

Anticipation in Families with MLH1-associated Lynch Syndrome

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Abstract **Background** Lynch syndrome (LS) is an autosomal-dominant, hereditary cancer predisposition syndrome caused by pathogenic variants (PVs) in one of the mismatch-repair genes *MLH1*, *MSH2/EPCAM*, *MSH6*, or *PMS2*. Individuals who have *MLH1* PVs have high lifetime risks of colorectal cancer (CRC) and endometrial cancer (EC). There is controversy regarding whether a younger age at diagnosis (or anticipation) occurs in *MLH1*-associated LS. The objective of this study was to assess anticipation in families with *MLH1*-associated LS by using statistical models while controlling for potential confounders. **Methods** Data from 31 families with *MLH1* PVs were obtained from an academic registry. Wilcoxon signed-rank tests on parent–child-pairs as well as parametric Weibull and semiparametric Cox proportional hazards and Cox mixed-effects models were used to calculate hazard ratios or to compare mean ages at CRC/EC diagnosis by generation. Models were also corrected for ascertainment bias and birth-cohort effects. **Results** A trend toward younger ages at diagnosis of CRC/EC in successive generations, ranging from 3.2 to 15.7 years, was observed in *MLH1* PV carrier families. A greater hazard for cancer in younger generations was not precluded by the inclusion of birth cohorts in the model. Individuals who had *MLH1* variants with no Mlh1 activity were at a 78% greater hazard for CRC/EC than those who retained Mlh1 activity. **Conclusions** The current results demonstrated evidence in support of anticipation in families with *MLH1*-associated LS across all statistical models. Mutational effects on Mlh1 activity influenced the hazard for CRC/EC. Screening based on the youngest age of cancer diagnosis in *MLH1*-LS families is recommended.

Keywords: LS, syndrome, CRC

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1. Introduction

Lynch syndrome (LS) is an autosomal-dominant, hereditary cancer predisposition syndrome due to a pathogenic variant (PV) in one of the mismatch-repair (MMR) genes *MLH1*, *MSH2/EPCAM*, *MSH6*, or *PMS2* [1] and manifests with increased lifetime risks of colorectal cancer (CRC) and endometrial cancer (EC) among other less common cancer types. [2] Of the variants in LS, 43% have been reported in *MLH1*, 40% have been reported in *MSH2*, and the remaining 17% have been reported in *MSH6* and *PMS2*. Penetrance estimates for CRC and EC in LS vary by gene, variant, sex, and geographic location. [3]

The tendency for cancer to develop at an earlier age in successive generations within a family was first reported in 1913 by Aldred Scott Warthin. [4] This defines the phenomenon of *anticipation* in which the age at onset of a disorder is reduced and/or the severity of the phenotype is

increased in successive generations. [5] Anticipation has been observed in familial melanoma; Li Fraumeni syndrome; breast, ovarian, and pancreatic cancers; and von Hippel–Lindau syndrome. [6] Statistical models for estimating the effects of anticipation on cancer range from a simple comparison of the mean or median ages at the onset of cancer in parent–child pairs (PCPs) to more complex models that follow randomized distributions with the inclusion of covariates. Several types of biases, such as ascertainment bias, birth-cohort effects, and right truncation effects, can potentially influence the estimation of anticipation. [7]

In LS, studies on anticipation have indicated decreases in the age of onset in successive generations by 1–2 years for most MMR genes and up to 7 years for those with *PMS2* PVs. [6], [8,9,10,11] [12,13,14,15] These anticipation effects reportedly disappeared after correcting for birth cohort, [7,13,16] except in one study. [17] The presence and magnitude of anticipation effects in LS in these studies have depended on the type of model used, the choice of

covariates included in the model, and the methods used to correct for bias. [18]

Given the mixed results of anticipation effects in LS, the objective of this study was to estimate genetic anticipation in families with *MLH1* PVs. Families with *MLH1*-associated LS were selected because of their higher incidence and strong association with CRC and EC. We applied three statistical models to examine the effects of generation on age at first diagnosis of CRC or EC while correcting for ascertainment bias and birth-cohort effects.

