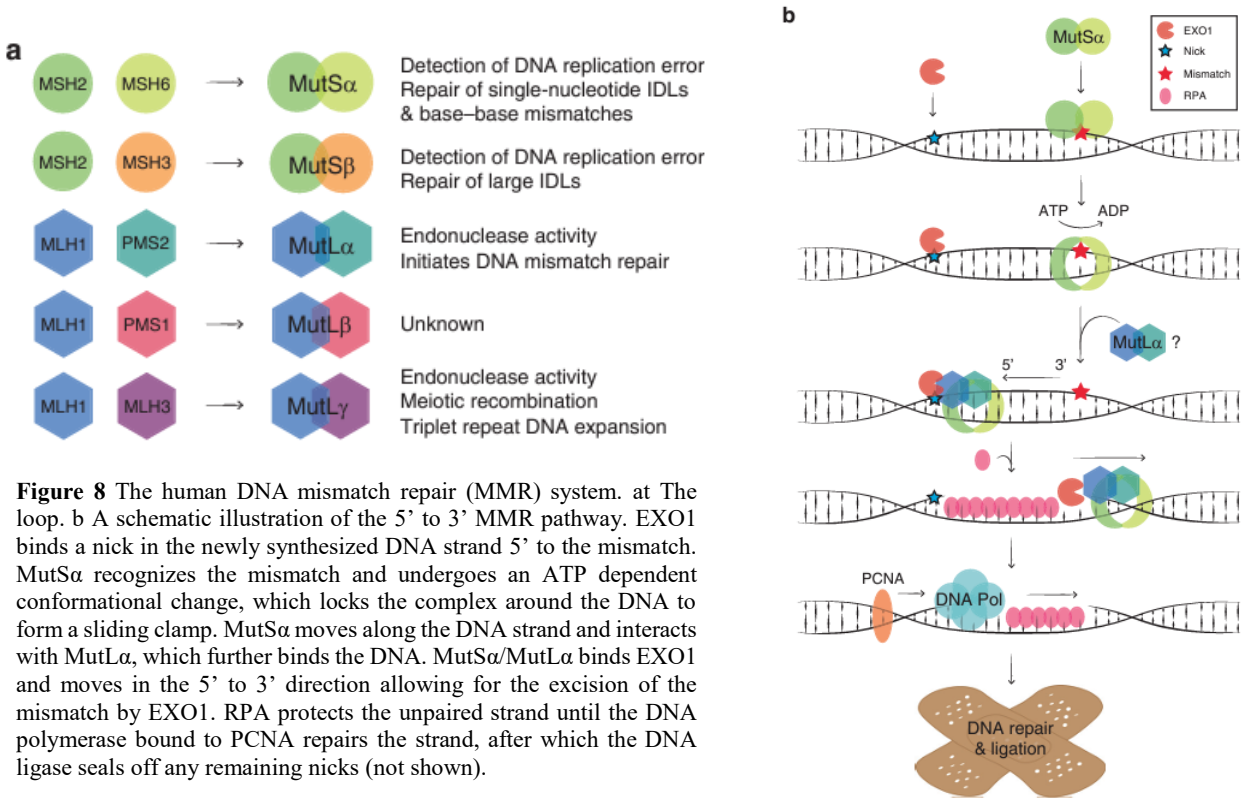

Figure 8 The human DNA mismatch repair (MMR) system. at The loop. b A schematic illustration of the 5' to 3' MMR pathway. EXO1 binds a nick in the newly synthesized DNA strand 5' to the mismatch. MutSα recognizes the mismatch and undergoes an ATP dependent conformational change, which locks the complex around the DNA to form a sliding clamp. MutSα moves along the DNA strand and interacts with MutLα, which further binds the DNA. MutSα/MutLα binds EXO1 and moves in the 5' to 3' direction allowing for the excision of the mismatch by EXO1. RPA protects the unpaired strand until the DNA polymerase bound to PCNA repairs the strand, after which the DNA ligase seals off any remaining nicks (not shown).

In humans, five MutS homologs (MSH2, MSH6, MSH3, MSH4, and MSH5) and four MutL homologs (MLH1, PMS2, PMS1, and MLH3) have been identified which can form heterodimers in different combinations (Fig. 8). The main mismatch-binding factor in humans is hMutSa, consisting of MSH2 and MSH6, which recognizes single-base mispairs and IDLs. Another mismatch-binding heterodimer is hMutSb, formed by MSH2 and MSH3, which mainly acts on IDLs. Upon mismatch binding, the hMutS complex undergoes an ATP-driven conformational change into a sliding clamp and a hMutL heterodimer is recruited. The main hMutL complex is hMutLa, consisting of MLH1 and PMS2 and participating in the repair of single-base mismatches and IDLs. Alternative hMutL heterodimers are hMutLc, composed of MLH1 and MLH3, which may predominantly contribute to IDL repair, and hMutLb (MLH1 and PMS1), which does not seem to participate in MMR. When the hMutS-hMutL complex encounters a strand discontinuity, an excision machinery is recruited, the mismatch-containing fragment is degraded, and a new strand is synthesized.[7]

The specificity of substrates for individual MMR proteins is mirrored in the varied MSI (Microsatellite Instability) phenotypes observed in tumors from patients with Lynch Syndrome (LS). Mutations in MSH2 and MLH1 are linked to a high degree of instability, affecting both mononucleotide and dinucleotide (as well as other types of short tandem) repeats.[22] This pattern is also seen in mutations of PMS2. [23] Conversely, mutations in MSH6 tend to be associated with a lower degree of MSI, predominantly affecting mononucleotide repeats.[24] In the case of MLH3 mutation carriers, the relevance of mononucleotide repeats may diminish in comparison to dinucleotide and tetranucleotide repeats, with reported phenotypes ranging from MSI-high to complete absence of MSI.[7]

EPCAM is not an MMR gene, but structural alterations in EPCAM may lead to LS as it is adjacent to the MSH2 gene. In LS, the deletion of heterozygote sequences at the 3’ end of EPCAM can lead to the inactivation of MSH2 in tissues expressing EPCAM due to its promoter hypermethylation. And what’s more, the EPCAM 3’s end deletion might extend to the first MSH2 exon, which includes the promoter region, resulting in the suppression of both EPCAM protein and MSH2 protein expressions without MSH2 hypermethylation. Therefore, MSH2-negative patients need to be tested for EPCAM deletions. In total, 19 different deletions were identified, varying in size from 2.6 to 23.8 kb. All deletions were located upstream of the MSH2 gene promoter and encompassed at least the last two exons of the EPCAM gene, (Fig. 9) leaving its 50 exons intact. Our breakpoint mapping data indicate that a wide variety of EPCAM deletions do occur in these Lynch syndrome families.[16, 25]

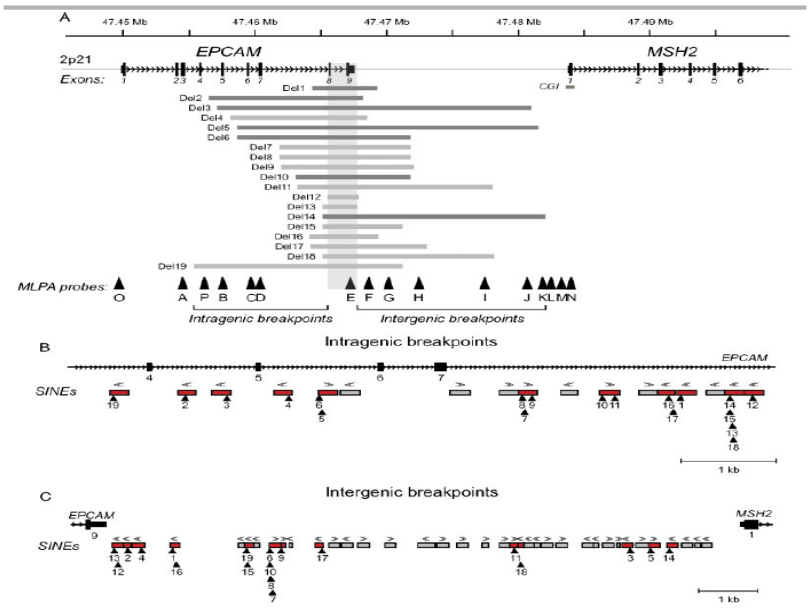

Figure 9 EPCAM deletions in Lynch syndrome patients. A: Schematic outline of the genomic region around EPCAM and MSH2, showing 19 different deletions (gray bars) identified in 45 families. All deletions include at least exons 8 and 9 of EPCAM. Deletions identified in multiple (apparently) unrelated families are indicated in dark gray. Positions of the MLPA probes used for deletion mapping are indicated by triangles. All intragenic (B) and intergenic (C) breakpoints are in Alu repeats (referred to as SINEs: short interspersed nuclear elements, red bars), of which eight are involved in several different deletions (indicated by arrows and numbers of the deletion). Arrowheads above the bars denote the orientation of the repeats.[16]

1.2.5 Gender and age distribution

The distribution of Lynch Syndrome is generally equal between genders. However, the spectrum and age of onset of associated cancers can vary. Males with Lynch Syndrome have a higher risk of developing colorectal cancer compared to women, whereas women have a significantly increased risk of endometrial cancer. The average age of onset for colorectal cancer in Lynch Syndrome patients is typically around 45 years, which is significantly younger than the general population's average age of onset for colorectal cancer. [26] Early onset of cancer is a critical characteristic of Lynch Syndrome and is a key factor in its diagnosis and management.

1.2.6 Familial Patterns and Inheritance

Lynch Syndrome is inherited in an autosomal dominant manner, meaning that having a single copy of the mutated gene significantly increases an individual's risk of developing associated cancers. Family members of individuals diagnosed with Lynch Syndrome have a 50% chance of inheriting the mutation. The familial clustering of cancers, particularly colorectal and endometrial cancer, is a hallmark of Lynch Syndrome. (Fig. 10) Genetic counseling and testing are recommended for at-risk family members to facilitate early detection and intervention. [27] The identification of Lynch Syndrome in families allows for targeted surveillance strategies, such as more frequent

colonoscopies, which have been shown to significantly reduce cancer incidence and mortality in this population. [4]

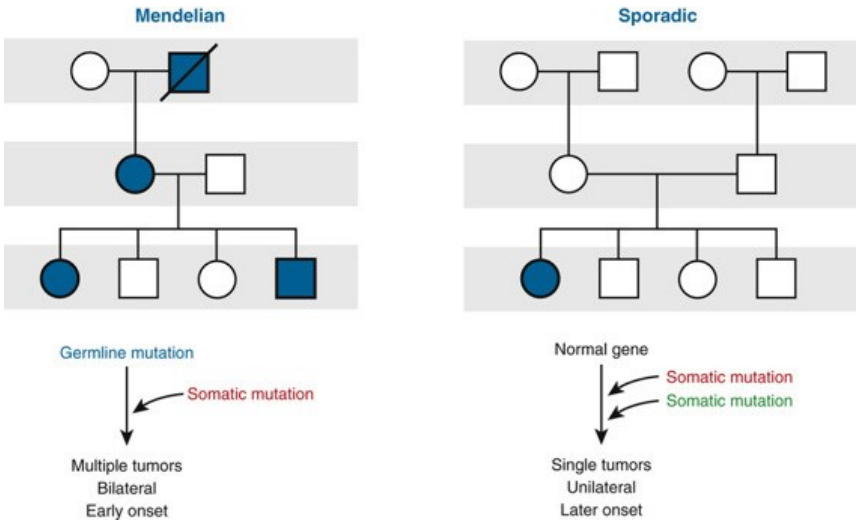

Figure 10 Comparison of mendelian and sporadic forms of cancers.

