Branching out: the clinical relevance of mismatch repair variants

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General discussion

BRIEF SYNOPSIS

This thesis explores three aspects of mismatch repair (MMR) deficiency.

Part I evaluates the effectiveness of universal tumor screening in identifying individuals with Lynch syndrome (LS). A systematic review (**Chapter 2**) of 58,580 colorectal cancer (CRC) cases found that MMR deficiency was present in 6.22%, while a germline pathogenic variant (PV) was identified in 2.00%, highlighting variability in diagnostic completeness. However, gaps remain, with 11.81% of immunohistochemistry (IHC) results missing and 0.61% of MMR-deficient tumors remaining unexplained. **Chapter 3** examines the challenges in diagnosing *MSH6*-associated LS, revealing that discordant IHC and microsatellite stability (MSS) phenotypes can lead to missed diagnoses. Importantly, further molecular analyses demonstrated that some of these discordant cases were due to sporadic tumors rather than LS, emphasizing the need for a comprehensive diagnostic approach that considers family history and broader germline testing.

Part II examines cancer risks in LS, with a specific focus on *MSH6* (likely) pathogenic ((L)P) variant carriers **Chapter 4,** based on a Dutch cohort of 1118 *MSH6* variant carriers, refines cancer risk estimates: by age 80, colorectal cancer risks were 36% (95% CI: 25-48%) for males and 21% (95%CI: 15-43%) for females, while the risk of endometrial cancer was 23% (95%CI: 15-43%). Elevated risks were also observed for ovarian, urinary tract, and biliary tract cancers, but not for prostate or breast cancer. These findings suggest a need for adjusted surveillance strategies, including delayed CRC screening and consideration of prophylactic surgery for females. **Chapter 5** broadens this assessment by summarizing extracolonic cancer risks in LS patients as a group. One of the key findings is the variability in cancer risks, both between and within families. Although the specific MMR gene carrying the (L)P variant is a significant factor, variations in cancer risks persist even among individuals with the same (L)P variant. **Chapter 6** further investigates genotype-phenotype correlations and the role of the parent-of-origin effect (POE). While no POE was found for *MSH6* cancer risks, RNA expression patterns appeared to influence endometrial cancer risk—though this observation requires further validation.

Part III (**Chapter 7**) assesses the potential of whole-exome sequencing (WES) and mutational signatures to classify MMR variants of uncertain significance (VUS). Although WES did not lead to direct reclassification of VUS in this study, it clarified phenotypic aspects such as age of onset and discordant MMR staining patterns. These findings underscore the value of integrating tumor sequencing with functional assays to improve variant interpretation. In this final chapter, the key findings are reviewed and discussed in depth, framed within the context of the existing literature to provide a comprehensive understanding of the broader implications of these studies.

Part I - Detection of Lynch Syndrome via Universal Tumor Screening

Understanding whether a tumor exhibits mismatch repair deficiency (MMRd) holds significant implications for both treatment planning and predicting the tumor's response to therapy.[1-4] MMRd can result from somatic or germline variants or a combination thereof in the mismatch repair (MMR) genes. The identification of a (likely) pathogenic germline MMR variant confirms the diagnosis of LS, a hereditary condition associated with an elevated risk of colorectal cancer (CRC), endometrial cancer (EC), and various extracolonic cancers.[5] LS patients are advised to undergo regular colonoscopies and other surveillance measures to mitigate their cancer risk.[6] Therefore, early detection of LS patients is important to offer appropriate surveillance. In the Netherlands, universal tumor screening involves immunohistochemistry of the MMR proteins in colorectal or endometrial cancer cases before the age of 70 years.[7]

