

ORIGINAL RESEARCH

Canonical and uncanonical pathogenic germline variants in colorectal cancer patients by next-generation sequencing in a European referral center

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Background: Despite increasing use of next-generation sequencing (NGS), data concerning the gain in germline pathogenic variants (PVs) remain scanty, especially with respect to uncanonical ones. We aimed to verify the impact of different cancer predisposition genes (CPGs) on colorectal cancer (CRC) in patients referred for genetic evaluation.

Materials and methods: We enrolled for NGS, by Illumina TruSight Cancer panel comprising 94 CPGs, 190 consecutive subjects referred for microsatellite instability (MSI) CRC, polyposis, and/or family history.

Results: Overall, 51 (26.8%) subjects carried 64 PVs; PVs coexisted in 4 (7.8%) carriers. PVs in mismatch repair (MMR) genes accounted for one-third of variant burden (31.3%). Four Lynch syndrome patients (20%) harbored additional PVs (*HOXB13*, *CHEK2*, *BRCA1*, *NF1* plus *BRIP1*); such multiple PVs occurred only in subjects with PVs in mismatch syndrome genes (4/20 versus 0/31; $P = 0.02$). Five of 22 (22.7%) patients with MSI cancers but wild-type MMR genes harbored PVs in unconventional genes (*FANCL*, *FANCA*, *ATM*, *PTCH1*, *BAP1*). In 10/63 patients (15.9%) with microsatellite stable CRC, 6 had *MUTYH* PVs (2 being homozygous) and 4 exhibited uncanonical PVs (*BRCA2*, *BRIP1*, *MC1R*, *ATM*). In polyposis, we detected PVs in 13 (25.5%) cases: 5 (9.8%) in *APC*, 6 (11.8%) with biallelic PVs in *MUTYH*, and 2 (3.9%) in uncanonical genes (*FANCM*, *XPC*). In subjects tested for family history only, we detected two carriers (18.2%) with PVs (*ATM*, *MUTYH*).

Conclusion: Uncanonical variants may account for up to one-third of PVs, underlining the urgent need of consensus on clinical advice for incidental findings in cancer-predisposing genes not related to patient phenotype.

Key words: colorectal cancer, colorectal cancer genes, DNA microsatellite instability, gene testing, inherited cancers

INTRODUCTION

Although colorectal cancer (CRC) remains among the big killers in Western countries, its understanding and management have greatly improved, also thanks to the unraveling of the genetic factors involved in its development.¹ Hereditary CRC syndromes such as Lynch syndrome (LS), familial adenomatous polyposis [FAP—attenuated FAP (aFAP)], *MUTYH*-associated polyposis (MAP), and other less frequent polyposis syndromes altogether are considered to account for $\approx 5\%$ of all diagnosed CRCs.^{2–4} These syndromes are caused by germline pathogenic variants (PVs) in CRC-related genes, i.e. the DNA mismatch

repair (MMR) genes (*MLH1*, *MSH2*, *MSH6*, *PMS2*, and *EPCAM*) in LS, *APC* in FAP—aFAP, and biallelic *MUTYH* PVs in MAP.⁵ Timely introduction of appropriate surveillance strategies, as recommended from international guidelines,^{6,7} is the most efficient measure to decrease CRC incidence and, therefore, its mortality in PV carriers.^{8,9}

Recent years have witnessed the development and increasing usage of next-generation DNA sequencing (NGS) technologies. These have allowed the ascertainment of germline variants by panels that simultaneously assess multiple genes. Consequently, uncanonical PVs in cancer predisposition genes (CPGs) apparently not related to CRC predisposition have been described in patients with CRC, both in selected [e.g. for early onset (EO), familial or personal history of cancer] and unselected populations.^{10–12}

These findings had already been postulated more than a decade ago,¹³ yet recent data broaden the spectrum and frequency of potentially predisposing germline PVs

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detectable in CRC patients up to 10% in unselected CRC series.¹⁰ Yield and cost-effectiveness are properly balanced when the analyses are carried out in high-risk populations, and international recommendations have been established on these bases.⁷ However, narrowing the search to such populations may underestimate the true prevalence of inherited factors in the global CRC population.¹⁰

Applying a panel assessing 25 genes in a cohort of patients undergoing genetic analysis for suspected LS, Yurgelun et al. had a 14.6% yield of PV detection, which included 3% of alterations in high- or moderate-penetrance non-MMR CPGs.¹¹ Employing the same methodology in a statewide cohort of patients with EO CRC, Pearlman et al.¹² found germline PVs in 16% of the patients, including 3% with alterations in genes not traditionally associated with CRC. The same approach has been applied to an unselected consecutive series of CRC patients: germline PVs were detected in 10%, and in 3% they affected genes not typically implicated in CRC.¹⁰ In addition, 0.5%-2% of patients with a positive test result in these studies harbored highly and moderately penetrant variants in more than one gene.