1.3 CLINICAL FEATURES AND DIAGNOSIS OF LS

1.3.1 Common Clinical Manifestations

Lynch Syndrome is most associated with an increased risk of colorectal cancer and endometrial cancer. Other cancers associated with Lynch Syndrome include gastric, ovarian, urinary tract, small bowel, and brain tumors, as well as sebaceous gland adenomas and keratoacanthomas as part of Muir-Torre syndrome (before the age of 50), and for women, a higher risk of endometrial and ovarian cancers often at a younger age compared to the general population. Additionally, tumors in Lynch Syndrome often exhibit distinctive pathological features such as mucinous or signet-ring cell differentiation and lymphocytic infiltration. [18, 28, 29]

1.3.2 Criteria for Diagnosis (Amsterdam Criteria, Bethesda Guidelines)

1. Amsterdam Criteria: Developed in 1991 and focused primarily on a family history of colorectal cancer. They include at least three relatives with Lynch Syndrome-associated cancers, one being a first-degree relative of the other two, at least two successive generations affected, and at least one diagnosis before the age of 50.

2. Revised Bethesda Guidelines: Updated in 2004, these guidelines are broader and include personal and family history of Lynch Syndrome-associated cancers, presence of tumors exhibiting MSI, and patients with colorectal cancer diagnosed before age 50, among other criteria. These guidelines are used to identify individuals who should undergo further testing for MMR gene mutations. [30]

1.4 ROLE OF GENETIC TESTING AND COUNSELING

Genetic testing for Lynch Syndrome involves screening for mutations in the MMR genes. It is recommended for individuals who meet the Amsterdam or Bethesda criteria. Pre-test and post-test genetic counseling is crucial to help individuals understand the implications of testing, the inheritance pattern, and management strategies for cancer risk reduction. Genetic testing not only guides clinical management for the individual but also has implications for at-risk family members. [31, 32]

1.4.1 Genetic Laboratory Techniques

Next Generation Sequencing (NGS): NGS has revolutionized the genetic analysis of Lynch Syndrome. This technique allows for the simultaneous sequencing of multiple genes, which is particularly beneficial given the genetic heterogeneity of Lynch Syndrome. NGS is efficient in identifying both known and novel variants in mismatch repair (MMR) genes associated with the syndrome. Its comprehensive approach makes it an invaluable tool for diagnosing Lynch Syndrome, especially in cases where the genetic background is complex.[33]

Multiple Ligation-dependent Probe Amplification (MLPA): MLPA is particularly useful for detecting large genomic rearrangements that cannot be identified by sequencing methods like NGS or Sanger sequencing. These rearrangements are significant in Lynch Syndrome as they account for a notable proportion of mutations in the MMR genes. Therefore, MLPA complements other genetic testing methods by covering a wider range of possible genetic alterations associated with Lynch Syndrome.

Sanger Sequencing: Traditionally used for genetic testing, Sanger Sequencing has been a standard in confirming and characterizing specific mutations in Lynch Syndrome. However, with the advent of NGS, its role has evolved. While NGS provides a broad overview of genetic variants, Sanger Sequencing is often employed to validate and confirm the findings from NGS, particularly in complex cases or where discrepancies arise. Despite the rise of NGS, Sanger Sequencing remains relevant for its accuracy and reliability in certain diagnostic scenarios.[34]

1.5 COLORECTAL CANCER IN LYNCH SYNDROME

Colorectal cancer (CRC) is the most common cancer associated with Lynch Syndrome, characterized by an early onset, typically before the age of 50. The lifetime risk for CRC in individuals with Lynch Syndrome ranges from 40% to 80%, depending on the specific gene mutation. These cancers often develop in the proximal colon and are more likely to be poorly differentiated and mucinous, with an increased lymphocytic infiltrate, compared to sporadic CRC cases. Despite their aggressive histological features, CRCs in Lynch Syndrome often have a better prognosis due to their unique pathobiology. (Fig. 11) [29, 35]

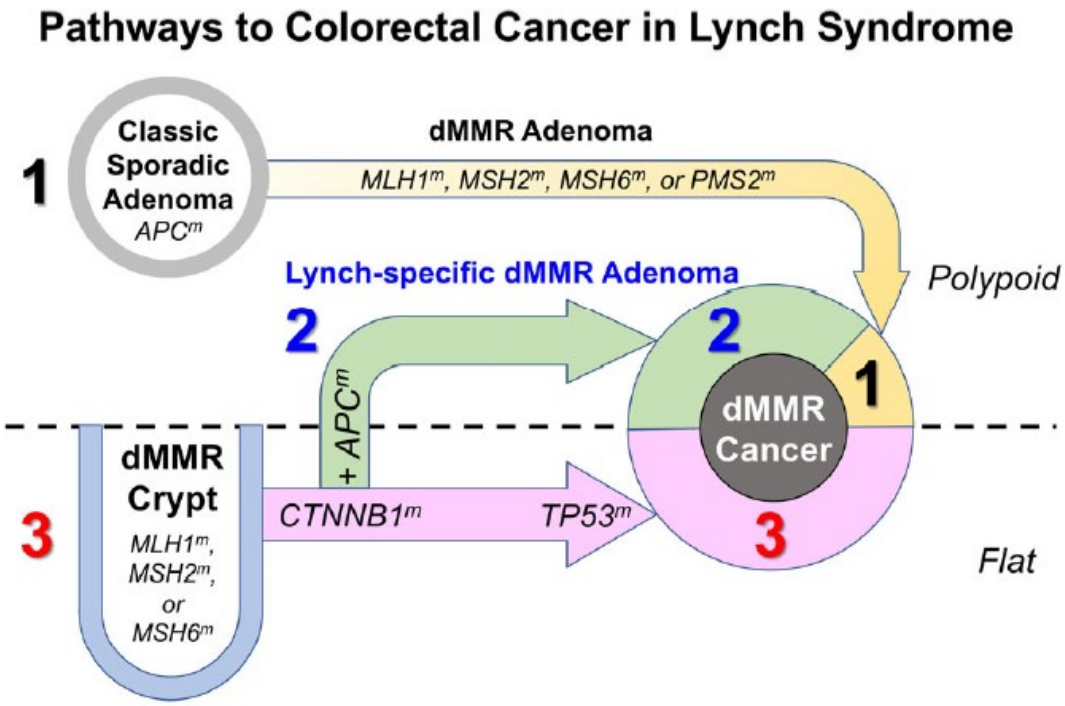

Figure 11 Three Pathways to Colorectal Cancer in Lynch Syndrome

1.5.1 Other Associated Cancers (Endometrial, Gastric, Ovarian, etc.)

Individuals with Lynch Syndrome face an elevated risk of several non-colorectal cancers, notably endometrial, gastric, and ovarian cancers (Tab. 01). The lifetime risk for endometrial cancer in these individuals can be as high as 60%, while the risks for gastric and ovarian cancers are about 13% and 9%, respectively. Other associated cancers include urinary tract, small bowel, hepatobiliary tract, brain, and skin cancers, such as sebaceous carcinomas. The variability in cancer risk is influenced by specific MMR gene mutations, underlining the importance of personalized surveillance and management strategies. [36]

Males				
Cancer type	***MLH1***	***MSH2***	***MSH6***	***PMS2***
Any cancer	71% [63–81%]	75% [66–86%]	42% [25–67%]	See below
Colorectal (bowel)	57% [49–68%]	51% [41–65%]	18% [8–43%]	See below
Stomach, small bowel, bile duct, gallbladder, and pancreas	22% [16–30%]	20% [14–28%]	8% [3–30%]	See below
Ureter and kidney	5% [3–10%]	18% [13–25%]	2% [<1–24%]	See below
Urinary bladder	7% [4–13%]	13% [8–21%]	8% [3–30%]	See below
Prostate	14% [9–22%]	24% [17–33%]	9% [3–31%]	5% [<1–68%]
Brain	0.7% [<1–5%]	8% [4–15%]	2% [<1–24%]	See below
Females				
Cancer type	***MLH1***	***MSH2***	***MSH6***	***PMS2***
Any cancer	81% [74–88%]	84% [77–91%]	62% [47–78%]	See below
Colorectal (bowel)	48% [41–57%]	47% [39–55%]	20% [12–41%]	See below
Endometrium	37% [30–47%]	49% [40–61%]	41% [29–62%]	3% [5–50%]
Ovaries	11% [7–20%]	17% [12–31%]	11% [4–39%]	3% [<1–43%]
Stomach, small bowel, bile duct, gallbladder, and pancreas	11% [7–17%]	13% [9–19%]	4% [3–30%]	See below
Ureter and kidney	4% [2–8%]	19% [14–27%]	6% [2–27%]	See below
Urinary bladder	5% [3–11%]	8% [5–14%]	1% [<1–23%]	See below
Brain	2% [<1–5%]	3% [1–8%]	1% [<1–23%]	See below

Both Sexes combined	
Cancer type	***PMS2***
Any cancer	34% [19–60%]
Colorectal (bowel)	10% [3–41%]
Stomach, small bowel, bile duct, gallbladder, and pancreas	4% [1–34%]
Ureter and kidney	4% [<1–34%]
Urinary bladder	<1% [0–31%]
Brain	<1% [0–31%]

Table 1 the incidence of different types of cancers in the presence of germline mutations in the different genes. [5]

1.5.2 Pathophysiology of Cancer Development in Lynch Syndrome

The pathophysiology of cancer in Lynch Syndrome is primarily driven by inherited mutations in the mismatch repair (MMR) genes. The most frequently mutated genes in this syndrome include MLH1, MSH2, MSH6, PMS2, and EPCAM. These genes play a crucial role in repairing DNA replication errors. When mutated, they lead to microsatellite instability (MSI), a condition characterized by the accumulation of length variations in microsatellite DNA sequences. MSI is a distinctive feature of tumors in Lynch Syndrome and contributes to genomic instability. This instability is the primary driver for the development of various cancers associated with Lynch Syndrome, leading to the rapid accumulation of mutations in critical genes involved in cell cycle

control and apoptosis. Such widespread genomic alterations are central to the early onset and increased risk of multiple cancers in individuals with Lynch Syndrome. [37, 38]

1.6 MANAGEMENT AND TREATMENT STRATEGIES FOR LS

1.6.1 Surveillance Strategies for Early Detection

Surveillance in Lynch Syndrome is essential for early cancer detection. Updated guidelines recommend colonoscopy starting at age 20-25 or 2-5 years earlier than the youngest case in the family, with subsequent screenings every 1-2 years. For endometrial and ovarian cancer, annual screening with transvaginal ultrasound and endometrial biopsy from age 30-35 is advised. Urinalysis is suggested annually for urinary tract cancer, and upper gastrointestinal endoscopy is recommended every 3-5 years for gastric and small bowel cancer. [39, 40]

1.6.2 Surgical Interventions

In Lynch Syndrome, surgical options are considered both for treatment and as a prophylactic measure. For colorectal cancer, options include segmental colectomy or total colectomy, based on disease extent and patient factors. Women with Lynch Syndrome may consider prophylactic hysterectomy and bilateral salpingo-oophorectomy after completing childbearing to reduce the risks of endometrial and ovarian cancers. [41, 42]

1.6.3 Pharmacological Treatments and Chemoprevention

Aspirin is used in chemoprevention to reduce the risk of colorectal and potentially other cancers in Lynch Syndrome. Ongoing research aims to determine the optimal aspirin dosage and duration. Additionally, immunotherapy has shown promise in treating Lynch Syndrome-related cancers, especially those with high microsatellite instability, by stimulating the immune system to attack cancer cells. [43, 44]

1.6.4 Latest Advancements in Immunotherapy

Recent studies have shown promising results in the use of immunotherapy for Lynch Syndrome. A study by Memorial Sloan Kettering Cancer Center published in Nature Medicine in October 2023 suggests that immunotherapy drugs, specifically checkpoint inhibitors, may prevent serious tumors in people with Lynch Syndrome. This innovative approach is based on past patient data and proposes future clinical trials to test immunotherapy as a preventive treatment for cancer in Lynch Syndrome patients.