Our analysis[8], which includes meta-analyses and -regression of data from 56 studies involving 58,580 cases of colorectal cancer (CRC), highlights that the effectiveness of universal tumor screening depends significantly on the thoroughness of the diagnostic process. A (likely) pathogenic germline variant was identified in 2.00% of CRC cases diagnosed in individuals under the age of 70, with this percentage rising to 7.27% when the diagnosis occurred at or before 50 years of age. This suggests that age-based restrictions on testing may lead to a higher rate of pathogenic variants being detected. Additionally, BRAF mutations or *MLH1* hypermethylation were found in 3.6% of cases, while genetic testing was only performed in 76% of the patients. Double somatic variants were observed in 0.24% of cases. The key determinant for these findings was whether somatic tumor testing had been conducted. These results emphasize the importance of a comprehensive approach that includes not only genetic testing but also somatic tumor testing and careful interpretation of results to enhance diagnostic accuracy and treatment effectiveness.

These findings underscore the critical importance of adopting a comprehensive approach to maximize the benefits of universal tumor screening. field. Recent studies in the field further confirm this need for a comprehensive approach.[9,10]

Challenges in diagnosing LS with immunohistochemistry and MSI analysis

It is acknowledged that individuals carrying (likely) pathogenic *MSH6* variants may, at times, present with a microsatellite stable (MSS) tumor exhibiting normal staining.[11,12] This occurrence is often linked to the sporadic nature of certain tumors, consistent with the lower penetrance associated with (likely) pathogenic *MSH6* variants. However, intriguingly, these tumors may also manifest at a young age, introducing uncertainties about the underlying mechanisms governing their early onset.

In addition to these observations, there are instances of discordant microsatellite instability/immunohistochemistry (MSI/IHC) results. While some cases have identifiable somatic explanations, a significant number remain unexplained. Motivated by these complexities, our study sought to uncover the diverse causes contributing to discordant phenotypes in CRC and EC within the context of *MSH6*-related LS. Notably, we identified

a discordant staining pattern in 77 cases, constituting 36% of the known MSI/IHC results.

Next-generation sequencing (NGS) was performed in 12 cases to delve deeper into the discordance. Upon revision, two out of three MSI/IHC cases were found to be in concordance with the *MSH6* variant. Intriguingly, NGS unveiled that four discordant IHC results indicated sporadic tumors rather than LS-associated ones. In a specific case, somatic events were identified as the explanatory factor for the discordant phenotype.[12]

These findings underscore a critical consideration: relying on reflex immunohistochemistry (IHC) mismatch repair testing, a general practice in many Western countries, may potentially lead to misdiagnosing individuals carrying germline *MSH6* variants. This observation aligns with the lower penetrance described in *MSH6*-related Lynch syndrome.[13] However, the intriguing aspect is the occasional early onset of these tumors, for which a definitive explanation has yet to be elucidated.

Part II - Phenotypic Characterization and Risk stratification in Lynch Syndrome

Early clinical criteria, such as the 'Amsterdam I' (1991) and 'Amsterdam II' (1999), were developed to identify families with MMR variants[14,15] but later found to miss families with *MSH6* and *PMS2* variants. This limitation led to the introductions of broader criteria, like the Bethesda guidelines[16,17], and eventually universal tumor screening for colorectal en endometrial cancer.[7,18] Notably, pathogenic variants in *MSH6* and *PMS2* are more frequently detected than in *MLH1* or *MSH2*, reflecting their higher prevalence in the population, despite *MLH1* and *MSH2* PV carriers presenting with higher cancer incidence.[19]

The current cancer risk estimates for *MSH6* variant carriers are largely based on highly selected, clinic-based families, which tend to represent a smaller subset of the broader population. Consequently, these previous studies, including ours, have limitations due to the inherent biases in selecting families based on clinical criteria. While such studies have been valuable, they may not fully capture the diverse range of cancer risks in *MSH6* PV carriers, particularly those from more general or population-based settings.