Uncertainties remain about the clinical significance of the un/non-canonical PVs detected by NGS.¹⁴ Despite these intriguing findings, data remain limited, mostly unreplicated outside US academic centers.¹⁵

In order to verify the impact of different CPGs on CRC, we systematically assessed the prevalence of germline PVs, using a panel of 94 candidate CPGs in a consecutive series of patients referred to genetic evaluation for MMR-deficient (dMMR) tumors, personal and/or familial history of CRC, or polyposis.

MATERIALS AND METHODS

Patients

Patient recruitment occurred through the Hereditary Cancer Genetics Clinic at the Humanitas Research Hospital, Rozzano (Milan, Italy) from February 2017 to October 2019.

Patients were referred for consultation based upon clinical criteria (i.e. EO CRC if diagnosed at an age ≤ 50 years, occurrence of synchronous or metachronous cancers, positive family history, or the occurrence of colonic polyposis whenever presenting with ≥ 10 polyps). According to current recommendations enforcing universal screening for MMR status in CRC,⁷⁻¹⁶ patients with dMMR CRCs were referred for consultation, except those with *MLH1/PMS2*-deficient cancers and the *BRAF* p.V600E somatic mutation. Overall, 190 consecutive cases (92 males and 98 females; age range 22-84 years, mean age 56.6 ± 13.7 years) were evaluated and enrolled in the study, comprising 179 patients, and 11 subjects evaluated for family history only.

Ethics statement

All participants provided written informed consent as per the institutional ethics guidelines, regarding execution genetic testing and collection of data within research projects.

Next-generation sequencing

Blood samples were screened for germline variants by using the TruSight Cancer panel (Illumina, San Diego, CA) covering 94 CPGs (Supplementary Table S1, available at <https://doi.org/10.1016/j.esmooop.2022.100607>), and then run on the Illumina MiSeq platform according to the manufacturer's standard protocol (Illumina Inc.).

Fifty nanograms of genomic DNA was fragmented and tagged by the addition of sequencing adaptors and indices by polymerase chain reaction. Sample libraries were pooled and denatured into single-stranded DNA, before being hybridized to biotin-labeled probes specific to the targeted region. The pool was further enriched by adding streptavidin beads that bind to the biotinylated probes. Biotinylated DNA fragments bound to the streptavidin beads were subsequently pulled down and eluted from the beads and hybridized for a second enrichment reaction followed by polymerase chain reaction amplification. The targeted library was loaded on to the MiSeq platform for cluster generation and subsequent sequencing.

Bioinformatic analyses and variant characterization

Primary and secondary data analyses, including quality and coverage information, were carried out using the onboard MiSeq Reporter software (Illumina Inc.). The mean sequencing coverage for the regions targeted by the TruSight panel was $366.2\times$. The fraction of targeted region with $\geq 30\times$ coverage was on average 94.6% across all samples, and 89.1% of all regions targeted by the TruSight panel had $\geq 100\times$ coverage.

Sequencing data were aligned using Burrows-Wheeler Aligner (BWA) software (BaseSpace Labs Apps, Illumina Inc, San Diego, CA). Genetic variants were identified using GATK software (Genome Analysis Toolkit, The Broad Institute, Cambridge, MA) and were subjected to further analysis if the genotype quality calculated was ≥ 99 , and if the site was identified as a heterozygous site or a homozygous variant site. Detected variants were subsequently annotated using ANNOVAR, Variant Interpreter (Illumina), and Sophia DDM (Sophia Genetics, Lausanne, Switzerland). Polymorphisms at $>1\%$ frequency were removed using Genome Aggregation Database (<https://gnomad.broadinstitute.org/>), 1000 Genomes (<https://www.internationalgenome.org/home>), and Exome Sequencing Project (<https://evs.gs.washington.edu/EVS/>). Variants were classified according to the American College of Medical Genetics and Genomics/Association for Molecular Pathology criteria.¹⁷ To this aim, in line with the Standards and Guidelines for the Interpretation and Reporting of Sequence Variants in Cancer,¹⁸ we reviewed the data for each variant through different repositories, namely Varsome, Human Genome Mutation Database, ClinVar, LOVD, and Insight. Variants classified as pathogenic and likely pathogenic were considered for further analyses, for class 4 only if their classification was matched in at least two repositories. Variants classified as pathogenic and likely pathogenic or