Another study highlighted in 2023 by the UCLA Health Department of Medicine discussed the response of sarcoma in Lynch Syndrome patients to immunotherapy. This indicates the expanding role of immunotherapy in treating various cancers associated with Lynch Syndrome.

These findings are part of the broader landscape of advancing cancer treatments, where immunotherapy is increasingly playing a pivotal role in managing cancers associated with genetic

conditions like Lynch Syndrome. The new approach suggests that immunotherapy may not only treat but also potentially prevent the development of more serious cancers in these patients.

These latest developments underline the importance of personalized medicine in cancer care, especially for genetically predisposed conditions like Lynch Syndrome. The ongoing research and clinical trials will continue to shape the future of treatment strategies for Lynch Syndrome, offering hope for more effective and targeted therapies.[45] [46]

1.7 PSYCHOLOGICAL AND SOCIAL IMPLICATIONS OF LS

1.7.1 Impact on Patients and Families

Lynch Syndrome significantly impacts both individuals and their families. Understanding the genetic risk of cancer can lead to increased anxiety and stress, as well as uncertainty about the future. The psychosocial impact is profound, who noted considerable emotional distress related to the risk of transmitting the condition to offspring and the burden of constant medical surveillance. These factors can influence major life decisions, such as family planning, and affect family dynamics and relationships. [47]

1.7.2 Genetic Counseling and Ethical Considerations

Genetic counseling is crucial in managing Lynch Syndrome, addressing both the dissemination of medical information and the psychological and ethical issues faced by patients and families. Counselors guide individuals through decisions about genetic testing, disclosure of genetic status to relatives, and reproductive choices. Ethical challenges include privacy, informed consent, and the potential for discrimination, requiring sensitive navigation. [48, 49]

1.7.3 Support Systems and Patient Advocacy

Support networks, including patient advocacy groups and counseling services, are vital for those with Lynch Syndrome. These groups offer emotional support, education, and resources, and play a key role in raising awareness, advancing research, and influencing healthcare policies for better management of Lynch Syndrome. [50, 51]

Recent advances in molecular genetics have significantly enhanced our understanding of Lynch Syndrome. Breakthroughs in genomic sequencing have identified new genetic variants and modifier genes that influence cancer risk and disease phenotype. Studies in 2023 have further elucidated the mechanisms of mismatch repair (MMR) deficiency and its role in tumorigenesis. This has led to a more refined stratification of cancer risk and has begun to explain the variability in cancer incidence among individuals with Lynch Syndrome. [52, 53]

Ongoing research is focusing on improving diagnostic methods, including non-invasive screening tools and more precise genetic testing techniques. Clinical trials are currently exploring the efficacy of various chemopreventive agents, such as aspirin, and their optimal dosing and duration for cancer prevention in Lynch Syndrome. Additionally, immunotherapy trials are investigating the potential of checkpoint inhibitors in treating Lynch Syndrome-related cancers, especially those with high microsatellite instability (MSI-H). [54, 55]

The future of Lynch Syndrome management is advancing toward personalized medicine, with a significant focus on gene editing and artificial intelligence. Gene editing, particularly with CRISPR-Cas9, holds promise for correcting mismatch repair gene defects. However, this approach requires careful navigation due to the complexity of potential off-target effects and unintended genetic alterations. Simultaneously, artificial intelligence is emerging as a powerful tool in personalized medicine. Its application in neoplasms is proving to be effective in enhancing risk assessment, diagnosis, and the development of individualized treatment plans. These technological advancements are poised to revolutionize the prevention, diagnosis, and treatment of hereditary cancers, offering new hope for individuals with Lynch Syndrome. [56, 57]

2. MATERIALS AND METHODS

Diagnosis of Lynch Syndrome (LS) involve a combination of genetic testing and clinical assessments to identify individuals at risk and confirm the diagnosis. The diagnosis process often begins with a detailed review of personal and family medical history, focusing on incidences of colorectal cancer, endometrial cancer, and other LS-associated cancers.

Two primary methods are used to screen for these genetic alterations:

1. Immunohistochemistry (IHC) Testing: This involves staining tissue samples from tumors to check for the presence or absence of MMR proteins. The absence of one or more MMR proteins suggests a potential LS diagnosis and warrants further genetic testing.

2. Microsatellite Instability (MSI) Testing: MSI testing identifies tumors characterized by MSI-High (MSI-H) status, a condition common in LS-associated cancers. This PCR-based test assesses the length of DNA segments known as microsatellites and is used to detect abnormalities in MMR function. (Fig. 12) [58]

3. Targeted sequencing including MLH1, MSH2, MSH6, PMS2, and EPCAM. This method allows for a more efficient and cost-effective analysis than broader sequencing approaches, facilitating the detection of mutations that contribute to the disease. (Fig. 13)

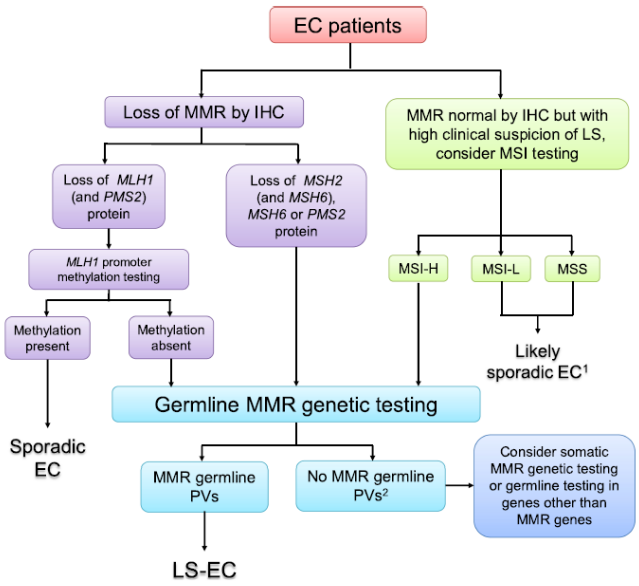

Figure 12 Algorithm for screening patients with EC for LS. Universal screening or selective screening according to high-risk factors for LS is recommended in EC patients. MMR-IHC of tumor tissues should be performed at first, If the expression of MMR proteins is normal by IHC without clinical suspicion of LS, it is likely sporadic EC and no further MSI testing is considered based on limited economic conditions, but MSI testing can be considered if economically feasible. The result of MLH1 (and PMS2) protein loss is followed by MLH1 promoter methylation testing. If results indicate MSI-H status or loss of MMR protein without MLH1 promoter methylation, germline MMR genetic testing is supposed to be performed. LS-EC will be diagnosed if there are MMR germline PVs found. 1Negative result cannot rule out LS definitively and that the results should be interpreted with the clinical information. 2If MSH2 and MSH6 are unmutated, consider EPCAM sequencing. EC, endometrial cancer; MMR, mismatch repair; IHC, immunohistochemistry; MSI, microsatellite instability; MSI-H, microsatellite instability-high; MSI-L, microsatellite instability-low; MSS, microsatellite stable; LS-EC, Lynch syndrome-associated endometrial cancer; PVs, pathogenic variants [Color figure can be viewed at wileyonlinelibrary.com]

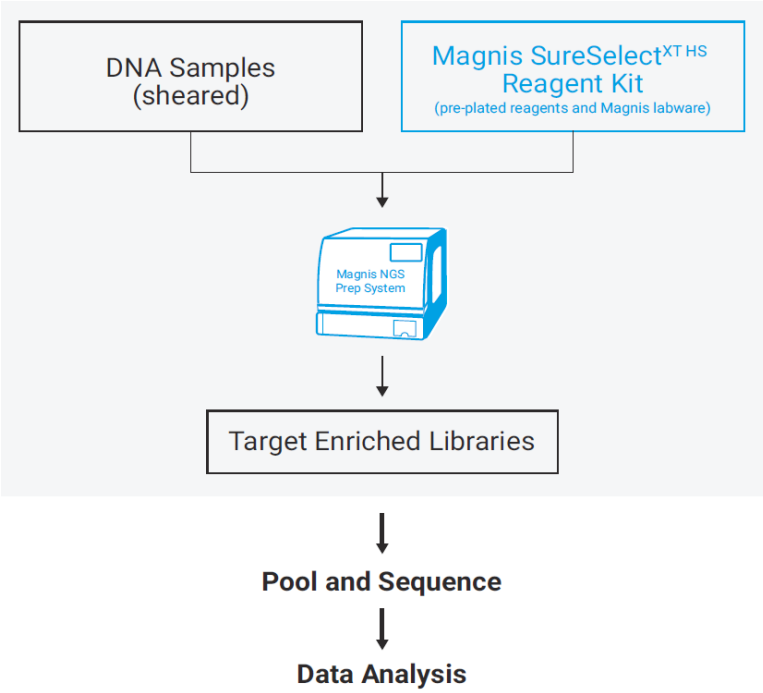

Figure 13 The workflow for SureSelect XT HS target enrichment using the Magnis NGS Prep. Once the sheared genomic DNA samples and the pre-plated reagents and labware are loaded, the Magnis NGS Prep System performs all SureSelectXT HS library preparation and target enrichment liquid handling and incubation steps. After the Magnis NGS Prep System run is complete, the target-enriched libraries are ready to be pooled for multiplexed NGS sample preparation and sequence analysis using Illumina HiSeq, MiSeq, NextSeq 1000 or NovaSeq 6000 sequencers. [59]

2.1 PATIENTS' ENROLMENT

A total of 46 patients suspected of colorectal cancer syndrome have been enrolled and underwent Targeted sequencing for genetic test of Lynch Syndrome. All the genetic analysis have been performed in the Laboratory of Genetics (SCDU Biochimica Clinica) of AOU Maggiore della Carità (Novara).

All the patients involved in this study have consented to perform genetic analyses after having signed an informed consent, that was received along with medical referral and corresponding clinical anamnesis.

2.2 GENOMIC DNA EXTRACTION

Patients DNA was extracted from 200µl of whole blood (anticoagulated with EDTA) using ReliaPrep™ Blood gDNA Miniprep System. [60] The DNA was eluted in a final volume of 50 µl of DNAse-free water, and subsequently quantified with NanoDrop One to assess the extraction quality. [61] Indexes of a good DNA extraction quality are the absorbance ratios 260/280 and 260/230, that should present values ranging between 1.8-2.0, and a DNA concentration ≥ 30 ng/µl.

2.2.1 Targeted Sequencing: Library preparation, Sequencing and Data analysis

Library preparation was performed with Magnis NGS Prep System [62] using SureSelect Enzymatic Fragmentation Kit [63], Magnis SureSelect XT HS NGS target enrichment kit [59] for Illumina multiplexed sequencing (Fig. 14), and the Agilent SureSelect Custom Constitutional Panel 17 Mb (Multiple Panel Gene) (Tab. 03) [64], according to manufacturer instructions. Then, the obtained libraries are pooled and quantified with Tapestation 4150 [65] to asses quality and the average fragment size; subsequently, the final pooled libraries are loaded in the P2 Reagent/Flow-cell Cartridge, in which Illumina sequencing will occur.

Figure 14 Magnis NGS Prep System from Agilent-Technologies and Illumina NextSeq1000

Sequencing probes cover all coding exons ±20 bp flanking sequence from the intron-exon boundary of 86 genes (Tab. 02) correlated to cancer syndromes among which MLH1, MSH2, MSH6, PMS2, and EPCAM are examined. The exon-enriched library was subjected to a 150 bp paired-end sequencing on the platform Illumina NextSeq1000 (Fig. 14) [66].

