

## MOLECULAR BASIS OF INHERITED COLORECTAL CARCINOMAS IN THE MACEDONIAN POPULATION: AN UPDATE

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### ABSTRACT

Hereditary factors are assumed to play a role in ~35.0-45.0% of all colorectal cancers (CRCs) with about 5.0-10.0% associated with high penetrant disease-causing mutations in genes correlated to hereditary polyposis (HP) or hereditary non polyposis syndromes (HNPCC). Although inherited germline mutations in mismatch repair (MMR) and the *APC* genes contribute significantly to CRC, genetic diagnosis cannot yet be obtained in more than 50.0% of familial cases. We present updated data of 107 probands from the Macedonian population with clinically diagnosed HP ( $n = 41$ ) or HNPCC ( $n = 66$ ) obtained by next generation sequencing (NGS) with three different gene panels covering the coding, flanking and promoter regions of 114 cancer predisposition genes. Using this approach, we were able to detect deleterious mutations in 65/107 (60.7%) patients, 50.4% of which were in known well-established CRC susceptibility genes and 10.2% in DNA repair genes (DRG). As expected, the highest frequencies of deleterious variants were detected in familial adenomatous polyposis (FAP) and in HNPCC patients with microsatellite instabil-

ity (MSI) tumors (93.8 and 87.1%, respectively). Variants of unknown significance (VUS) were detected in 24/107 (22.4%) patients, mainly in HNPCC patients with microsatellite stable (MSS) tumors or patients with oligopolyposis. The majority of VUS were also found in DRG genes, indicating the potential role of a double-strand break DNA repair pathway deficiency in colorectal cancerogenesis. We could not detect any variant in 18/107 (16.8%) patients, which supports the genetic heterogeneity of hereditary CRC, particularly in HNPCC families with MSS tumors and in families with oligopolyposis.

**Keywords:** Hereditary colorectal cancer (CRC); Macedonian population; mutations.

### INTRODUCTION

Colorectal cancer (CRC) is one of the most frequent cancers, especially in well-developed countries and it is one of the major public health concerns worldwide. Hereditary factors are assumed to play a role in approximately 35.0-45.0% of all CRCs [1]. Our understanding of the genetic basis, as well as the guidelines for clinical management of hereditary CRC syndromes continue to evolve rapidly, so it is crucial for clinicians to recognize the unique features in the diagnosis and management of these syndromes. A precise understanding of the genetics of inherited CRCs is important for identifying at-risk individuals, improving cancer surveillance and prevention strategies and developing better diagnostic and therapeutic approaches [2,3].

Approximately 5.0-10.0% of hereditary CRCs develop due to highly penetrant mutations in genes associated with well-characterized inherited Mendelian cancer syndromes. The most commonly affected genes are the MMR genes [*MLH1* (OMIM: 120436); *MSH2* (OMIM: 609309); *MSH6*

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(OMIM: 600678) and *PMS2* (OMIM: 600259)] in patients with the Lynch syndrome, and the *APC* gene (OMIM: 611731) in patients with familial adenomatous polyposis (FAP). Current data indicate that the molecular defects in these syndromes are very heterogeneous, and that certain mutations might occur with considerable frequencies in particular populations [3]. In the remaining ~30.0% of inherited CRCs, the etiology is still not completely understood. The recent expansion of new sequencing technologies based on massive parallel next generation sequencing (NGS), including whole-genome and whole-exome sequencing, as well as copy number approaches, offered opportunities for identification of new high-penetrant genes that could explain the aggregation of CRC in high-risk families [4]. New insights in this field open considerable challenges concerning variant interpretations even for the known CRC susceptibility genes. Next generation sequencing data provide a huge number of variants in both coding and non coding regions; thus, identifying a disease-causing variant from this large number of variants poses a serious task.

Another possible explanation for the aggregation of CRCs in certain families is that the heritability is not due to a single monogenic defect but a multifactorial condition, caused by the conjunction of moderate-risk or low-risk genetic variants, possibly in combination with environmental or lifestyle risk factors. Evidence from recent studies shows that the accumulation of risk variants is associated significantly with an increased risk of CRC in individuals with a family history of the disease [5].

We have previously reported our initial data on the molecular characterization of FAP and HNPCC in our population [6,7]. We now present updated results of the study, which was performed on a larger cohort of patients with a clinical diagnosis of hereditary CRCs using an extended panel of genes related to cancer predispositions.