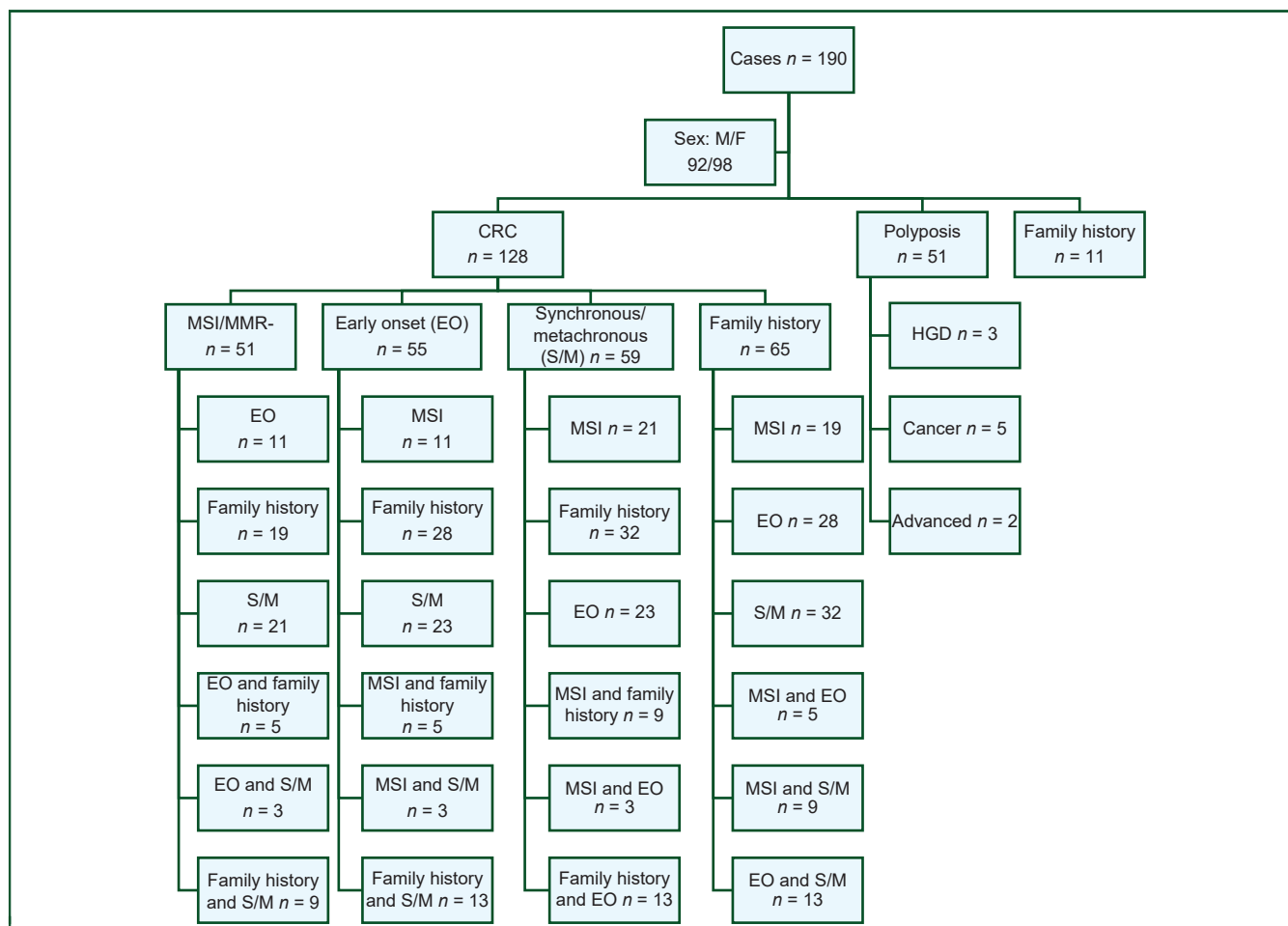


Figure 1. Characteristics suggestive of inherited predisposition in 190 subjects undergoing NGS for CRC.

CRC, colorectal cancer; EO, early onset; F, female; M, male; MMR, mismatch repair; MSI, microsatellite instability; NGS, next-generation sequencing.

novel (not previously reported at the time of our analysis) were confirmed by Sanger sequencing.

The 94 genes analyzed are categorized as high or moderate penetrance based on estimates of cancer risks. In the employed panel, 'high-penetrance' genes involve a lifetime risk of cancer $\geq 40\%$, while the risk associated with 'moderate' genes is usually $< 40\%$, although this notion does not invariably apply to fully penetrant CPGs with a lower lifetime risk (e.g. *NF1*).^{19,20} We first retrieved NGS sequencing data for CPGs that are firmly associated with CRC. Thereafter, irrespective of the clinical picture, the entire panel was interrogated, and both PVs and variants of uncertain significance (VUS) were annotated.