AIP	DICER1	MEN1	PTEN	TSC2
ALK	DIS3L2	MET	RAD51C	VHL
APC	DST	MLH1	RAD51D	WHSC1
ARID1A	EGFR	MLH3	RB1	WRN
ATM	EPCAM	MSH2	RET	WT1
BAP1	ERCC2	MSH6	RHBDF2	XPA
BLM	ERCC3	MTOR	ROS1	
BMPR1A	ERCC5	MUTYH	RUNX1	
BRCA1	EXT1	NF1	SBDS	
BRCA2	EXT2	NOTCH1	SDHAF2	
BRIP1	EZH2	NSD1	SDHC	
CDH1	FANCM	PALB2	SDHD	
CDK4	FLCN	PHOX2B	SMAD4	
CDKN1C	GPC3	PIK3CD	SMARCB1	
CDKN2A	HNF1A	PMS1	STK11	
CEBPA	HRAS	PMS2	SUFU	
CEP57	KIT	POLE	TAF1	
CHEK2	KMT2D	PRF1	TAF1L	
CTNNB1	KRAS	PRKAR1A	TP53	
DDB2	MAX	PTCH1	TSC1	

Table 2 Gene Panel

Sequencing begins with cluster generation, in which all the library fragments are isothermally amplified to obtain millions of fragment clusters through bridge amplification. (Fig. 15) Subsequently, reverse strands are cleaved and washed away, the 3'-ends locked, and the priming step with Read-1 sequencing primer occurs, initiating the first read. The extension of the first sequencing primer produces the first read, leading to forward-strands sequencing for each fragments-cluster. With each cycle, fluorescently tagged nucleotides compete for addition to the growing chain. Only one is incorporated based on the sequence of the template. After the addition of each nucleotide, clusters are excited by a light source, and a characteristic fluorescent signal is emitted (Sequencing-by-Synthesis).

After the completion of the first read, the read product is washed away, and Index1-read occurs. Then the 3'-ends of the template are deprotected, and the template folds over the second oligo on the flow cell leading to Index2-read and extension, forming a double-stranded bridge for the second read. This dsDNA is then linearized, the 3'-ends blocked, and the original forward strands cleaved and washed away. Follow the second read, which is performed as the previous one but with Read-2 sequencing-primer instead, leading to reverse-strands sequencing for each fragments-cluster.

The number of cycles determines the length of the read. The emission wavelength, along with the signal intensity, determines the base call. For a given cluster, all identical strands are read simultaneously. Hundreds of millions of clusters are sequenced in a massively parallel process.

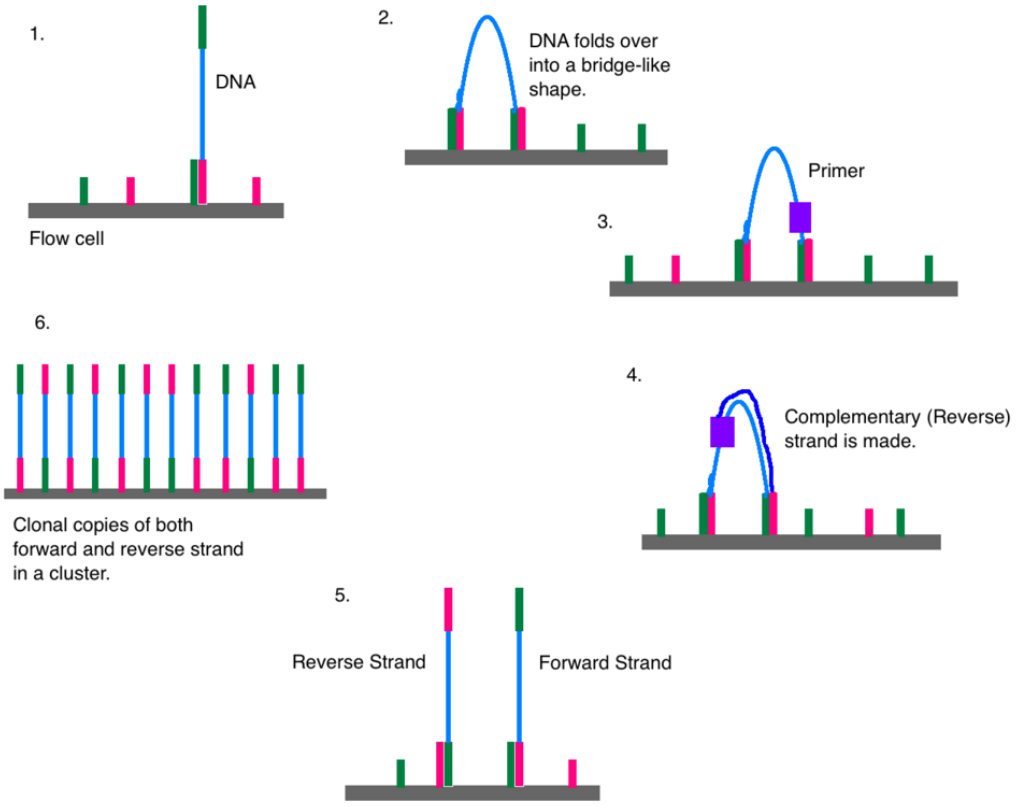

Figure 15 Illustration of the bridge amplification

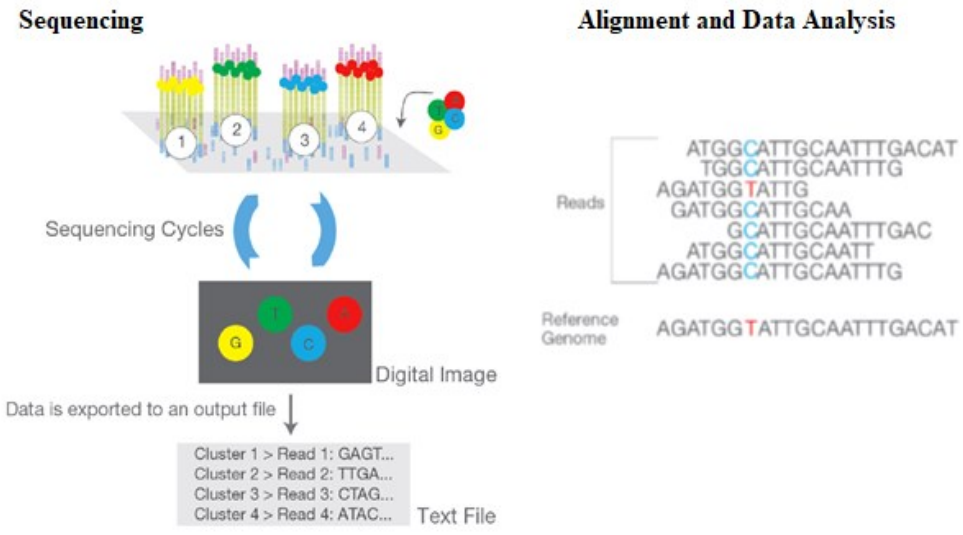

Figure 16 Schematic representation of Sequencing process and Data Analysis.

Data analysis: This entire process generates millions of reads, representing all the fragments. Sequences from all the sample libraries are separated based on the unique indices introduced during library preparation (Figure 15). For each sample, sequencing reads passing quality filters were aligned to the human reference genome build (Figure 16), and variant calling was performed using the SureCall v3.5 software (Agilent Technologies). Then, VCF files were annotated in Excel with the wANNOVAR tool [70]. Finally, data was analyzed using a personalized bioinformatics pipeline.

More in-depth, the chemistry at the basis of the Multiple panel gene library preparation is Hybridization-capture Target Enrichment. The panel consists of a targeted genome sequencing of 86 genes, to screen for mutations in loci of established clinical significance. This requires targeted enrichment of genomic regions of interest (ROIs), thus their focused augmentation of thousands of folds from the extensive entire genome background, allowing efficient and accurate NGS sequencing. This is necessary to ensure a sufficient sequencing depth of ROIs to confidently identify variants of interest while decreasing the risk of incidental findings with potential ethical complications. [67]

In target enrichment throughout Hybridization-capture, the input gDNA is fragmented using enzymatic or acoustic methods and then ligated at both ends with adapter sequences (containing unique barcode indices, sequencing-binding site, flow-cell oligos complementary sequences, and PCR amplification binding-site), following PCR amplification of the total gDNA, generating the genomic library. Subsequently, this library is denatured, and the ROIs are enriched by in-solution hybridization using biotin-labeled target sequence-specific single-stranded oligonucleotides probes, followed by capture of the hybridized probes using Streptavidin magnetic beads. Any nonspecific genomic background bound to the beads is eliminated by several stringent washing steps. The captured ROIs are then isolated by eluting from the streptavidin beads, following which a second PCR step is run, which further amplifies the enriched DNA before sequencing. [67]

2.3 MLPA

MLPA assay was performed with MRC Holland Probemix P003 MLH1/MSH2 and Probemix P008 PMS2 Applying the MLPA MRC Holland general protocol, according to manufacturer instructions. Subsequently, raw data analysis has been performed with Coffalyser software. About the molecular bases of MLPA: it is a multiplex PCR assay that utilizes a set of sequence-specific oligonucleotide probes, to evaluate the relative copy number of each DNA sequence or the presence of a specific mutation (small indels – SNV).

The MLPA reaction can be described into five steps: (1) DNA denaturation and probes hybridization; (2) ligation; (3) PCR amplification; (4) amplicons electrophoresis; (5) data analysis (Fig. 17)

First, the DNA is denatured and then incubated with a mixture of MLPA probes. Subsequently, the two half probes (5'-LPO and 3'-RPO of each probe) recognize contiguous target-specific sequences, and only in the presence of a perfect match without a single gap, after hybridization, can the two half-probes be ligated and amplified. PCR amplification is performed using only one PCR primer pair, one of which is fluorescently labeled. Because only ligated probes will be amplified, the number of probe ligation products is a measure of the number of target sequences in the sample. PCR products are then separated by size using Capillary Electrophoresis under denaturing conditions. The height or area of the PCR-derived fluorescence peaks is measured, quantifying the amount of PCR product after normalization, and comparing it with control DNA samples (Male and Female), thus indicating the relative amount of target DNA sequence in the input DNA sample.

Data analysis: the obtained raw data are subsequently processed and analyzed through bioinformatic software, such as Coffalyser. Homozygous or hemizygous deletions are evidenced by the absence of the specific peaks for the target gene, in the presence of a normal amplification of control probes. Conversely, heterozygous deletions, duplications, and CNVs produce a different height and/or area of the relative peaks. The presence of different efficiencies of the PCR reaction among the different probes, and sample-to-sample variations, could complicate the interpretation of results.