Our research[20], however, adds significant value by providing a more comprehensive and detailed understanding of cancer risks in *MSH6* variant carriers, while acknowledging the clinic-based nature of the cohort. The primary contribution of this study lies in the larger sample size of 360 Dutch *MSH6* families with (likely) pathogenic variants, representing a broader and more diverse clinic-based population compared to prior studies. Importantly, the scale of our cohort allows for more robust and precise estimates of cancer risks, offering greater clarity on the cumulative risks (CRs) for both colorectal and endometrial cancers. Our findings show significant elevations in cancer risks compared to the general population, with the CR for colorectal cancer (CRC) by age 80 estimated at 35.7% (95% CI: 25.4%-47.7%) for males and 21% (95% CI: 13.4%-32%) for females, and a CR of 23.4% (95% CI: 15.4%-43%) for endometrial cancer (EC). These results offer new insights into surveillance strategies in this group. While current guidelines[6,21] recommend starting CRC surveillance at age 25, our data suggest that a later start—potentially at age 30 or 35—could be more

appropriate, particularly for males. Given the relatively low CRC risk in individuals under 50, delaying the start of screening may help reduce unnecessary procedures and the associated psychological burden without significantly increasing cancer risk. This strategy aligns with evolving individualized surveillance recommendations and aims to balance effectiveness with reduced burden.

Furthermore, our data provide more robust estimates for extracolonic cancer risks associated with *MSH6* variants. The CR for ovarian cancer was 5.6% (95% CI: 2.4%-13.6%) by age 70, and risks for biliary tract and urinary tract cancers also showed notable increases, with respective CRs of 4.9% (95% CI: 2.2%-10.8%) and 10.1% (95% CI: 5.2%-18.8%) in males by age 80. Interestingly, the risk for small bowel cancer remained relatively low, with a CR of 0.2% (95% CI: 0-2.8%), consistent with prior findings[5,22]. These insights further validate the clinical relevance of extracolonic cancer surveillance in *MSH6* variant carriers.

In contrast to the more definitive data on CRC and EC, the evidence regarding breast cancer risks in *MSH6* carriers remains less clear. Recent studies on breast cancer risk in *MSH6* carriers have yielded conflicting results. For instance, Dorling et al.[23] observed a modestly increased risk for breast cancer, but the cumulative risk is considerably lower than what was suggested in some earlier studies. However, our findings indicate hazard ratios that are more aligned with population averages, with cumulative risks at age 80 of 6.4% (95% CI: 3-14.8%) for females. These previous studies may have been based on smaller or non-representative sample sizes, which can introduce statistical biases and lead to overestimated cancer risks. This helps explain why the elevated breast cancer risks reported earlier may not be as accurate as previously thought. Our study, with its larger and more diverse cohort, provides a more balanced and accurate understanding of the risks of breast cancer in *MSH6* variant carriers.

Prostate cancer risks showed a modest elevation with a CR of 16.6% (95% CI: 8.4%-30.6%) by age 80, suggesting the need for further studies to clarify the implications for male carriers.

Modifiers of cancer risk

Understanding the intricacies of carcinogenesis is essential for comprehending cancer risks in individuals with LS. Internal and external factors play key roles in developing malignant tumors.

Internal risk factors

Disparities in cancer risks exist both within and between LS families. One significant determinant is which MMR gene has the pathogenic variant[5]. However, even when the same mutated MMR gene is considered, disparities in cancer risks persist among individuals within and between families. Moreover, regional disparities in cancer risks have been noted across continents.[24] Despite several proposed hypotheses, such as the parent-of-origin effect and geno-phenotype correlation, no singular explanation has emerged as definitive. [24-33]

In our geno-phenotype study,[ref] we were unable to validate the existence of a parent-of-origin effect in *MSH6* (likely) pathogenic variant carriers. However, we did observe a possible correlation between genotype and phenotype. It is worth noting that only one other study reported a similar observation, which was exclusively in carriers of pathogenic variant in the *MLH1* gene.[28] While genotype-phenotype correlations are of interest in understanding LS, the inherent complexity and heterogeneity of the condition and limitations in study design and data analysis can make it challenging to identify consistent and robust associations between specific MMR gene mutations and clinical outcomes. Nonetheless, ongoing research and larger, well-designed studies may provide further insights into these relationships.