Statistical analysis

The relative frequencies of pathogenic and likely pathogenic variants between different groups were compared by Fisher's exact test. The distribution of class 4 and 5 germline variants according to features suggestive of inherited predisposition has been assessed by multivariable logistic regression analysis employing STATA software, version 13 (Stata Corp, College Station, TX).

RESULTS

In the enrollment period, 190 consecutive subjects who were referred for consultation entered in the present study. Among these, 128 patients (67.4%) had developed CRC. Microsatellite (MS) status [by molecular or immunohistochemical (IHC) analysis; [Supplementary Table S2](https://doi.org/10.1016/j.esmooop.2022.100607), available at <https://doi.org/10.1016/j.esmooop.2022.100607>] was available for 114 (89.0%) cancers, 51 (45.1%) showing microsatellite instability (MSI). Among these, 28 (54.9%) cases had lost MLH1-PMS2 (all with wild-type BRAF; one case had loss of PMS2 only), and 17 (33.3%) had lost MSH2-MSH6 (4 cases had loss of MSH6 only). Six (11.7%) MSI CRCs were not assessed by IHC. Non-mutually exclusive features suggestive of an underlying inherited predisposition in our series of patients, and their relationships, are depicted in [Figure 1](#) and summarized in [Supplementary Table S3](#), available at <https://doi.org/10.1016/j.esmooop.2022.100607>. Overall, 55 patients (42.9%) had EO CRC, 59 patients (46.1%) presented with personal history of synchronous or metachronous cancers, and 65 (34.2%) had a positive family history of CRC and 28 (14.7%) of LS-related tumors. Fifty-one patients (26.8%) had a personal history of polyposis, which was consistent with an attenuated phenotype in 40 patients (78.4%) (8 of whom also had

polyps with serrated histology in addition to adenomas); 9 (17.7%) had at least one polyp with high-grade dysplasia, and 3 (5.9%) also developed CRC. Finally, 11 unaffected subjects (5.8%) requested consultation for positive family history, i.e. at least one first-degree relative with CRC plus another relative with CRC or LS-related tumors.

After sequencing, detected variants were challenged against their classification in multiple repositories; among class 4 variants (Supplementary Table S4, available at <https://doi.org/10.1016/j.esmooop.2022.100607>), only those classified as such by two repositories were considered for further analysis (Table 1). Accordingly, 77 (40.5%) subjects showed no variants or class 1-2 ones. Overall, 64 pathogenic [i.e. class 5; $n = 57$ (89%)] and likely pathogenic [class 4 or 4 and 5 by two independent classifications; $n = 7$ (11%)] germline variants were identified in 51 (26.8%) patients (listed in Table 1).

Among those carrying PVs, eight (15.7%) had two pathogenic *MUTYH* alleles (four being homozygous PV), and coexisting germline class 4 or 5 variants were detected in 4 (7.8%) subjects.

Pathogenic germline variants clustered in MMR genes, altogether accounting for one-third of the whole burden of variants (20/63, 31.7%). Of the germline variants accounting for LS, 8 (40.0%) occurred in *MSH2*, 5 (25.0%) in *MLH1*, 4 (20.0%) in *MSH6*, and 3 (15.0%) in *PMS2*. In four LS cases (20.0%), a coexisting additional germline variant was detected (*MSH2* plus *HOXB13*, *MSH2* plus *BRIP1*, *MSH6* plus *CHEK2*, and *MSH6* plus *BRCA1*), a condition occurring only in patients with germline alterations in MMR genes and in none of those carrying PVs in other genes, a statistically significant difference (4/20 versus 0/31; $P = 0.02$ by Fisher's exact test). Furthermore, 5 out of 51 (9.8%) patients with dMMR CRC had PVs in uncanonical genes (*FANCL*, *FANCA*, *ATM*, *PTCH1*, *BAP1*) but no PVs in LS genes (Table 1).

PVs were detected in 10 out of 63 patients with microsatellite stable CRC (15.9%) (summarized in Figure 2). In this set, *MUTYH* was the most frequently affected gene, with variants identified in four patients (two showing biallelic mutation). All the other carriers exhibited uncanonical germline variants (i.e. *BRCA2*, *BRIP1*, *MC1R*, and *ATM*). In patients with CRC of unknown MS status, we detected PVs associated with LS in three cases, plus one *APC* I1307K variant in a patient with EO CRC and one monoallelic *MUTYH* PV.

PVs were detected in 13 (25.5%) patients with polyposis: 5 patients (9.8%) harbored *APC* variants, 6 (11.8%) had biallelic *MUTYH* PVs (i.e. MAP), and 2 (3.9%) showed variants in other genes (*FANCM*, *XPC*).

In the group of unaffected subjects screened for a positive family history, we detected two carriers (2/11, 18.2%) of pathogenic monoallelic variants in *ATM* and *MUTYH*.