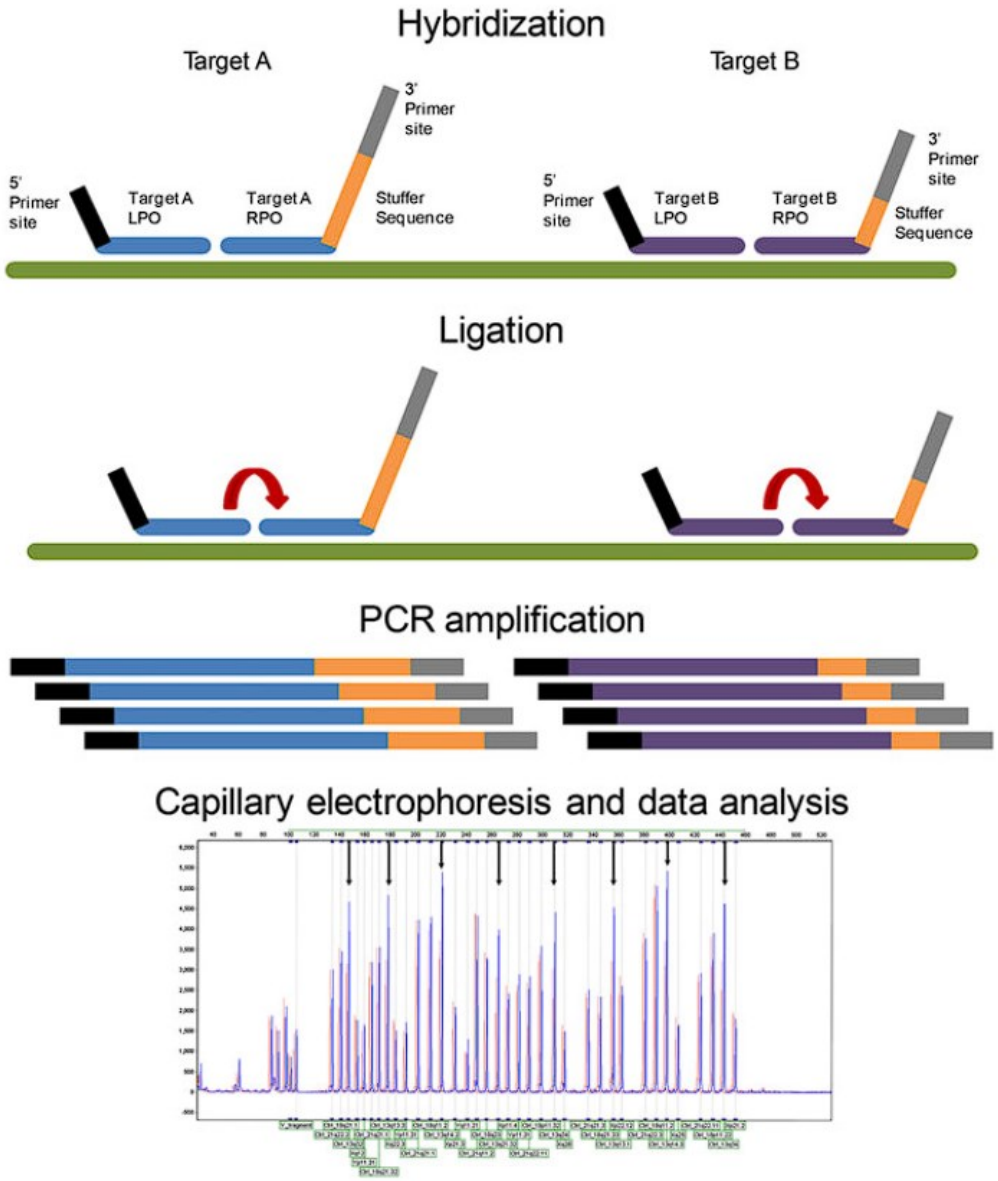

Figure 17 Schematic diagram of the MLPA process. (1) Hybridization of the probes to the target DNA sequence. (2) Ligation of adjacent bound probes. (3) PCR amplification of the ligated probe sequences using the universal primer set. (4) Capillary electrophoresis and (5) data analysis. The patient sample is shown in blue, and the control is shown in red for the sake of comparison. The data shows an increase in peak size for selected fragments indicating a gain in copy number for those locations. The arrows mark the peaks where the sample has increased signal over the control, indicating a gain in copy number in the region covered by the probes.

3. **RESULTS**

3.1 DATA ANALYSIS AND FILTERING

A total of 46 individuals suspected of having Lynch syndrome, including 21 males and 25 females, with an average age of 60 years were sent to the Genetic Laboratory for genetic testing.

The analysis was performed on a DNA specimen obtained from a peripheral blood sample using next-generation sequencing (NGS) technology. This analysis targeted the coding regions and adjacent 20 intronic bases surrounding the exons of the genes MLH1, MSH2, MSH6, and PMS2. No pathogenic point mutations were detected through this method. Additionally, a separate examination using the Multiplex Ligation-dependent Probe Amplification (MLPA) technique was conducted. This utilized the SALSA MLPA Probemix P003-D1 MLH1/MSH2 kit, focusing on the exons of the MLH1 and MSH2 genes as well as exon 9 of the EPCAM gene. Notably, this approach also facilitates the identification of a 10 Mb inversion impacting the short arm of chromosome 2, resulting in disruption and deactivation of the MSH2 gene.

To filter the clinically relevant variants in the 5 genes involved in Lynch Syndrome we proceeded as follows:

Firstly, only exonic/splicing variants with a MAF (Minor Allele Frequency) <1% in public databases (1000 Genome Project, ExAC, and GnomAD) were considered, and all synonymous variants were excluded. Among the selected variants priority was given to those that caused frameshift, stop codon, splicing, or amino acidic changes predicted as pathogenic.

Pathogenicity of variants was determined following the consensus guidelines of the American College of Medical Genetics and Genomics (ACMG), the Association for Molecular Pathology, enGenome eVai software (evai.engenome.com), The International Society for Gastrointestinal Hereditary Tumours (InSiGHT) [68], FranklinGeenox [69] and VarSome [70].

Moreover, also Omim [71] database and Orphanet [72] have been utilized to investigate the Gene-Phenotype relationships.

Among the 46 patients,18 carried pathogenic variants: 6 carried deletions, and the remaining 12 point mutations. (Tab. 03).

Code	Sex	Age	Point mutation	In-Dels	Gene	Zigosity	ACMG Classification	Clinical case
1244-21	M	76	Negative	Del exon 1-14	PMS2	het	Pathogenic	Sporadic
689-22	M	50	Negative	Del Whole gene	MLH1	het	Pathogenic	Familiar
1670-22	F	46	Negative	Del Whole gene	MLH1	het	Pathogenic	Familiar
1671-22	F	74	Negative	Del Whole gene	MLH1	het	Pathogenic	Familiar
690-22	M	77	c.1687 c>t p.Arg 563Ter (stop-gain)	Negative	PMS2	het	Pathogenic	Familiar
1621-22	M	53	Wild Type	Negative	PMS2	wt	/	Familiar
728-22	F	45	Negative	Del Ex 08	MSH2	het	Pathogenic	Familiar
1056-23	F	50	Negative	Del Ex 08	MSH2	het	Pathogenic	Familiar
1026-22	M	33	c.116+1 G>T (splicing)	Negative	MLH1	het	Pathogenic	Familiar
921-23	M	31	c.116+1 G>T (splicing)	Negative	MLH1	het	Pathogenic	Familiar
219-23	F	60	c.116+1 G>T (splicing)	Negative	MLH1	het	Pathogenic	Familiar
1162-22	F	60	⁞1del p.Asn444IlefsTer47	Negative	MLH1	het	Pathogenic	Familiar
105-24	F	83	Wild Type	Negative	MLH1	wt	/	Familiar
1668-22	M	74	C.677+3 A>G	Negative	MLH1	het	Likely Pathogenic	Sporadic
1667-22	M	48	C.1731+1 C>G (splicing)	Negative	MLH1	het	Pathogenic	Sporadic
636-23	F	59	c.1951G>T p.Glu651Ter (stop-gain)	Negative	MLH1	het	Likely Pathogenic	Sporadic
763-23	M	37	c.1216 C>T p.Arg 406Ter (stop-gain)	Negative	MSH2	het	Pathogenic	Sporadic
1019-23	M	55	Negative	Del Ex 08	MSH2	het	Pathogenic	Sporadic
124-21	M	56	Negative	EPCAM Del ex9 + MSH2 (1-8) exon	EPCAM + MSH2	het	Pathogenic	Sporadic
654-21	M	54	C.1865 T>C p. Leu622pro (non-synonimous SNV)	Negative	MLH1	het	Pathogenic	Familiar
1010-21	F	23	C.1865 T>C p. Leu622pro (non-synonimous SNV)	Negative	MLH1	het	Pathogenic	Familiar
1127-21	F	56	Wild Type	Negative	MLH1	wt	/	Familiar
1243-21	F	41	c.3001 G>T p.Glu1001Ter (stop-gain)	Negative	BRCA1	het	Pathogenic	Sporadic
1245-21	F	73	Negative	Del ex 06	MLH1	het	Pathogenic	Sporadic
1422-21	M	51	Negative	Del ex 08	MSH2	het	Pathogenic	Sporadic
4-22	F	66	c.1046 C>G p.Pro349Arg (non-synonimous SNV)	Negative	MSH2	het	Pathogenic	Familiar
303-22	M	30	Wild type	Negative	MSH2	wt	/	Familiar
299-22	M	61	›9dup p.Ter757Leuext*33 (stop-loss)	Negative	MLH1	het	Pathogenic	Familiar
143-23	M	23	Wild type	Negative	MLH1	wt	/	Familiar
144-23	M	63	›9dup p.Ter757Leuext*33 (stop-loss)	Negative	MLH1	het	Pathogenic	Familiar
924-23	M	58	Wild type	Negative	MLH1	wt	/	Familiar

Table 3 Patients with detected pathogenic variants

Pathogenic variants were identified in 39.1% of the patients in the investigated genes (Fig. 18). All the patients were analyzed for all the panel genes that also include no-LS genes. This might lead to the identification of mutations in non-typical Lynch Syndrome genes. In fact, we detected a mutation in a patient in *BRCA1* which is normally involved in breast/ovarian cancer. These can be considered either an incidental finding or an atypical cause of colorectal cancer.

The distribution of specific genetic mutations is detailed in our second pie chart (Fig), which categorizes the mutations into five distinct genes: MLH1, MSH2, PMS2, EPCAM, and BRCA1. Among these, MLH1 and MSH2 mutations are the most prevalent, appearing in 8 and 6 patients respectively. This is followed by PMS2 mutations in 2 patients, and EPCAM and BRCA1 mutations, each found in 1 patient. This distribution is pivotal for understanding the genetic landscape of the cohort and indicates a higher incidence of mutations in the MLH1 and MSH2 genes, which are strongly linked to Lynch Syndrome (Fig. 19).

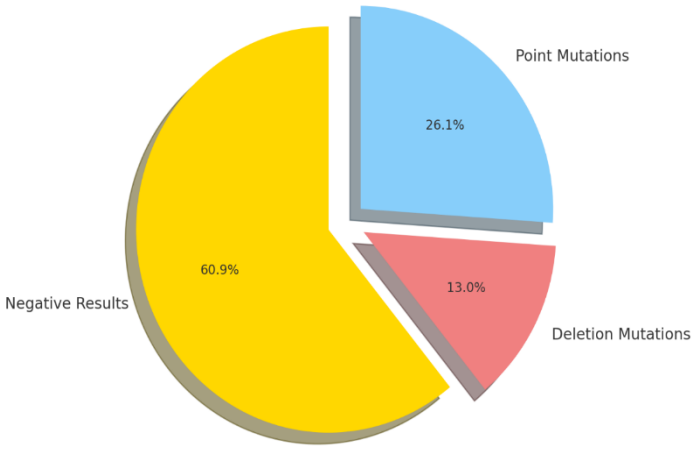

Figure 18 Test Result Distribution among patients

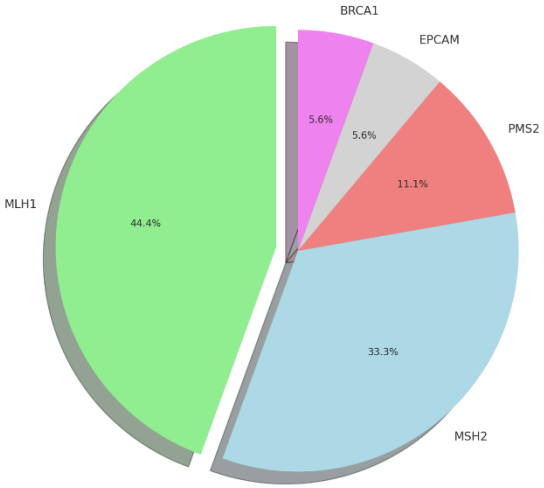

Figure 19 Distribution of Gene Mutations among patients

3.2 CASES RESOLVED WITH TARGETED NGS SEQUENCING

Patient #1244-21

This patient was a 73-year-old man who suffered from COPD (Chronic Obstructive Pulmonary Disease) and hypercholesterolemia, with no further significant clinical issues beyond the oncological history. The patient had abdominal pain and was diagnosed with G3 adenocarcinoma of the large intestine. Furthermore, he has diagnosed for diagnosis of urothelial bladder carcinoma. After checking his medical family history, we could not find any familial cancerous issues.