## MATERIALS AND METHODS

A total of 107 probands included in this study were recruited from the University Clinics for Digestive Surgery and for Radiology and Oncology, Skopje, RN Macedonia. Sixty-six patients comply with Amsterdam criteria for clinical diagnosis of HNPCC and 41 patients were diagnosed with multiple polyps reminiscent of FAP (>1000 adenomatous polyps through the large bowel, attenuated FAP (<100 adenomatous polyps) or juvenile polyposis (multiple polyps with hamartomatous component). Informed consent was obtained from all participants. The research protocol was approved by the Ethics Committee of the Faculty of Pharmacy in Skopje, RN Macedonia.

Whole blood (3 mL with EDTA as anticoagulant) and fresh frozen tumor tissue or formalin-fixed paraffinembed-

ded (FFPE) blocks were used for DNA isolation. DNA was extracted using the standard phenol/chloroform method and quantified using the spectrophotometer NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Microsatellite instability in tumor samples was analyzed with a multiplex fluorescent polymerase chain reaction (PCR), followed by capillary electrophoresis (CE) on a 3500 Automated Genetic Analyzer (Life Technologies, Carlsbad, CA, USA). Microsatellite instability (MSI) status was determined using nine short tandem repeat (STR) markers: BAT25, BAT26, D2S123, D5S107, D5S346, D17S250, D18S58, D18S61 and D18S535, derived from the panel of microsatellite loci defined by the National Cancer Institute and the protocols described previously [7,8]. The tumor samples were classified as MSI-H (MSI-high) if instability was present at more than 30.0% of the loci screened, MSI-L (MSI-low) if at least one but fewer than 30.0% of the loci showed instability or MSS (microsatellite stable) if all loci were stable.

Four different multiplex ligation-dependent probe amplification (MLPA) analyses were used for screening for extended germline rearrangements in the *MLH1*, *MSH2*, *MSH6*, *PMS2* and *APC* genes. The HNPCC samples were ligated and amplified using the SALSA MLPA P003 (*MLH1/MSH2*) SALSA MLPA P072 (*MSH6*) and SALSA MLPA P008 (*PMS2*) probe mixes according to the manufacturer's recommendations [Microbiology Research Centre Holland (MRC-Holland), Amsterdam, The Netherlands]. In patients manifesting oligopolyposis, the whole *APC* gene (exon 1-18) and a substantial part of the *MUTYH* gene (exons 1, 4 and 9) were screened for large deletions/duplications using the SALSA MLPA P043 (*APC*) probemix. Data normalization and analysis for both analyses were conducted using Coffalyser.Net software (MRC-Holland; <http://www.mlpa.com>).

The presence of methylation in the *MLH1* gene promoter was analyzed in DNA isolated from tumors of all patients with MSI positive non polyposis familial CRC. For this analysis, 1 µg of DNA was converted by bisulfite modification using EZ DNA Methylation-Lightning Kit (Zymo Research, Irvine, CA, USA) following the procedure recommended by the manufacturer. A total of 50-100 ng of converted DNA was subjected to real-time PCR analysis using primers and fluorescent probes specific for methylated DNA in the *MLH1* gene promoter region [9]. The efficacy of the bisulfite modification was evaluated by a control reaction, which was run in parallel for every sample with primers and probe located in the *ACTB* gene that does not contain CpG islands and is not subjected to methylation.

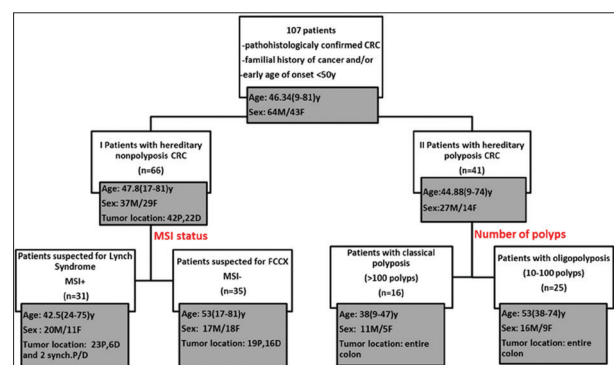
Two different platforms were used for NGS: Ion Torrent PGM™ (Thermo Fisher Scientific) and MiniSeq (Illumina Inc., San Diego, CA, USA). For the Ion Tor-