2. Materials and Methods

Study cohort

Families with confirmed *MLH1* PVs or likely PVs that included at least two first-degree relatives with cancer were identified in a prospective, Institutional Review Board-approved registry collected from 1992 through 2021 at The University of Chicago. Pedigree data collected on these families included age at diagnosis of cancer, year of birth, age and sex of family members, genetic test results, and site of cancer (Figure 1A).

Generations and birth cohorts

The proband was the individual indicated as such on the pedigree. Generations were created with respect to the proband for each family as the proband's *parents*, *grandparents*, and *children* (Figure 1B). Proband and their siblings or cousins were designated as the proband's generation, henceforth referred to as *probands* to distinguish these from the proband from a ped. PCPs were created with individuals who had both a parent and their child diagnosed with CRC or EC. Birth cohorts were created at 20-year and 10-year intervals starting with those born before 1920 through 2000. Birth cohorts were also created for affected PCPs who had children born before and after 1945. Assuming that proactive screening of unaffected family members from age 25 years began with publication of the Amsterdam I criteria [19] in 1991, two birth cohorts of those born before and after 1966 were also created.

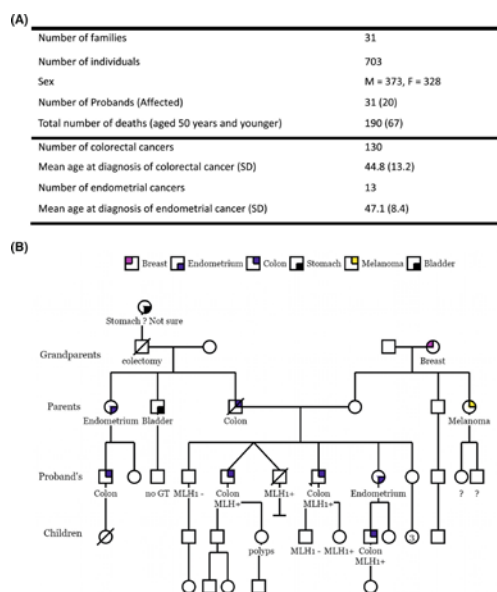


Figure 1. Descriptive statistics

Descriptive statistics of (A) the study cohort and (B) an example pedigree depicting the creation of generations with respect to the proband. Individuals who did not fall into one of the four generations shown or who had cancers other than colorectal or endometrial were not included in the analysis. F indicates female; GT, genetic test; M, male; *MLH1*−, negative for *MLH1* mutation 1; *MLH1*+, positive for *MLH1* mutation 1; SD, standard deviation.

For individuals with missing age information, it was assumed that both parents of the proband were born in the same year, that the years of birth differed by 25 years in each generation, and that the year of birth of siblings was the same. [20] For individuals who had died and were reported as affected with an unknown age of diagnosis, the age of death was considered as the age of diagnosis. [20] Year of birth, if not available, was calculated from the last age reported on the pedigree and the year the pedigree was recorded or updated.

Variant effects and Mlh1 proficiency

Germline *MLH1* PVs were known in 28 of 31 families and were recorded as *MLH1*-positive in the remaining three families. Variants were classified based on their effect on the protein as a single-residue variation (SRV), in which only a single amino acid residue in the Mlh1 protein was altered; a termination (T) effect if the variant resulted in a longer or shorter form of protein because of displacement of the termination codon; and a splicing effect (SP), in which the variant was known through in-silico or functional studies to cause in-frame exon skipping, resulting in an altered transcript (see Supporting Information S1). Variants were also classified as Mlh1-proficient or Mlh1-deficient based on published literature on functional studies (see Supporting Information S1). Mlh1 was considered MMR-proficient if it demonstrated an MMR level activity above that of controls. Variants were also categorized into truncating and nontruncating as in a previous study. [21] Variants that could not be classified unambiguously were excluded from the analyses.