The genetic test performed on the tumor tissue identified the deletion involving exons 1-14 of the PMS2 gene which correlates with the diagnosis of Lynch Syndrome. While previous sequencing could not identify any point mutation the MLPA analysis allowed the identification of the PMS2 deletion

The PMS2 gene mutations increase the risk of colon cancer (8.7%-20% lifetime risk). Other associated but less prevalent tumors include urinary tract (up to 3.7%), stomach, and small intestine cancers, as well as skin and nervous system tumors. Males with this mutation face a higher prostate cancer risk.

Patient #689-22, #1670-22, and #1671-22

These individuals are members of the same family: Patient #689-22, a 48-year-old male diagnosed with colorectal cancer, where a colonoscopy and biopsy revealed colon adenocarcinoma. He exhibits high microsatellite instability (MSI-H) and is BRAF wild type. The MLPA analysis has highlighted the presence of a heterozygous deletion of the entire MLH1 gene compatible with a diagnosis of Lynch Syndrome. Patient #1671-22 is his 72-year-old mother, who transmitted the variant to her son. His sister (#1670-22), has inherited the MLH1 deletion.

Patients #690-22 and #1621-22

Patient #690-22 is the 75-year-old father and #1621-22 is the 51-year-old unaffected son. The father's medical history includes surgical treatment for aortic coarctation and pacemaker implantation in 1988, followed by a diagnosis of an aortic aneurysm in 2017. Additionally, colon wall thickening led to the discovery and surgical removal of NAS G2 Adenocarcinoma, with molecular analysis revealing high-frequency microsatellite instability (MSI) but negative for the BRAF mutation.

The genetic assessment through targeted sequencing revealed a heterozygous point mutation in exon 11 of the PMS2 gene (Fig. 20) (PMS1 homolog 2, the component of mismatch repair system; c.1687C>T. This mutation leads to the formation of a premature termination codon (p. Arg563Ter). This variant has not been listed in databases of polymorphisms (such as dbSNP, 1000 Genomes Project, ExAC Browser, ESP, gnomAD) and is predicted pathogenic based on the guidelines provided by the ACMG Laboratory Practice Working Group (Richards S. et al. Genet Med 2015; 17:405–424).
The segregation analysis revealed that #1621-22 did not exhibit the pathogenic variant and this finding allowed us to determine the low risk for this individual.

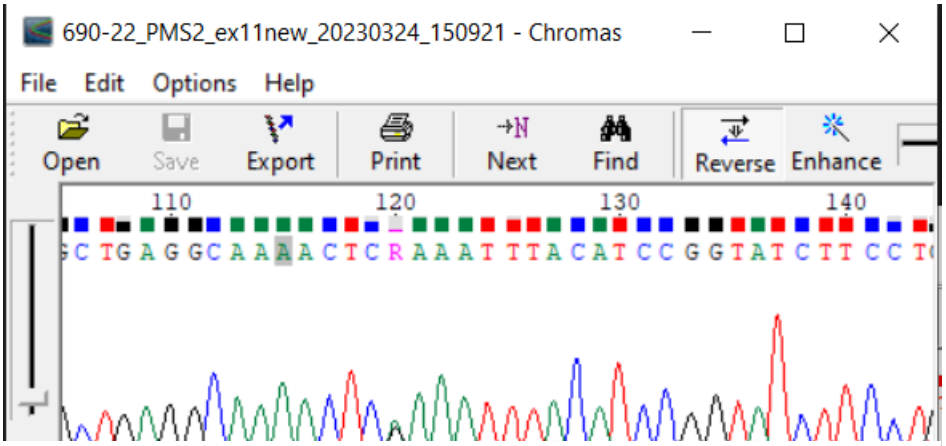

Figure 20 PMS2: c.1687 c>t (stop-gain)

Patient #728-22 and #1056-23

Patient #728-22 is a 42-year-old woman who had diabetes since the age of 10. Furthermore, the patient has been diagnosed with NAS adenocarcinoma of the large intestine, associated with high-grade dysplasia, and a tubular adenoma with low-grade dysplasia. Additionally, molecular analysis indicated high-frequency microsatellite instability (MSI-H) but showed that the BRAF gene is wild-type. Patient #728-22 and her sister #1056-23 carried a deletion that includes exon 8 of the MSH2 gene, which is compatible with a diagnosis of Lynch Syndrome.

Patients #1026-22, #921-23, and #219-23

These patients are from the same family. Patient #1026-22 is a 31-year-old man diagnosed with Lynch syndrome and his brother #921-23 carries the same pathogenic variant inherited from their mother #219-23.

The clinical history of #1026-22 in 2022 revealed anemia and a positive fecal occult blood test (FOBT). A subsequent comprehensive colonoscopy detected a neoplastic lesion. A right hemicolectomy was conducted, with histological evaluation confirming adenocarcinoma of the large intestine. Molecular testing identified high-frequency microsatellite instability (MSI-H) and confirmed the BRAF gene as wild-type.

The genetic analysis detected the presence of a heterozygous germline splicing variant at the donor site in the first intron of the MLH1 gene c.116+1G>T. (Fig. 21) The variant is not reported in polymorphism databases (dbSNP, 1000 Genomes Project, ExAC Browser, ESP, gnomAD) and is classified as pathogenic according to the ACMG Laboratory Practice Working Group guidelines. Moreover, the same variant is reported in ClinVar as likely pathogenic.

The heterozygous germline splicing variant c.116+1G>T in the donor site of the first intron of the MLH1 gene is classified as pathogenic due to its critical impact on gene expression and function. This specific mutation occurs at a splice site, crucial for the correct processing of the MLH1 pre-mRNA into mature mRNA. As a result, the alteration can disrupt the normal splicing process, leading to abnormal protein production or loss of function. Given the MLH1 gene's vital role in DNA mismatch repair, such disruptions can significantly increase the risk of developing Lynch syndrome-associated cancers.

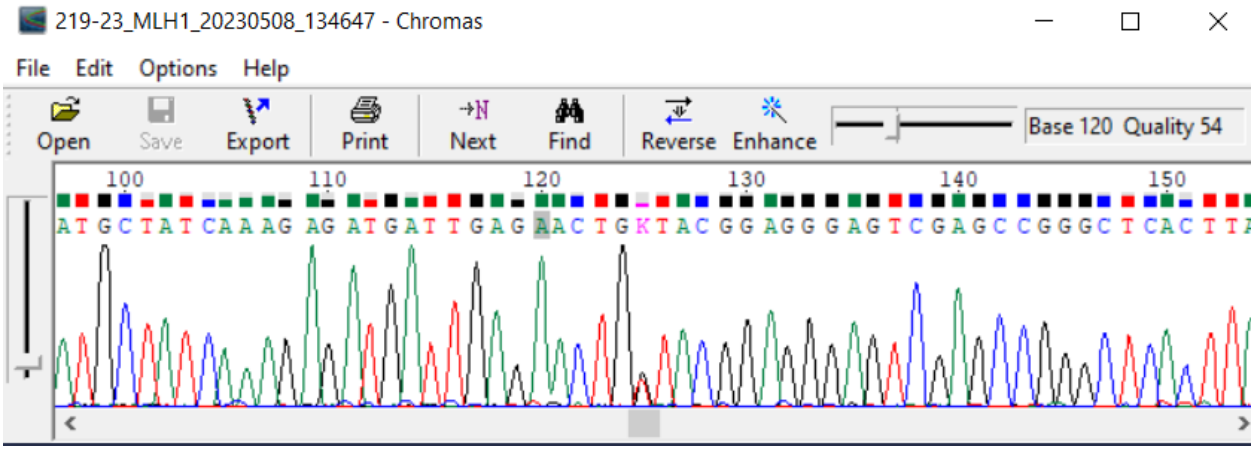

Figure 21 MLH1: c.116+1G>T (splicing)

Patients #1162-22 and #105-24

These patients are from the same family. Patient #1160-22 is a 58-year-old woman which diagnosed with mucinous adenocarcinoma of the colon in 2015, followed by a diagnosis of poorly differentiated G3 endometrioid adenocarcinoma of the uterine body with partial clear cell features in February 2022.

The genetic analysis (TS) revealed the presence of a germline frameshift variant in heterozygosity in exon 12 of the MLH1 gene (mutL homolog 1; NM_000249.4) c.1331del which results in a frameshift and the presence of a premature stop codon (p. Asn444IlefsTer47). The variant is not reported in the polymorphism databases (dbSNP, 1000 Genomes Project, ExAC Browser, ESP, gnomAD) and is classified as pathogenic according to the ACMG Laboratory Practice Working Group guidelines (Richards S. et al. Genet Med 2015; 17: 405–424).

When this gene is mutated, especially by a frameshift leading to a premature stop codon, it results in a truncated, nonfunctional protein. This inactivation impairs the MMR system, leading to an accumulation of DNA replication errors and an increased risk of developing cancer, particularly colorectal cancer and other types associated with Lynch Syndrome. The loss of MLH1 function due to such mutations is a well-documented cause of hereditary cancer syndromes, underlining the variant's pathogenic nature. MLH1 function due to such mutations is a well-documented cause of hereditary cancer syndromes, underlining the variant's pathogenic nature. The genetic analysis of #105-24 showed that she doesn’t carry this variant.

Patient #1668-22

This patient is a 72-year-old man diagnosed with adenocarcinoma of the colon. Immunohistochemical tests to assess the mismatch repair (MMR) complex proteins revealed the absence of hMLH1 and hPMS2, while hMSH2 and hMSH6 were present.

The DNA extracted from peripheral blood leukocytes was analyzed to assess the presence of the following pathogenic germline variant previously identified in a family member in the MLH1 gene c.677+3A>G; in intron 8 of the MLH1 gene. Based on ACMG classification, this variant is Likely pathogenic, and splicing mutation was identified in the MLH1 gene c.677+3A>G.

Patient #1667-22

This patient is a 46-year-old man with no notable past clinical issues who was diagnosed with ulcerated vegetating adenocarcinoma following tests for anemia and abdominal pain. The patient underwent a left hemicolectomy with laparoscopic total mesorectal (TT) mechanical anastomosis. Microsatellite instability testing revealed MSI-H, BRAF wild type. The patient is currently undergoing adjuvant chemotherapy. No familial history has been reported.

The analysis detected the presence of a germline splicing variant in heterozygosity at the donor site, in intron 15 of the MLH1 gene c.1731+1G>C. The variant is not reported in the polymorphism databases and is classified as pathogenic according to the guidelines of the ACMG Laboratory Practice Working Group. Moreover, the same variant is reported in ClinVar as pathogenic/probably pathogenic. The presence of the variant was confirmed in the patient by traditional sequencing using the Sanger method.

It’s confirmed the c.1731+1G>C variant in the MLH1 gene found in the patient is to be considered causative of the patient's clinical phenotype.

Patient #636-23

The patient, a 57-year-old, experienced various health issues over time, including abdominal pain leading to a left hemicolectomy for intestinal obstruction. Pathological examination revealed G3 adenocarcinoma of the descending-sigmoid colon with positive microsatellite instability. Subsequent routine check-ups detected low-grade endometrioid carcinoma of the endometrium, leading to a radical hysterectomy with bilateral salpingo-oophorectomy, where remnants of G1 endometrioid carcinoma were confirmed alongside microsatellite instability. Additionally, the patient underwent the removal of a basal cell carcinoma located near the right eyebrow.

The analysis has detected the presence of a heterozygous point germline variant in exon 17 of the MLH1 gene c.1951G>T, which results in the appearance of a premature stop codon (p.Glu651Ter). The variant is not reported in polymorphism databases (dbSNP, 1000 Genomes Project, ExAC

Browser, ESP, gnomAD) and is classified as probably pathogenic according to the guidelines of the ACMG Laboratory Practice Working Group. It is in the functional domain "DNA mismatch repair protein Mlh1, C-terminal" of the protein.