At multivariate logistic regression, the presence of germline class 4-5 variants was significantly associated with younger age ($P = 0.009$), positive family history of CRC ($P = 0.04$), MSI or dMMR molecular phenotype ($P < 0.001$), and barely ($P = 0.06$) with polyposis (Table 2).

Overall, 143 VUS were detected in 101 (53.2%) (Supplementary Table S5, available at <https://doi.org/10.1016/j.esmooop.2022.100607>) subjects in our cohort. VUS occurred with similar frequencies in subjects with PVs (29/51, 56.9%) and in those without (72/139, 51.7%; odds ratio 1.23; 95% confidence interval 0.64-2.34; $P = 0.5$). VUS occurred most frequently in MMR genes (20, 20%), followed by Fanconi anemia complementation group (15, 15%) and *ERCC* excision repair-associated family (9, 9%).

DISCUSSION

The rate of patients with pathogenic germline variants (PVs) detected by multigene panel in our consecutive series was almost one out of three tested subjects, higher than previously reported.^{10-12,21} Such different detection rates could be explained either by the number of tested genes or by the relative prevalence of inherited variants in pre-selected and unselected cohorts. The relevance of the number of tested genes is supported by the results of universal germline testing in unselected CRC patients. Recently, by using an 83-gene NGS panel the prevalence of PVs was 15.3%,²² while a 10% yield was obtained with a 23-gene panel.¹⁰⁻¹² Increasing the detection rate by multigene panels will maximize the identification of transmissible variants in CRC patients, and in cancer patients in general. On the other hand, such improvement should be weighted in the light of suitable strategies for risk management, which should reflect available evidence on the actionability.

In our series, two-thirds (44/64, 68.8%) of PVs were in canonical high-penetrance CRC genes,⁵ mostly MMR genes ($n = 20$, 31.3%), followed by polyposis genes, i.e. *APC* ($n = 6$, 9.4%) and *MUTYH* ($n = 14$, 21.9%). Monoallelic *APC* (I1307K) and biallelic *MUTYH* variants were also detected in three patients without polyposis: these had EO CRC, and one of them also had an important family history (the mother had CRC in the sixth decade and the sister in the fifth decade). Thus, employing a stringent phenotype-genotype approach, we would have missed carriers of variants in canonical CRC genes. In addition, we detected *BRCA1/2* PVs in two (3.1%) patients who did not show characteristics of the hereditary breast ovarian cancer syndrome. These two *BRCA1/2* carriers should be viewed cautiously: the *BRCA1* carrier also carried a pathogenic *MSH6* variant and presented with EO CRC, while the *BRCA2* carrier had EO CRC as well as a positive family history. *BRCA2* carriers are not currently considered at increased risk for CRC.^{5,23} However, based on family history, endoscopic surveillance beginning at age 40 years was recommended to first-degree family members of our index *BRCA2* patient.^{7,21,23}

Penetrance of PVs poses a further issue, as one-third (18/64, 28.1%) of such variants were in low/moderate-penetrance genes, which convey a lower, yet not negligible, contribution to increased CRC risk. *MUTYH* and *ATM* monoallelic variants were the most represented (9.3% and 4.7%, respectively). Monoallelic *MUTYH* variants have been associated with up to 1.5- to 2-fold increased risk of CRC,²⁴ and *ATM* pathogenic alleles with 0.7-fold increase.²⁵ For the latter, no specific guidelines have been proposed so far due