Statistical analysis

Differences in ages of diagnosis between PCPs were determined using the Wilcoxon pairwise signed-rank test (see Supporting Information S2). The study outcome variable in multivariate regression models was age at first diagnosis of CRC/EC. Follow-up was defined as the time elapsed from birth until the first CRC/EC diagnosis or censoring. An individual was censored either at the age of last contact with the family, as recorded in the pedigree, or at the age of death. The primary predictor variable of interest in multivariate regression models was the categorical variable *generation*, with sex, birth cohort, and the effect of *MLH1* mutation on protein as covariates. Hazard ratios (HRs) for CRC/EC between generations were determined using nonparametric Cox proportional-hazard models with (Cox mixed-effects) and without (Cox-PH) inclusion of families as random effects. Multivariate parametric regression with Weibull models was used to estimate the mean age of diagnosis for each generation. Cox-PH and Weibull models were run both with and without the inclusion of probands. Survival analyses were also performed with pre-1945 and post-1945, pre-1966 and post-1966, and 10-year birth cohorts as covariates in Cox-PH models (see Supporting

Information S1). The effect of Mlh1 protein activity (see Supporting Information S1) on age at diagnosis of CRC/EC was examined through univariate Cox-PH regression. Mutation effects were included as a covariate to assess their relevance to the effect of generation on age of diagnosis (see Supporting Information S1). Mutational probabilities were added as weights to all statistical models. One-way analysis of variance was used when the Cox-PH model indicated a poor fit to the data. Statistical significance was set at 95% confidence intervals (CIs; $\alpha = .05$) for all models. Values of $p < .001$, $p < .01$, $p < .05$, $p < .1$, and $p \geq 0.1$ were considered as very strong, strong, moderate, trend, and insufficient evidence, respectively. [22]

3. Results

Our data comprised a total of 703 members from 31 families that had at least one member ascertained to carry a PV in *MLH1* through genetic testing. A description of the cohort demographics is provided in Figure 1A. All generations except children had a lower mean age of diagnosis compared with their parent's generation (Table 1). Median ages of diagnosis of CRC/EC in generations did not show any specific trend. A consistent decrease in mean and median ages of diagnosis was observed from older to younger birth cohorts in individuals born after 1920 (Table 1).

Table 1. Distribution By Age Of Diagnosis Weighted By Mutation Probabilities For Individuals Diagnosed With Colorectal Or Endometrial Cancer By Generation, Birth Cohort, Mismatch Repair Proficiency, And Mismatch Repair Deficiency

	Total no.	No. affected (%)	Age of diagnosis: Mean $\pm$ SD, years	Age of diagnosis: Median [IQR], years
Generation, N = 668				
Grandparents	129	21 (16.3)	52.6 $\pm$ 14.0	48 [23]
Parents	193	48 (24.9)	46.3 $\pm$ 12.2	45 [15]
Probands	218	53 (24.3)	40.1 $\pm$ 11.9	40 [15]
Children	128	17 (13.2)	42.1 $\pm$ 8.4	40 [13]
Cohort, N = 656				
<1920	82	19 (23.2)	45.5 $\pm$ 11.0	42 [10]
1920–1939	140	38 (27.1)	53.1 $\pm$ 13.5	50 [13]
1940–1959	173	51 (29.0)	42.9 $\pm$ 10.3	44 [13]
1960–1979	164	26 (15.9)	39.6 $\pm$ 6.9	39 [10]
1980–2000	97	7 (7.2)	25.7 $\pm$ 6.4	24 [8]
MMR-proficient, N = 158				
Grandparents	31	5 (16.1)	58.5 $\pm$ 20.7	42 [26]
Parents	46	7 (15.2)	46.0 $\pm$ 12.6	45 [16]
Probands	56	3 (5.3)	36.6 $\pm$ 15.4	31 [26]
Children	23	0 (0.0)	—	—
MMR-deficient, N = 310				
Grandparents	50	5 (10.0)	52.3 $\pm$ 10.5	47 [3]
Parents	89	26 (29.2)	45.3 $\pm$ 10.7	44 [13]
Probands	86	28 (32.5)	42.9 $\pm$ 13.6	40 [13]
Children	56	12 (21.4)	43.9 $\pm$ 8.1	39.5 [9]

Abbreviations: IQR, interquartile range; MMR, mismatch repair; SD, standard deviation.