rent PGM platform, two custom AmpliSeq panels were designed using AmpliSeq Designer (Life Technologies). First, analyses of the genes most commonly affected in HNPCC and FAP (*MLH1*, *MSH2*, *MSH6*, *PMS2* and *APC*) were conducted and all negative samples were additionally analyzed using a second panel consisting of all genes implicated in HNPCC or familial polyposis (*POLE*, *POLD1*, *MUTYH*, *SMAD4*, *BMPRIA*, *STK11*, *GREM1*, *PTEN*, *SEMA4A*, *RPS20*, *FAN1*, *POT1*, *MRE11A*, *LIMK2*, *IL12RBI*). In both cases, DNA libraries were prepared using Ion AmpliSeq™ Library Kit 2.0 (Life Technologies), barcoded using Ion Xpress™ Barcode Adapters Kit (Thermo Fisher Scientific), and normalized with Library Equalizer™ Kit (Thermo Fisher Scientific), multiplexed according to the manufacturer's protocols, to an amount permitting a theoretical coverage of at least 100 reads per targeted sequence/patient. Template preparation and sample enrichment was conducted on Ion OneTouch™ 2 System (Thermo Fisher Scientific) using Ion PGM™ Hi-Q OT2 Kit (Thermo Fisher Scientific). The sequencing reactions were carried on an Ion torrent PGM™ platform using Ion PGM™ Hi-Q Sequencing Kit and Ion 316™ Chips v2 (Ion torrent; Life Technologies). The data were aligned using TMAP (Torrent Suite Software; Life Technologies) and annotated using the Variant caller (Torrent Suite Software; Thermo Fisher Scientific) and ANNOVAR (<http://annovar.openbioinformatics.org>) [10]. All patients in whom no causative mutations were found in the genes from the two custom panels, were analyzed using TruSight Cancer Panel (Illumina Inc.), which targets 94 genes and 284 SNPs associated with a predisposition to various cancers. DNA libraries were prepared and multiplexed using TruSight Rapid Capture kit, according to the manufacturer's instructions. The sequencing reactions were performed on a MiniSeq platform (Illumina Inc.) using MiniSeq Mid Output Kit (300-cycles). The data were aligned and annotated using the Variant Studio v.3 software (Illumina Inc.). The potential pathogenicity of the detected variants of unknown significance (VUS) was tested using the software for functional prediction [PolyPhen2 (<http://genetics.bwh.harvard.edu.pph2/>), SIFT (<https://sift.bii.a-star.edu.sg/>), FATHMM (<http://fathmm.biocompute.org.uk>), Mutation Taster (<http://www.mutationtaster.org>), PROVEAN (<http://provean.jcvi.org/index.php>), VARSOME (<https://varsome.com>)] and for their clinical impact [ClinVar; (<https://www.ncbi.nlm.nih.gov/clinvar>) and InSight (<https://www.insight-group.org/variants/databases/>)]. For final classification of the detected sequence variants, we used the recommended guidelines from the American Collage of Medical Genetics (ACMG) [11]. The variants classified by this approach as pathogenic or likely pathogenic, are referred to as deleterious variants in the text.

All potentially deleterious variants and low coverage regions were validated using standard protocols for Sanger sequencing. In addition, the region spanning exons 10-15 of the *PMS2* gene were initially amplified by two long range PCR reaction spanning exons 10 to 12 and 12 to 15, respectively, using primers that discriminate the *PMS2* gene from the highly homologous *PMS2CL* pseudogene sequences. These PCR products were used as templates for the amplification of individual exons of the *PMS2* gene. Prior to the sequencing reaction, the amplicons were purified using a low-melt agarose protocol [12]. Bidirectional sequencing was performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). Sequencing products were purified using BigDye X Terminator® Purification Kit (Thermo Fisher Scientific) and analyzed with CE on a 3500 Genetic Analyzer (Thermo Fisher Scientific). The reference sequences used for variant nomenclature are given in Supplementary Table 1.

## RESULTS

After a detailed review of the clinical/pathological data and familial segregation of CRC and other cancers, we selected 107 unrelated patients for molecular analysis, of which 66 probands complied with the Amsterdam criteria for clinical diagnosis of HNPCC and 41 had multiple polyps reminiscent of FAP or associated syndromes (Figure 1). Of the 66 HNPCC patients, 31 had the MSI+ phenotype and 35 had MSS tumors. The disease in HNPCC patients with MSI+ tumors developed predominantly in males, at a younger age (average 42.5 years, range 24-75 years) and with a preponderance for the proximal colon, whereas in HNPCC patients with MSS tumors, the onset of the disease was at an average age of 53 years (range 17-81 years), equally distributed in both genders and localizations. Of the 41 patients with polyposis, 16 patients, predominantly