Table 1. List of canonical and uncanonical gene mutation				
No. of patients	Gene (Ref.Seq.)	HGVS nomenclature ^a		
		c.DNA	Protein	Colonic phenotype
Canonical genes				
5	MLH1: (NM_000249.4)	c.1961C>T c.1731G>A c.676C>T c.544A>G c.1639_1643dup	(p.Pro654Leu) (p.Ser556ArgfsTer14) (p.Arg226Ter) (p.Glu153PhefsTer8) (p.Leu549TyrfsTer44)	MSI EO CRC CRC + family hx MSI EO CRC MSI CRC, synchr/metachr CRC, + family hx EO CRC + family hx
8	MSH2: (NM_000251.3)	c.2536C>T c.484G>A c.2536C>T c.942 + 3A>T c.2536C>T c.365dup c.367_?_645 + ?del c.1076 + 1dup	(p.Gln846Ter) (p.Gly162Arg) (p.Gln846Ter) (p.?) (p.Gln846Ter) (p.Ala123GlyfsTer10) (p.?) (p.Leu360IlefsTer29)	MSI CRC MSI, EO CRC MSI CRC + family hx MSI, EO CRC + family hx MSI CRC + family hx MSI CRC MSI CRC + family hx CRC, synchr/metachr cancers + family hx
4	MSH6: (NM_000179.3)	c.2569_2572del c.3173-1G>A c.872_875del c.2848_2849del	(p.Asp857PhefsTer10) (p.?) (p.Asn291ThrfsTer13) (p.Ser950ProfsTer15)	MSI CRC + family hx MSI CRC MSI CRC, synchr/metachr cancers + family hx EO CRC + family hx
3	PMS2: (NM_000535.7)	c.(2446 + 1_-2446-1)_(2589 + 1_-2589-1) c.2192_2196del c.137G>T	p.(Val816_Asn862delinsPro) (p.Leu731CysfsTer3) (p.Ser46Ile)	MSI, EO CRC MSI, EO CRC MSI CRC
6	APC: (NM_000038.6)	c.3920T>A c.221-2A>G c.4875del c.1177dup c.3183_3187del c.3615del	(p.Ile1307Lys) (p.?) (p.Gln1625HisfsTer25) (p.Ser393PhefsTer4) (p.Gln1062Ter) (Asn1206MetfsTer41)	EO CRC Polyposis Polyposis Polyposis Polyposis Polyposis
14	MUTYH: (NM_001128425)	c.536A>G c.734G>A c.1187G>A c.1187G>A c.1437_1439del c.1227_1228dup c.536A>G c.536A>G c.1187G>A c.1187G>A c.1437_1439del c.1187G>A c.312C>A c.1187G>A c.536A>G c.1187G>A c.1437_1439del c.1437_1439del c.1187G>A c.1187G>A c.536A>G c.536A>G c.1437_1439del	(p.Tyr179Cys) (p.Arg245His) (p.Gly396Asp) (p.Gly396Asp) (p.Glu480del) (p.Glu410GlyfsTer43) (p.Tyr179Cys) (p.Tyr179Cys) (p.Gly396Asp) (p.Gly396Asp) (p.Glu480del) (p.Gly396Asp) (p.Tyr104Ter) (p.Gly396Asp) (p.Tyr179Cys) (p.Gly396Asp) (p.Glu480del) (p.Glu480del) (p.Gly396Asp) (p.Gly396Asp) (p.Tyr179Cys) (p.Tyr179Cys) (p.Glu480del) (p.Glu480del) (p.Gly396Asp) (p.Gly396Asp) (p.Tyr179Cys) (p.Tyr179Cys) (p.Glu480del)	EO CRC, + family hx CRC, polyps EO CRC, + family hx CRC, polyps CRC + family hx Synchr/metachr CRC EO CRC + family hx Polyposis, CRC Polyposis Polyposis, CRC Polyposis, CRC Polyposis; + family hx Polyposis Family hx
Uncanonical genes				
3	ATM: (NM_000051.4)	c.4906C>T c.6671dup c.1212_1213del	(p.Gln1636Ter) (p.Met2224IlefsTer25) (p.Asn405Ter)	MSI CRC, synchr/metachr cancers + family hx Family hx EO CRC, + family hx
1	BAP1: (NM_004656.4)	c.38-16_38-1del	(p.?)	MSI CRC
1	BRCA1: (NM_007294.4)	c.3351dup	(p.Gln1118SerfsTer4)	EO CRC, ^b + family hx
1	BRCA2: (NM_000059.4)	c.7180A>T	(p.Arg2394Ter)	EO CRC, + family hx
2	BRIP1: (NM_032043.3)	c.1046_1049del ^f c.2392C>T	(p.Ala349ValfsTer9) (p.Arg798Ter)	CRC, synchr/metachr cancers other than CRC MSI, ^b EO CRC, + family hx
1	CHEK2: (NM_005228.5)	c.507del	(p.Phe169LeufsTer2)	MSI CRC ^b
1	FANCA: (NM_000135.4)	c.2851C>T	(p.Arg951Trp)	MSI CRC
1	FANCL: (NM_001114636.1)	c.1022_1024del	(p.Ile341_Cys342delinsSer)	MSI CRC
1	FANCM: (NM_020937.4)	c.5791C>T	(p.Arg1931Ter)	Polyposis
1	MC1R: (NM_002386.4)	c.451C>T	(p.Arg151Cys)	EO CRC
1	PTCH1: (NM_000264.4)	c.579dup	(p.Asn194GlnfsTer58)	MSI CRC, + family hx
1	XPC: (NM_004628.5)	c.2116-1G>A	(p.?)	Polyposis
1	HOXB13: (NM_006361.5)	c.251G>A	(p.Gly84Glu)	CRC, ^b synchr/metachr cancers + family hx

+ family hx, positive family history; CRC, colorectal cancer; EO, early onset; HGVS, Human Genome Variation Society; MMR, mismatch repair; MSI, microsatellite instability; PV, pathogenic variant; synchr/metachr, synchronous or metachronous cancers.

^aAs assessed on 1 June 2022.