Table 2. (Pseudo)-Median Of Differences In Age At Diagnosis Of Affected Parents And Their Affected Children With The Wilcoxon Signed-Rank Test, N = 20 Probands

P-C pairs	No. without probands	Pseudomedian, P-C pairs (95% CI)	<i>p</i>	No. with probands	Pseudomedian, P-C pairs (95% CI)	<i>p</i>
All P-C pairs	54	5.49 (0.50–11.00)	.03	62	6.00 (2.00–10.99)	< .01
Child born <1945	25	4.7*e-5 (4.99, 6.00)	.94	26	1.50 (-4.49, 7.50)	.66
Child born >1945	29	10.99 (2.50–21.00)	.12	36	9.99 (3.99–17.50)	< .01

Abbreviations: CI, confidence interval; P-C pairs, parent-child pairs.

Table 3. Estimated mean age at onset of cancer for each generation from parametric Weibull distribution models with and without the inclusion of probands and adjusted for birth cohort, sex, and *MLH1* variant effect

Generation	Without probands, N = 411		With probands, N = 438	
	Age of diagnosis: Mean $\pm$ SE, years	95% CI	Age of diagnosis: Mean $\pm$ SE, years	95% CI
Grandparents	78.2 $\pm$ 8.3	61.8–94.5	75.0 $\pm$ 7.8	59.6–90.3
Parents	62.5 $\pm$ 3.4	55.9–69.1	62.5 $\pm$ 3.3	56.0–69.0
Probands	59.3 $\pm$ 3.6	52.3–66.4	55.7 $\pm$ 2.7	50.5–61.0
Children	44.4 $\pm$ 3.9	36.7–52.0	45.4 $\pm$ 3.9	37.6–53.1

Abbreviations: CI, confidence interval; SE, standard error.

There were 62 PCPs in which both the parent and a child had been diagnosed with CRC or EC. The

pseudomedian of differences (see Supporting Information S2) between ages at diagnosis of the children compared

with their parents (age P – age C) among the PCPs was 6 years and was statistically significant ($p < .01$; paired, two-sided Wilcoxon signed-rank test; Table 3). When separated into birth cohorts, parents were found to be diagnosed at a median of approximately 10 years later compared with their affected children when children were born after 1945 (Table 2). The exclusion of probands showed a similar trend with a median increase of approximately 11 years in age at diagnosis of parents compared with their children born after 1945, but with lower statistical significance (Table 2).

Predicted mean ages of onset in different generations calculated from multivariate Weibull accelerated failure time models indicated that children, probands, and parents were diagnosed at an average of 10.3, 6.8, and 12.5 years earlier, respectively, compared with their previous generations (Table 3). When probands were excluded, children, probands, and parents showed a decline in age of diagnosis by 14.9, 3.2, and 15.7 years, respectively, compared with their previous generations (Table 3). These predicted mean ages of onset were estimated accounting for censoring, whereas the mean ages at diagnosis in Table 2 were calculated only among patients who were diagnosed with CRC or EC.

Cox-PH models with or without probands showed a

trend toward a greater hazard at any given age in younger generations (Figure 2). Probands were at 54% greater hazard for CRC/EC than their grandparents, similar to the hazard among parents, and at 150% lower hazard compared with children when probands were excluded from the multivariate Cox-PH model (Figure 2A). With the inclusion of probands, probands were at 60% greater hazard than grandparents, at 33% greater hazard than parents ($p = .06$; 95% CI, 0.44–1.01), and at 53% lower hazard than children ($p = .14$; 95% CI, 0.87–2.7; Figure 2B). The cumulative incidence of CRC/EC predicted by the Cox-PH model stratified by generation indicated that the median age at diagnosis of cancer was approximately 44, 46, 55, and 72 years in children, probands, parents, and grandparents respectively (Figure 3). The cumulative incidence of CRC/EC in children was lower before and greater after age 40 years compared with the incidence in probands (Figure 3).

Forest plots showing HRs for CRC/EC as estimated by the Cox-PH models (A) without probands and (B) with probands. CI indicates confidence interval; Cox-PH, Cox proportional hazards; CRC/EC, colorectal and/or endometrial cancers; HR, hazard ration; SP, splicing effect; SRV, single residue variation; T, termination effect.