External risk factors

Cancer risk in individuals with LS is not determined by genetic factors alone. External factors, such as lifestyle choices and viral infections, also play an important role in cancer development. Well-known risk factors like smoking, high body mass index (BMI), and poor dietary habits have been identified as contributors to tumor formation.[34] However, studying these factors presents challenges due to various biases in this research.

In addition to lifestyle factors, viral infections have gained attention as potential contributors to cancer risk. During the COVID-19 pandemic, there were reports of an increase in interval carcinomas among LS patients under surveillance.[35] However, it is essential to recognize that this rise may be due, at least in part, to reduced surveillance during the pandemic. COVID-19-related restrictions and less frequent screenings likely contributed to the increased occurrence of interval cancers.

LS patients have an impaired MMR pathway, which is also crucial for clearing certain RNA viruses, including influenza and coronaviruses, from host cells.[36] Recent studies have explored the potential consequences of viral infections, particularly SARS-CoV-2, on host DNA. These infections have been associated with immunological abnormalities, DNA damage, micronuclei formation, and telomere shortening – all of which can disrupt the DNA damage response network, a key player in maintaining genome stability and diversity.[37]

While viral infections, including SARS-CoV-2, can induce DNA damage and affect genomic stability, it is important to note that there is currently no evidence to suggest that COVID-19 itself increases cancer risk. Although these viruses can interfere with the cell cycle during replication in mammalian cells, there is no conclusive data linking COVID-19 directly to cancer development.[38]

Nonetheless, the potential impact of viral infections, such as SARS-CoV-2, on cancer risk in individuals with LS remains an area of interest. Further research would be valuable to better understand whether viral infections may influence cancer development in this vulnerable group, particularly considering their impaired DNA repair mechanisms.

In addition, recent research has started to investigate the role of the gut microbiome in LS. While findings vary, growing evidence suggests microbial imbalances may contribute

to cancer development in LS.[39,40] Distinct changes in the microbiome of LS patients compared to healthy controls are identified[41], with certain bacteria like *Streptococcus* and *Actinomyces* being more prevalent in those with LS-associated CRC, while others, such as *Faecalibacterium*, are depleted.[39] These alterations in microbiome composition hint at a possible link to the elevated cancer risk in LS. Also, animal models have shown that specific bacteria can influence tumor development. For instance, transferring mice to pathogen-free conditions led to a significant reduction in tumor growth, possibly due to the absence of inflammation and epithelial proliferation driven by a conventional microbiome.[40] This suggests that the microbiome could play a key role in cancer development in LS, although further research is needed to fully understand the underlying mechanisms.[40,41]

Environmental and occupational exposures also contribute to cancer risk. For instance, exposure to carcinogens like chromium VI (Cr(VI)), commonly found in industries like metallurgy and welding, has been linked to cancers of the lung, nasal, and other organs.[42] Genetically predisposed individuals with LS may face an even higher risk when exposed to such carcinogens, as these substances could cause additional mutations or DNA damage. Although more research is needed to establish a direct link between chromium VI exposure and LS specifically, the interaction between genetic susceptibility and environmental factors is clearly important in cancer development.

Another example of occupational cancer risk involves pilots and other aviation personnel, who have been identified as a group potentially at higher risk for cancer. One of the risks for aviation personnel is exposure to ionizing radiation at high altitudes. This radiation, mainly from cosmic rays, is known to increase the risk of certain cancers, such as skin cancer, rectal cancer, and breast cancer. Studies have shown that aviation personnel have a higher incidence of these cancers compared to the general population.[43,44] Besides ionizing radiation, factors like irregular work hours, and jet lag may also contribute to this elevated risk.[45-48] Ongoing research suggests that the combination of environmental and occupational hazards significantly contributes to the increased cancer risk in aviation personnel. It is logical to assume that individuals with LS face an even higher risk of developing cancer compared to those without LS when exposed to these external risk factors given their genetic predisposition.