^bCoexisting with a PV in MMR system.

^cPreviously unreported.

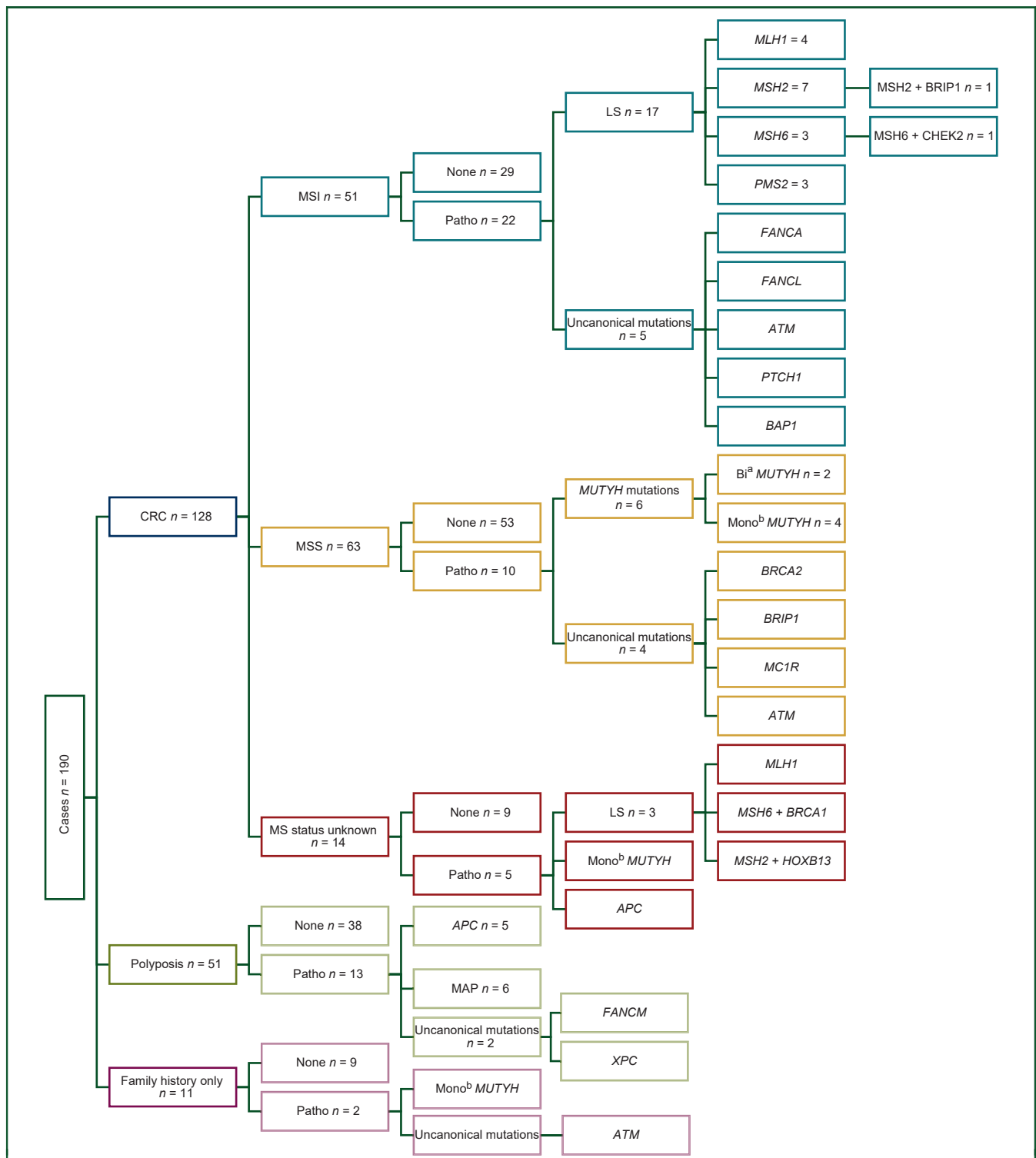


Figure 2. Pathogenic variants identified with reference to indication for genetic evaluation.

CRC, colorectal cancer; LS, Lynch syndrome; MAP, *MUTYH*-associated polyposis; MS, microsatellite; MSI, microsatellite instability; MSS, microsatellite stability; PV, pathogenic variant.

^aBi, biallelic PV in *MUTYH*.