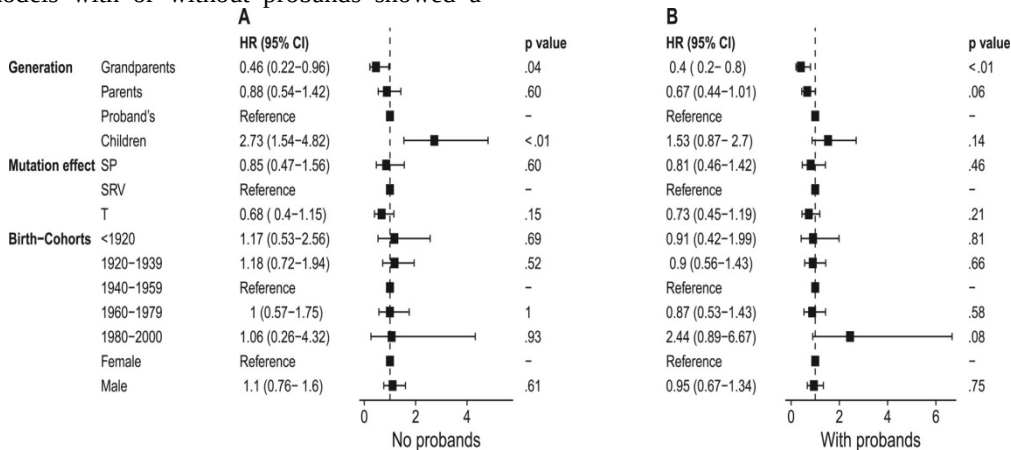


Figure 2. CRC/EC

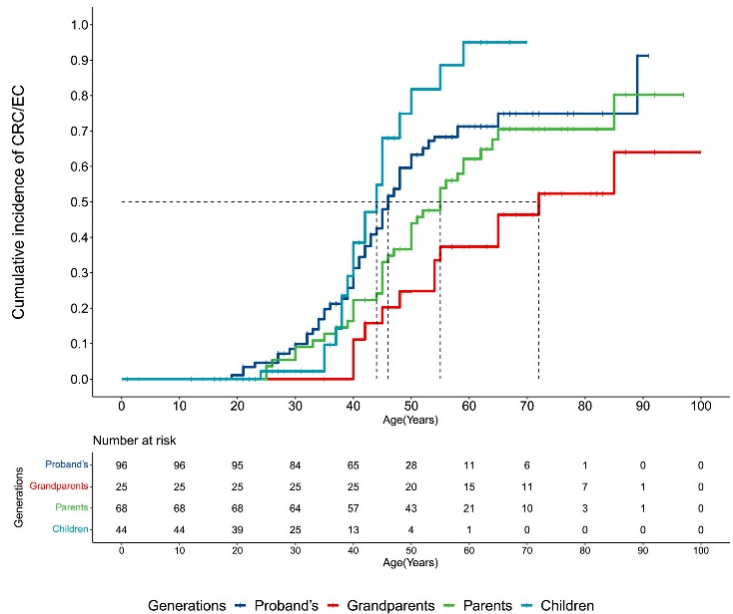


Figure 3. Cumulative incidence of CRC/EC by age

Cumulative incidence of CRC/EC by age as predicted by the multivariate COX-PH model stratified by generation, probands included. The median age at diagnosis for each generation is indicated. Censoring is represented by vertical bars. Cox-PH indicates Cox proportional hazards; CRC/EC, colorectal and endometrial cancers.

The hazard for CRC/EC with different mutation effects appeared slightly lower in SP and T mutation effects compared with SRV effects, but the difference was statistically insignificant (Figure 2). There were no significant differences in the hazard for truncating variants compared with nontruncating variants (HR, 0.9; $p = .67$; 95% CI, 0.56–1.45). MMR activity was known in 19 families, of which seven had mutations that were MMR-proficient. MMR deficiency showed a 78% increased hazard for CRC/EC compared with variants that retained MMR proficiency (HR, 1.78; $p = .03$; 95% CI, 1.05–3.04) through univariate Cox-PH analysis. Mean ages of diagnosis weighted by mutation probabilities decreased by 12.5 and 9.4 years between grandparents, parents, and probands in the MMR-proficient group (Table 1), with no CRC/EC in children. In the MMR-deficient group, the corresponding decrease in mean age of diagnosis were 7.0 years and 2.4 years between grandparents, parents and probands, while it increased by a year in children compared to probands. Cox-PH analysis in the MMR-deficient group showed no significant differences in HRs over generations except for grandparents (see Supporting Information S1). The Cox-PH analysis model for the MMR proficient group was a poor fit (likelihood ratio test, 0.72; degrees of freedom [df] = 3; $p = .80$). However, an analysis of variance indicated a significant effect of generation on age at diagnosis in the MMR-proficient group (df = 3; $F = 6.91$; $p < .01$), whereas it was not significant in the MMR-deficient group (df = 3; $F = 1.62$; $p = .18$).