Part III - Functional Interpretation of Variants of Uncertain Significance

The uncertainty surrounding the pathogenicity of MMR variants necessitates a range of tests for thorough investigation, with functional analyses serving as the primary method for classifying VUSs in MMR genes.[49-51] Despite these efforts, the ambiguity persists, stemming from conflicting results across clinical, molecular, and functional data. To address this, we conducted a pilot study, exploring whether WES could offer an additional layer of evidence to clarify the pathogenicity of MMR variants with conflicting interpretations or serve as an initial step in their evaluation. Our study, building on previous findings that mutational signatures can distinguish MMRd tumors from sporadic tumors[52], revealed that, within our

cohort, WES did not significantly reclassify VUS but provided clarification on phenotypic aspects, including age of onset and explanations for abnormal tumor research.[53]

While the generality of our results awaits validation through future research, our study has laid a foundation for further investigations. Currently, the use of WES/TMS to evaluate the potential pathogenicity of a VUS remains limited, necessitating additional evidence sources, such as functional assays, population frequency data, and clinical information, for a comprehensive interpretation. Future studies may overcome current limitations and assess the (cost-) effectiveness of WES/TMS analysis on a broader scale.

An advantageous aspect of WES analysis lies in its potential to unveil alternative explanations when clinical and functional data pose challenges for interpretation. This includes the identification of other genetic alterations that can shed light on the tumor's phenotype, offering insights into factors such as age of onset, secondary MMR deficiency, and high mutational burden. Additionally, exploring mutational signatures themselves is increasingly valuable. These signatures, which represent patterns of mutations driven by specific mechanisms, can reveal important insights into the underlying processes of tumor development. Even in the absence of clear pathogenic variants, analysing these mutational signatures can provide crucial information regarding the tumor's origin, progression, and response to treatment. Investigating mutational signatures further could, therefore, enhance our understanding of tumor biology and contribute to more precise, personalized approaches to cancer management.

SUMMARY AND CONCLUDING REMARKS

This thesis addresses three key aspects of MMR deficiency. First, it investigates the effectiveness of universal tumor screening for LS, focusing on MMR protein staining patterns. Second, it examines cancer risks in LS, with a particular emphasis on *MSH6* pathogenic variant carriers, considering both colorectal and extracolonic cancers, and exploring genotype-phenotype correlations. Third, this thesis explores the potential of mutational signatures from whole exome sequencing (WES) to assess the pathogenicity of MMR variants of unknown significance (VUS).

In **Part I,** the thesis demonstrates that the success of universal tumor screening for LS depends on a comprehensive and thorough diagnostic approach, as evidenced by the analysis of colorectal cancer cases. Challenges in diagnosing LS, especially in *MSH6* pathogenic variant carriers, highlight the need for careful interpretation of IHC and MSI analysis results. The study stresses the importance of re-evaluating testing practices to improve diagnostic accuracy.

In **Part II**, the thesis underscores the complex nature of cancer risk in LS, where internal genetic factors interact with external influences such as lifestyle and viral infections. The variability in cancer risks among *MSH6* carriers, both within and between families, suggests

that other yet-to-be-identified factors contribute to this phenomenon. The exploration of genotype-phenotype correlations and the potential influence of external factors represents an important step in understanding cancer risks in LS.

Part III delves into variant interpretation, focusing on the use of WES to assess VUSs in MMR genes. The findings of the pilot study indicate that while WES did not significantly reclassify VUSs, it provided valuable insights into phenotypic aspects, including age of onset and explanations for abnormal tumor characteristics. Despite the limitations of current research, the study lays a foundation for future investigations into the role of mutational signatures in enhancing our understanding of tumor biology and improving personalized approaches to cancer management.

In conclusion, this thesis contributes valuable insights into the detection, risk assessment, and variant interpretation of Lynch syndrome, advancing our understanding of LS and providing a basis for future research. It highlights the need for comprehensive diagnostic strategies and the consideration of both internal and external factors in cancer risk assessment, ultimately paving the way for more personalized and effective clinical management of LS patients.

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