^bMono, mono-allelic PV in *MUTYH*.

to limited data,^{5,26} while carriers of monoallelic *MUTYH* mutations who have an affected first-degree relative have sufficiently high risks of CRC to warrant preventive approaches²⁷ aimed at adenoma removal to effectively drop the risk of CRC after 40 years of age.⁵

Alike MMR genes and *MUTYH*, most uncanonical genes harboring PVs, including *BRCA1/2*, play a role in DNA repair processes. Their activities encompass activation of DNA damage response (i.e. *ATM*, *XPC*, *FANCM*) and DNA damage correction (i.e. *BRIP*, *FANCA*), or the participation in the

Table 2. Multivariable logistic regression for the likelihood of pathogenic germline variants according to features suggestive of inherited predisposition in 179 consecutive patients

Feature	Absent		Present		Odds ratio	95% Confidence interval		P value
	WT	MUT	WT	MUT				
Age	<i>Continuous variable</i>				0.94 ^a	0.90	0.98	0.009
Family history								
CRC	67	24	62	26	2.14	1.02	4.49	0.044
Other tumors	74	25	55	25	1.52	0.72	3.22	0.27
Early onset ^b	93	33	36	17	0.55	0.16	1.93	0.35
S/M ^c	87	33	42	17	1.74	0.75	4.02	0.19
Polyposis	91	37	38	13	3.48	0.95	12.66	0.06
MS status								
MSS			53	10	Ref. 1.00			
Undetermined			9	5	1.52	0.46	5.03	0.49
MSI			29	22	9.43	3.31	26.86	<0.001

CRC, colorectal cancer; MS, microsatellite; MSI, microsatellite instability; MSS, microsatellite stability; MUT, mutated; PV, pathogenic variant; S/M, synchronous/metachronous; WT, wild type.

^az, -2.61.

^bFifty-eight patients with age <50 years include three polyposis patients.

^cSixty-six patients with sync/metachronous CRC include six cases with polyposis and one patient with an ileal tumor.

regulation of cell growth and division (i.e. *PTCH1*, *BAP1*).^{25,28} PVs in some of these genes occur in syndromes with increased susceptibility to the development of specific tumor types (i.e. basal cell carcinoma in carriers of *PTCH1* PVs). Noticeably, the carrier of *PTCH1* PVs did not show the phenotypic hallmarks of the associated syndrome, nor did their relatives.

Polymorphisms in genes like *XPC* have been associated with modest increases in CRC risk.²⁹ However, the role of rare PVs in CRC, which is currently not part of their recognized clinical spectrum, remains to be defined. Under this respect, it might be of interest to assess whether the assets of CRC-associated genetic variants could affect the association of rare PVs with CRC development.²⁸ Finding uncanonical PVs outside their specific syndromic context poses clinical and ethical dilemmas for the communication with patients, which should be discussed within the frame of informed consent before the test.

Another relevant finding in carriers of PVs concerns the coexistence of additional, uncanonical ones in almost 8% of the cases, all bearing one variant in MMR genes. Among carriers of PVs, coexisting ones have been reported by Yurgelun et al. in 3/185 (1.6%) patients who had undergone genetic testing for LS¹¹ and in 5/105 (4.7%) unselected CRC patients,¹⁰ similar to the findings of Pearlman and colleagues in those with EO CRC (3/72, 4.1%).¹² Recently, Pearlman et al. reported the coexistence of one additional PV in 10/142 (7.0%) unselected patients with EO CRC harboring PV in MMR genes. The higher prevalence in our cohort of patients with PVs in MMR genes (4/20, 20%) should be taken cautiously, due to the small size of our series and the clinical selection of patients.³⁰ At any event, our results definitely testify that PVs other than those in MMR genes coexist in a fraction of patients with LS, and further studies will be important to assess their impact on risks and outcomes.

We also detected unconventional PVs in patients with Lynch-like syndrome (LLS). In this condition, MSI CRC

develops in the absence of germline mutations affecting MMR genes or of somatic changes which may explain the molecular phenotype, like *MLH1* somatic hypermethylation.³¹ A subgroup of cases with LLS harbored PVs in non-MMR genes (i.e. *FANCA*, *ATM*, *PTCH1*, *BAP1*). While many cases of LLS are associated with double somatic mutations in MMR genes,^{32,33} the presence of germline PVs in other genes has also been reported.³⁴⁻³⁶ In our series and in those studied by Pearlman et al.,^{12,34} such fraction was $\geq 10\%$ and NGS might help to exclude other syndromes before establishing a diagnosis of LLS.

In conclusion, this is the first European study reporting the highest prevalence of germline PVs in patients undergoing germline NGS for an increased CRC risk by clinical criteria.¹⁵ The high prevalence of PVs in canonical CRC genes is a strength calling for adoption and implementation of this technology, in the future also including in the panel-specific genes involved in rare polyposis syndromes. The frequent occurrence of uncanonical PVs with unclear clinical actionability poses the issue of identifying effective preventive strategies, as well as of devising adequate communication for patients and their relatives. Collection of additional data will be important to refine risk assessment and to define the appropriate gene content of the panels to deploy in this field.

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DISCLOSURE

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