HRs between males and females were not significant in any of the Cox models (Figure 2). HRs from multivariate Cox mixed-effects regression with families included as random effects were similar to HRs in the Cox-PH model (see Supporting Information S1).

HRs in birth cohorts in Cox-PH models did not follow a specific trend, with only the 1980–2000 birth cohort showing a 144% greater hazard than the 1940–1959 cohort when probands were included (Figure 2B). HRs between birth cohorts were statistically insignificant in Cox-PH models with 10-year, pre-1945 and post-1945, or pre-1966 and post-1966 birth cohorts (see Supporting Information S1).

4. Discussion

The results from our study show evidence of anticipation in families with *MLH1*-associated LS. At any given age, the hazard for CRC/EC was consistently lower in older generations across all models, and this effect was not precluded by the inclusion of birth cohorts. Younger mean ages at diagnosis (Weibull model) for younger generations aligned with their increase in hazard (Cox-PH model). We also observed that mutations causing MMR deficiency resulted in a greater hazard for CRC/EC compared with those that retained MMR activity.

Our results are in agreement with previous studies of *MLH1*-associated LS that reported anticipation effects ranging from 2.8 [8,9] to 6 years [17] or an increased hazard of cancer in subsequent generations. [6] Our study augments previously observed effects of birth cohorts by demonstrating dependence of anticipation effects on the criteria for defining birth cohorts. For example, in comparing PCPs in our study, children born before 1945 had similar ages of cancer onset compared with their parents, whereas those born after 1945 were diagnosed approximately 10 years earlier compared with their parents. In the general population, a birth-cohort effect for CRC is hypothesized to be caused by exposures, such as sedentary lifestyle, obesity, alcohol intake, and consumption of processed meat, among others, that began in the 1950s [23,24] and may explain the earlier onset of CRC in post-1945 birth cohorts in the current study. Studies similar to ours that reported the year of birth as a likely explanation of anticipation in LS [7,16] did not report the HRs associated with birth cohorts, which, in our study, were observed to be of negligible effect size and statistically insignificant. Another study reporting evidence against genetic anticipation in familial CRC also suggested that anticipation could not be explained by a secular trend. [13] This was evident in our models also, none of which showed a trend or significant difference in ages of diagnosis among birth cohorts irrespective of how they were defined (see Supporting Information S1). Birth-cohort effects are implicated in an increase in the worldwide incidence of cancer, [25] and well established risk factors for CRC have been associated with early onset CRC and later onset CRC to similar degrees, [26] which manifest as an increase in incidence but not a younger age at diagnosis. Several new risk factors for CRCs, such as exposures during fetal development and the gut microbiome, have continued to emerge. [25,27] The chronological occurrences of this array of risk factors is highly variable, making the categorization of birth cohorts by secular effects challenging. The occurrence of mutations in MMR genes in patients with pediatric cancer, in whom exposure to environmental effects is limited, also favors genetic rather than environmental effects in anticipation. [28,29,30,31,32,33]

Ascertainment bias can overestimate disease risk and penetrance in clinical genetic variant–cancer association studies [34] because of an overrepresentation of young onset cases in probands and right truncation effects, [35] and is corrected by excluding probands from analysis. Age at cancer diagnosis of affected family members was reported during family history-taking irrespective of whether individuals were seen at clinic, and all family members with CRC/EC were included in the analysis. This prevents ascertainment bias, so that the exclusion of probands is not necessary. Also, 11 of the 31 probands in our study were not affected by CRC/EC and did not contribute to younger ages of diagnosis. The exclusion of probands from the regression models unnecessarily discards reliable data with high mutation probabilities and may be less accurate than models with probands included.