The specific mutation mentioned, p.Glu651Ter, results in a premature termination of the protein sequence, leading to a truncated, and usually nonfunctional, protein. This type of mutation can disrupt the normal function of the MLH1 protein in DNA repair. Normally, MLH1 works together with other proteins to repair mistakes made during DNA replication. When MLH1 is not functioning properly due to a mutation, errors accumulate in the DNA, leading to increased mutations in other genes, particularly those that control the growth and division of cells. This increased rate of mutations can lead to cancer.

Patient #763-23

This patient is a 36-year-old man who was diagnosed with colorectal cancer. He experienced hematochezia, leading to the discovery of a sessile polyp with high-grade dysplasia through rectosigmoidoscopy, and a vegetative lesion in the proximal rectum identified by pan colonoscopy. Histological analysis confirmed high-grade dysplasia with MSI-H and BRAF wild type. The patient underwent neoadjuvant chemotherapy, followed by surgery with colorectal anastomosis, which revealed a histological diagnosis of G2-G3 adenocarcinoma of the large intestine. Subsequent treatments included radiation therapy (RT) and adjuvant chemotherapy.

The analysis revealed the presence of a germline point variant in heterozygosity in exon 7 of the MSH2 gene c.1216C>T, which results in the appearance of a premature stop codon (p.Arg406Ter). The variant is not reported in polymorphism databases (dbSNP, 1000 Genomes Project, ExAC Browser, ESP, gnomAD) and is classified as pathogenic according to the ACMG Laboratory Practice Working Group guidelines. It is in the functional domain "DNA mismatch repair protein MutS, core" of the protein. The variant is also reported in ClinVar as pathogenic and is described in the literature in patients suffering from colorectal cancers (CRC).

The MSH2 gene produces a vital protein for the DNA mismatch repair (MMR) mechanism, which fixes replication errors like base mismatches and insertion/deletion loops. The c.1216C>T mutation converts an arginine (Arg) into a stop codon (Ter) at the protein's 406th position (p.Arg406Ter), creating a likely non-functional or less effective truncated protein.

the c.1216C>T (p.Arg406Ter) variant in the MSH2 gene identified in the patient can be considered responsible for the patient's clinical phenotype.

Patient #1019-23

This patient is a 54-year-old man with diabetes mellitus who underwent tests due to anemia, leading to a colonoscopy that found abnormal growths in the colon. Surgery revealed

adenocarcinomas with mucinous traits in different parts of the colon. Tumor tissue examinations indicated high microsatellite instability and the presence of KRAS and KIT mutations, with no BRAF mutations detected.

The genetic analysis performed revealed the presence of a heterozygous deletion involving exon 8 of the MSH2 gene (Fig. 22) compatible with a diagnosis of Lynch Syndrome. Furthermore, no deletions/duplications were detected in the MLH1, PMS2, and MSH6 genes and exon 9 of the EPCAM gene.

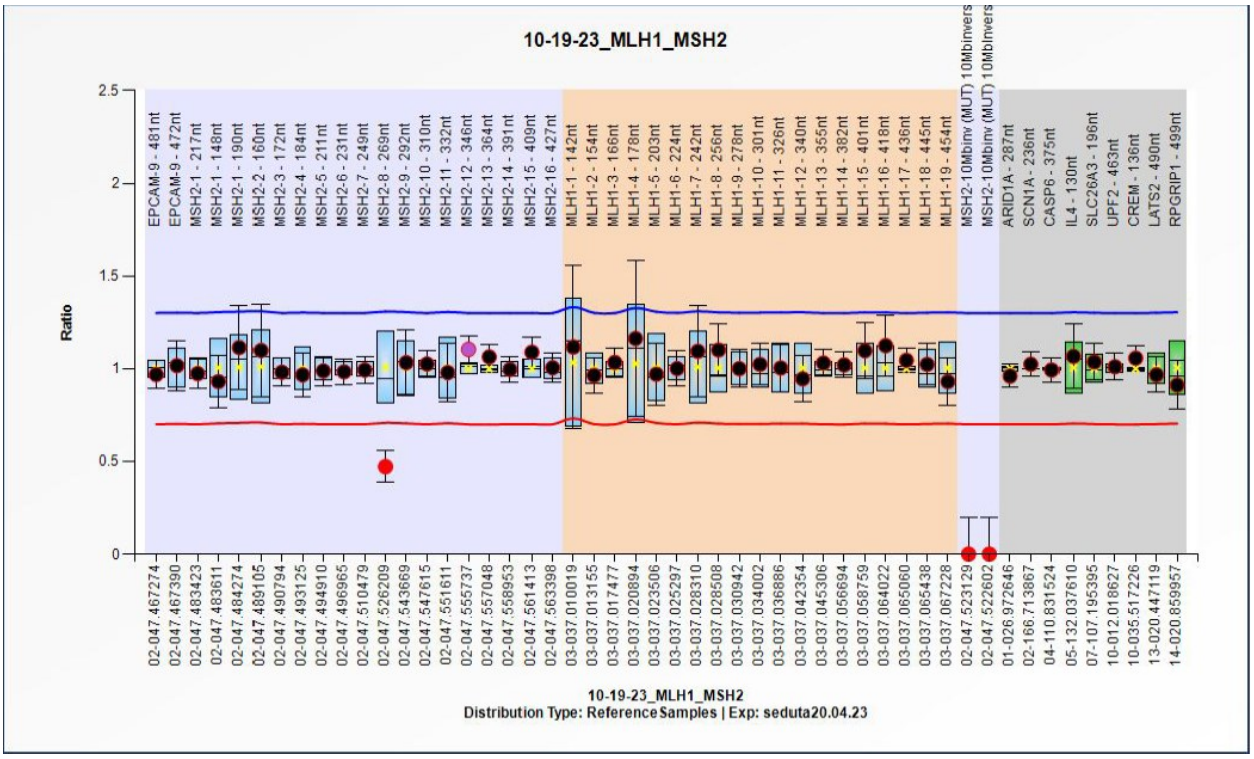

Figure 22 MLPA result: deletion exon 8 of the MSH2

Patient #124-21

This was a 52-year-old man diagnosed with LS. Furthermore, the patient has hypertension and has been under treatment for 3 years, moreover, he was diagnosed with high-grade dysplasia and a second moderately differentiated G2 colonic adenocarcinoma by colonoscopy in 2020. At the age of 35, he underwent a resection of the right ureter for transitional cell carcinoma of the ureter. In his medical family history, there is no reported HNPCC, however, from his paternal family, his grandmother has died from reported colon carcinoma.

The analysis was performed using MLPA (Multiplex Ligation-dependent Probe Amplification) with the SALSA MLPA Probemix P003-D1 MLH1/MSH2 kit on the exons of the MLH1 and MSH2 genes and exon 9 of the EPCAM gene on a new sample.

The deletion involving exon 9 of the EPCAM gene and exons 1-8 of the MSH2 gene disrupts the normal functioning of these genes. For the EPCAM gene, deletions can lead to reduced expression or complete loss of the gene product, impacting cell adhesion and potentially leading to increased cell proliferation. More critically, deletions in the MSH2 gene disrupt the mismatch repair system, increasing the likelihood of mutations in other genes, especially those related to cell cycle control and apoptosis, which can lead to uncontrolled cell growth and cancer.

Together It's confirmed the presence of a heterozygous deletion that includes exon 9 of the EPCAM gene and exons 1-8 of the MSH2 gene, compatible with a diagnosis of Lynch Syndrome.

Patients #654-21, #1010-21, and #1127-21

These three individuals belong to the same familial lineage. Patient #645-21, is a 51-year-old male diagnosed with colorectal carcinoma at approximately 50 years of age, exhibiting microsatellite instability within the tumor tissue. An examination of the family history has revealed a pattern of oncological conditions within the paternal side of the family. Patient #1010-21 is the daughter 20-year-old who inherits the same pathogenic variant, while patient #1127-21, his sister is 53 years old and displays the wild-type genotype, indicating she does not carry the significant familial mutation.

The genetic test analysis detected the presence of a non-synonymous germline variant at the heterozygous state in exon 16 of the MLH1 gene c.1865T>C, which results in the amino acid substitution of Leucine at position 622 of the protein with Proline (p.Leu622Pro). The variant is not reported in the polymorphism databases (dbSNP, 1000 Genomes Project, ExAC Browser, ESP, gnomAD) and is classified as pathogenic according to the guidelines of the ACMG Laboratory Practice Working Group.

Based on the investigation variant c.1865T>C (p.Leu622Pro) in the MLH1 gene found in the patient can be considered responsible for the patient's clinical phenotype.

Patient #1243-21

This patient was a 38-year-old woman who was investigated for the BRCA1 and BRCA2 genes and genes associated with Lynch Syndrome (MLH1, MSH2, MSH6, PMS2). Since 2020, findings of hematochezia and mild anemia with iron deficiency attributed to hemorrhoids led to the detection of an anal plaque lesion with a heteroplastic appearance through the pan colonoscopy which was histologically examined as Adenomacarinoma. In 2021 poorly differentiated (G3) infiltrating ductal carcinoma of the breast was detected and concurrent with G2 colonic adenocarcinoma leading to Immunohistochemistry on tumor tissue. The absence of PMS2 has been reported. Also, No family history of oncological issues has been reported.

The genetic test performed (TS) showed the presence of a heterozygous point germline variant in exon 10 of the BRCA1 gene c.3001G>T which results in the appearance of a premature stop codon

(p.Glu1001Ter). This variant is classified as pathogenic according to the ACMG Laboratory Practice Working Group guidelines.

Most tumors occur sporadically, influenced by various genetic and environmental factors. However, a minority are hereditary, primarily due to mutations in the BRCA1 or BRCA2 genes, significantly increasing the risk of developing breast and ovarian cancers. Individuals with these genetic mutations face higher cancer risks compared to the general population, characterized by an earlier onset, higher rates of bilateral tumors, and more family members affected. For women carrying BRCA mutations, comprehensive breast cancer screening programs and preventive surgeries like bilateral prophylactic mastectomy are available options that substantially reduce cancer risk. Additionally, there are specific gynecological surveillance strategies for detecting early signs of ovarian cancer, aimed at those with BRCA gene mutations.

Based on current knowledge, the variant c.3001G>T (p.Glu1001Ter) in the BRCA1 gene found in patient #1243-21 can be considered responsible for the patient's clinical phenotype. Furthermore, the presence of deletions/duplications in the MLH1 and MSH2 genes and exon 9 of the EPCAM gene was not revealed. It is necessary to conduct further investigation into this patient's case to elucidate the potential correlation between BRCA1 gene mutations and hereditary nonpolyposis colorectal cancer (HNPCC).

Patient #1245-21

This patient is a 70-year-old woman who has had multiple health issues over the years, starting with a diagnosis of right colon adenocarcinoma at the age of 52. Following this, the patient underwent surgery for a benign condition that required a hysterectomy and oophorectomy. Years later, the patient was diagnosed with moderately differentiated peripheral cholangiocarcinoma, followed by lung adenocarcinoma. Most recently, the patient was diagnosed with ductal carcinoma in situ of the left breast, which was estrogen receptor-positive at 90% and progesterone receptor-negative. Her personal and family history was suggestive of a predisposition to tumor development on a genetic basis; in particular, the patient had received a diagnosis of colon tumor at the age of 52 with a family history of colon cancer (father and two brothers) which indicated a possible Lynch Syndrome, also noted were diagnoses of breast carcinoma, cholangiocarcinoma, and lung carcinoma in the patient, and a family history of pancreatic, renal/urinary tract, and lung tumors.

The analysis was performed on a DNA sample extracted from a peripheral blood draw using next-generation sequencing (NGS) applied to the coding region and the 20 intronic bases flanking the exons of the MLH1, MSH2, MSH6, and PMS2 genes. This analysis did not reveal any pathogenic point variants.

An analysis was performed using Multiplex Ligation-dependent Probe Amplification (MLPA) with the SALSA MLPA Probemix P003-D1 MLH1/MSH2 kit on the exons of the MLH1 and MSH2 genes and exon 9 of the EPCAM gene. The kit also allows for the detection of a 10 Mb inversion affecting the short arm of chromosome 2 resulting in the breakage and inactivation of

the MSH2 gene. This analysis revealed the presence of a heterozygous deletion involving exon 6 of the MLH1 gene.

The analysis highlighted the presence of a heterozygous deletion that includes exon 6 of the MLH1 gene compatible with a diagnosis of Lynch Syndrome.

Patient #1422-21

This patient was a 48-year-old man who was diagnosed with colon carcinoma and prior he was affected with abdominal pain and hyperpyrexia because of mucinous adenocarcinoma, also he had an acute episode of pancreatitis in 2010. The medical family history does not show any specific hereditary syndrome, especially HNPCC. However, some of the family members have died and are affected by different types of cancers.

The genetic analysis conducted by NGS applied to the exons of the genes MLH1, MSH2, MSH6, and PMS2, which did not reveal any pathogenic point variant. However, for confirmation, we performed MLPA with the SALSA MLPA Probemix P003-D1 MLH1/MSH2 kit on the exons of the MLH1 and MSH2 genes and exon 9 of the EPCAM gene.

The deletion of exon 8 of the MSH2 is a pathogenic variant that disrupts the normal functioning of these genes related to the mismatch repairing system and this analysis indicates the presence of a heterozygous deletion that includes exon 8 of the MSH2 gene compatible with a diagnosis of Lynch Syndrome.

Patients #4-22 and #303-22

These two patients are part of the same family (mother: patient #4-22; son: patient #303-22). In particular, the mother is a 63-year-old diagnosed with Lynch Syndrome. She has colon bilateral renal pelvis carcinoma, endometrial and ovarian carcinoma, and colon carcinoma. In 2017 she was diagnosed with adenocarcinoma in the colon, Immunohistochemical investigation shows the presentation of Mlh1 and PMS2 and the absence of MSH2 and MSH6. Instead, her son (28 years old - patient 303-22) is in good health. No significant clinical issues were reported.

The genetic analysis (Targeted exome sequencing) revealed the presence of a heterozygous non-synonymous germline variant in exon 6 of the MSH2 gene c.1046C>G, which results in the amino acid substitution of Proline at position 349 of the protein with Arginine (p.Pro349Arg). The variant is not reported in polymorphism databases (dbSNP, 1000 Genomes Project, ExAC Browser, ESP, gnomAD) and is classified as pathogenic according to the guidelines of the ACMG Laboratory Practice Working Group.

It is confirmed that the variant c.1046C>G (p.Pro349Arg) in the MSH2 gene found in the patient can be considered responsible for the patient's clinical phenotype. No presence of deletions/duplications in the MLH1 and MSH2 genes and exon 9 of the EPCAM gene was revealed during MLPA.

Patients #299-22, #143-23, #144-23, and #924-23

These four patients are part of the same family (Uncle of #143-23, Nephew of #299-22, Brother of #299-22, Brother of #299-22). Patient #299-22 is a 58-year-old which diagnosed with colon cancer. This family exhibits a hereditary predisposition to colorectal carcinoma, evidenced by a significant history of the disease within the paternal lineage, including the patient's father, aunt, two cousins, and brother (patient #144-23). He has been diagnosed with adenocarcinoma of the large intestine, microsatellite instability, and no expression of MLH1 and PMS2 after IHC examination. The patient #143-23 and #924-23 didn’t show a significant variant. On the other hand, an investigation on patient #144-23, a 61-year-old brother detected the presence of a significant pathogenic variant.

The genetic test analysis (TS) has detected the presence of a heterozygous frameshift variant in exon 19 of the MLH1 gene c.2269dup, which results in the loss of the stop codon, leading to an elongation of the protein (p.Ter757Leuext*33). The variant is not reported in polymorphism databases (dbSNP, 1000 Genomes Project, ExAC Browser, ESP, gnomAD) and is classified as pathogenic according to the guidelines of the ACMG Laboratory Practice Working Group. Furthermore, the same variant is reported in ClinVar as pathogenic.

The heterozygous frameshift variant in exon 19 of the MLH1 gene, c.2269dup, is implicated in Lynch syndrome. (Fig. 23) This condition is predominantly linked with elevated lifetime risks of colorectal (46-61%) and endometrial cancer (34-54%) in females. Other malignancies associated with lower incidences include ovarian (4-20%), urinary tract (0.2-5%), pancreatic and biliary tract (1.9-6.2%), gastric and small bowel cancers, and cutaneous and central nervous system tumors. An augmented risk for prostate cancer in males and a tentative increase in breast cancer risk have also been observed.

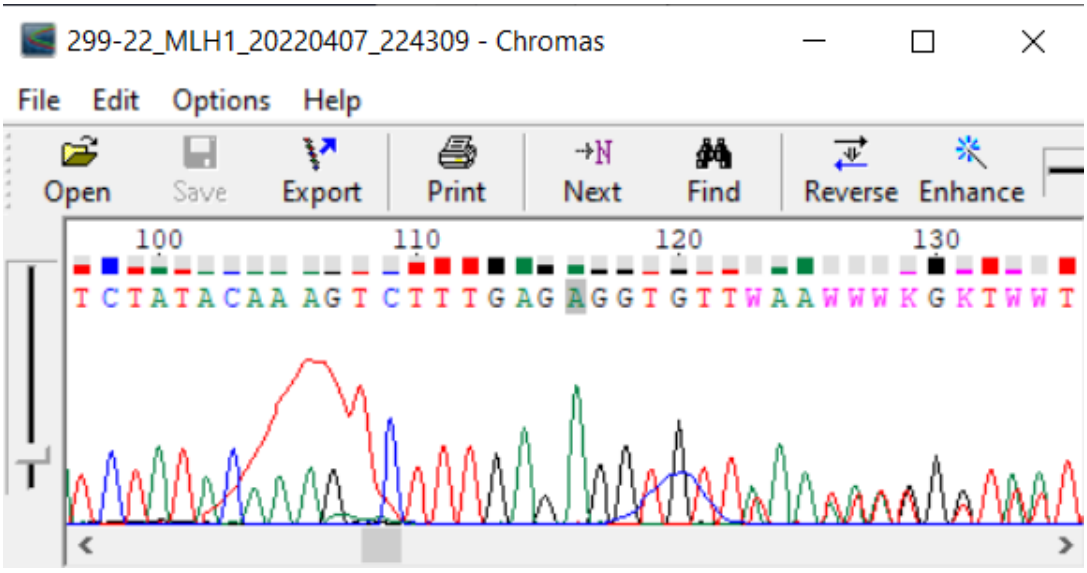

Figure 23 MLH1: c.2269dup in exon 19

4. DISCUSSION

Overall pathogenic variants were detected in 18 patients, 66.67% male and 33.33% female, all diagnosed with colorectal cancer (CRC). Targeted sequencing of the coding region and the 20 intronic bases flanking the exons of the MLH1, MSH2, MSH6, and PMS2 genes, MLPA for MLH1/MSH2 and deletion of exon 9 in EPCAM was performed. The majority of the genetic alterations identified by Targeted Sequencing were deletions in *MSH2*, *MLH1*, *PMS2,* and *EPCAM)* while only 4 were stop gain (in *PMS2*, *MLH1*, *MSH2*, and *BRCA1*). Three patients showed splicing mutations in *MLH1*. Two patients exhibited non-synonymous single nucleotide variations (SNVs), in *MLH1* and *MSH2.* Additionally, a frameshift and stop loss mutation was detected in 2 patients in *MLH1* (Fig. 24).

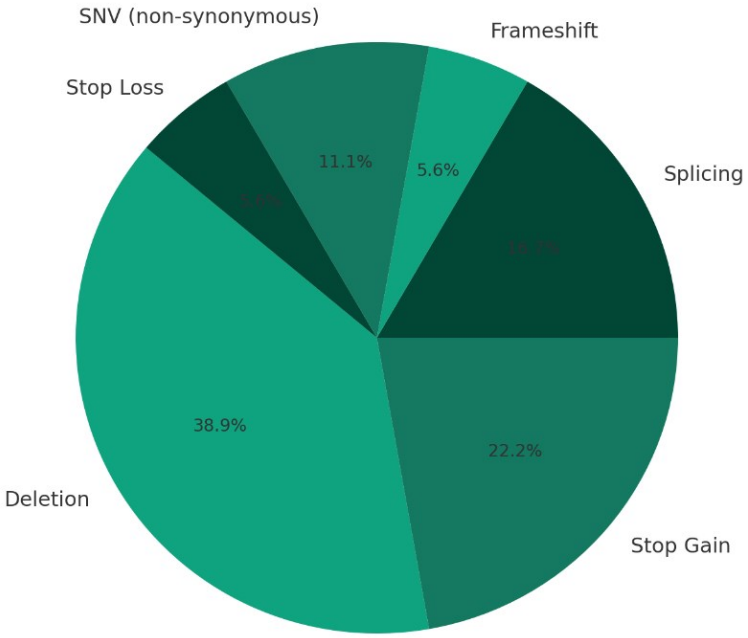

Figure 24: Distribution of mutation types in patients

Our data showed a higher prevalence of MLH1 and MSH2 mutations with respect to the other genes. Comparing our results to other studies our data align closely with already published data [73], reinforcing the significant contribution of MLH1 and MSH2 in Lynch syndrome whereas in another studies [74] MSH2 was the principal mutated gene.

Our results reinforce the value of genetic screening as an indispensable tool in the early detection and management of hereditary cancers. The individuals who tested negative to our genetic test develop a condition that is clinically identical to Lynch syndrome but is not caused by the known genetic mutations associated with the syndrome. There might be many reasons fot the negative results. For example the disease might be due to other genes which not included in the NGS panel (Tab. 02). The other possibility is an epigenetic mutation, for example, loss of MLH1 in these cancers can often be due to an epigenetic change, specifically the methylation of the MLH1 gene promoter. This methylation prevents the gene from being expressed as a protein[75] and this condition is not hereditary. Standard NGS is not capable of evaluating this kind of epigenetic deficiency, because related to DNA or chromatin modifications that impact gene expression but not on the sequence. Moreover introns and regulatory regions are not included in the targeted panel and this is an important limitations that NGS might not cover these areas as mutation that affect the splicing or the gene expression regulation might occur rwithin these regions.

A limitation of NGS is that variant-call accuracy of small insertions or deletions (indels) is lower than that of single nucleotide variants (SNVs) and sequence reads of genes for which there are also pseudogenes within the genome or large gene families, may be difficult to map, impairing the ability to call variants for these regions. Furthermore, trinucleotide expansion disorders cannot be detected by NGS, because the pathogenic expanded repeats are too long to be assessed by any of the currently available short-read NGS technologies. Thus, NGS sensitivity is not 100 %, and it may not be able to detect all the genetic causes of the disease.

It's remarkable that in a patient we identify an "unexpected" high-penetrance germline mutation in BRCA1, that do not seem concordant with the clinical histories raising the question as to whether hereditary cancer syndromes should be defined based on genotypic data, phenotypic data, or both. Based on prior studies usually there is no increased CRC risk in BRCA1/2 probands. [13] Lynch Syndrome (LS) and Hereditary Breast and Ovarian Cancer (HBOC) are considered distinct syndromes with different clinical manifestations. However, both syndromes are acknowledged to share a common risk factor, as they both lead to an increased risk of ovarian cancer. In this study, however, *BRCA1* mutation in #1243-21 had phenotypes that were markedly more "Lynch-like" than "HBOC-like". This patient will be thus followed up for the presence of other tumors related to BRCA1 and the family screening will be performed to identify at risk individuals.

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