It is interesting that the cumulative incidence of cancer in the children's generation is lower than that of probands as well as parents at ages younger than 40 years. A possible explanation is screening followed by family

members who carry the familial *MLH1* mutation. Screening through colonoscopy or sigmoidoscopy has been shown to reduce the risk and incidence of CRC. [36,37] Early detection of adenomas followed by polypectomy would prevent the transition to adenocarcinoma, postponing the onset of CRC/EC to an older age, which possibly explains lower incidence in younger individuals of the children's generation. Early detection of an existing CRC would lead to early diagnosis, which, in our data, aligns with the rapid increase in incidence of CRC/EC in children older than 40 years. This also indicates that more frequent screening after age 35 years may be desirable.

To our knowledge, this is the first study that has modeled mutation effects on Mlh1 protein as a covariate in a Cox-PH model to study anticipation effects in pedigree data. A previous study that stratified LS cancer risks by gene and variant type found significantly older median ages at the onset (lower hazard) of EC for *MLH1*-truncating variants compared with splice-site variants and large rearrangements. [38] However, a recent, prospective LS database study that examined the effect of type of variant in greater than 5000 carriers of MMR PVs found no difference in penetrance between truncating and missense/aberrant splicing variants in *MLH1* and *MSH2*. [21] Lastella et al. [39] demonstrated that missense variants in *MLH1* can have in-vivo effects different from those predicted by in-silico analysis, so that variant types may not always be correlated with Mlh1 protein activity. An international study has proposed the presence of risk modifiers in LS that may not be variant-specific, and the authors have proposed estimating penetrance according to the effect of a variant on protein function. [3] Our categorization of *MLH1* variants as SRV, SP, and T was based on published functional and in-silico evidence and augmented generational effects on age of diagnosis. Admittedly, some of these categories may approximate, but cannot replace, Mlh1 activity determined through functional assays.

Our study provides evidence for the influence of Mlh1 protein activity on age at onset of CRC/EC. Differences in the proportion of affected individuals and mean ages at diagnosis of CRC/EC over generations were notable between the MMR-proficient and MMR-deficient groups. Pathogenicity because of *MLH1* mutations is attributed to protein instability as well as catalytic dysfunction, [40] and an *MLH1* variant that produces active protein may still be pathogenic if its expression is below a certain threshold. [41] Also, the expression of immune-related genes has been identified as distinct in cancer-free patients with LS and those with CRC. [42] A substantial number of LS cases are associated with genomic rearrangements, susceptibility to which may be determined by Alu density in *MLH1*. [43] This complex interplay of *MLH1* cis-acting factors, epistasis, and the level of Mlh1 activity may be an essential part of the mechanism of anticipation, but further studies are needed to confirm this hypothesis. Statistical models with larger pedigree and functional data grouped by Mlh1 activity will help discern the role of the Mlh1 activity level in anticipation.

To the best of our knowledge, this is the first regression analysis in *MLH1*-associated LS that includes mutation effects as a covariate while adjusting for ascertainment

bias and birth-cohort effects. Despite the applied corrections, the possibility of other uncontrolled biases and skewness because of calculated ages for older generations cannot be excluded. Our study also limited cancer diagnoses to CRC or EC, which are strongly associated with *MLH1* mutations, while excluding other cancers known to be associated with *MLH1*, such as gastric, bladder, pancreatic, prostate, and brain cancers. [44,45] Inclusion of these cancer types in anticipation studies would present a more realistic picture of generational differences in ages of diagnosis of *MLH1*-associated cancers. Another limitation was the insufficient number of affected individuals with MMR proficiency. Future studies on a larger number of families with *MLH1*-associated LS caused by MMR-proficient mutations are needed to discern its effect on age of diagnosis.

In conclusion, we report evidence in support of an anticipation effect in families with *MLH1*-associated LS that cannot be explained by birth-cohort effects. Our study supports screening for CRC/EC 2 to 10 years earlier than the youngest age of diagnosis of *MLH1*-associated LS in the family. We noted that variant types, when defined by their effects on Mlh1 protein activity, constitute a contributing factor to alterations in cancer risk. Risk assessment for CRC/EC in families with *MLH1*-associated LS should factor in information on MMR proficiency or deficiency when available.

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