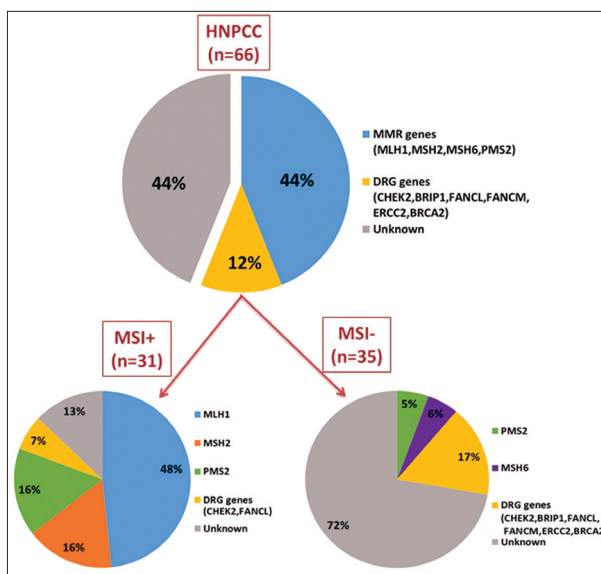


**Figure 1.** Number of patients in different subgroups based on the number of polyps and MSI status of their tumors at diagnosis. M: male; F: female; P: Proximal colon (caecum, ascendens, transversum); D: distal colon (descendens, sigma, rectum).

males (11/16), presented with a classical FAP phenotype (>1000 polyps) at an average age of 38 years (range 9-47 years) at diagnosis, while 25 patients with oligopolyposis (<100 polyps) were diagnosed at an average age of 53 years (range 38-74 years). Extracolonic cancers were present in 17/31 families with MSI+ HNPCC (primarily endometrial and gastric cancers), in 23/35 families with MSS HNPCC (primarily breast/ovarian, endometrial and gastric cancers and leukemias); in 2/16 families with FAP (both with gastric cancer) and in 7/25 families with oligopolyposis (primarily breast and pancreatic cancers). The clinical and pathological data of these patients are summarized in Table 1.

**Molecular Analysis of Patients with HNPCC.**

Overall, deleterious variants were detected in 37/66 (56.0%) patients with HNPCC, of which 29/66 (44.0%) in MMR and 8/66 (12.0%) in DRG (DNA repair pathway) genes (Figure 2). The majority of patients with MSI+ tumors (25/31; 80.6%) carried deleterious mutations in the MMR genes. Generally, we have identified seven different pathogenic variants in the *MLH1* gene in 15 unrelated patients, three different pathogenic variants in the *MLH2* gene in five patients, and three different pathogenic variants in the *PMS2* gene in five patients (Table 1). It is worth noting that five variants were present in >50.0% of the MSI+HNPCC patients [*MLH1*: c.392C>G p.(Ser131Ter) in six families; *MLH1*: c.244A>G p.(Thr82Ala), *MSH2*: c.2211-2A>C p.(?) and *PMS2*: c.(803+1\_804-1)\_(\*1\_?)del p.(?) each in three families, and *MLH1*: c.896\_897insC p.(Pro300SerfsTer7) in two families]. Apart from these, all other identified variant were found in individual families; two missense mutations [c.62C>T p.(Ala21Val) and c.683T>C p.(Leu228Pro)], one frameshift variant [c.1602del p.(Asn535IlefsTer56)] and one splice variant [c.1667+1del p.(?) in the *MLH1* gene, two in-frame deletions [c.1786\_1788 del p.(Asn596del) and c.209\_211+11del p.(?) in the *MSH2* gene and two small insertions/deletions [(c.2192\_2196 del p.(Leu731Cys fsTer3) and c.1327del p.(Arg443Glu fsTer5)] in the *PMS2* gene. The MSI+ phenotype in the remaining six patients from this group was due to the presence of *MLH1* promoter methylation (six patients) or bi-allelic somatic inactivation of the *MSH2* gene (one patient). In one of these patients we detected germline VUS [c.418G>A p.(Ser128 Leu)] in the *PMS2* gene, in another two patients we found deleterious germline variants in the *CHEK2* [c.470T>G p.Ile157Ser] and *FANCL* [c.2T>C p.Met1Thr] genes, and one patient had a VUS in the *FANCM* gene [c.643G>A p.(Glu215Lys)]. We did not detect any VUS/pathogenic anomalies in the 114 cancer predisposition genes tested in only two patients from the MSI+HNPCC group. It is worth noting that the mutation c.1799T>A p.Val600Glu in the *BRAF* gene was present



**Figure 2.** Distribution of deleterious variants in 66 patients with HNPCC. The distribution of the mutations in different genes in the two subgroups of HNPCC patients divided by the MSI status of their tumors is also shown.

only in the patient with the *FANCL* pathogenic variant from this subgroup. Overall, of the 14 pathogenic and two VUS detected in this group of patients, five and one variants, respectively are novel, and nine and one variants, respectively, have been reported previously and are present in the relevant databases (Table 1).

Deleterious variants were detected in 10/35 (28.0%) HNPCC patients with MSS tumors (Figure 2 and Table 1), of which three variants in the MMR genes in four patients (11.4%) [c.(?\_-152)\_260+1\_261-1) p(?) and c.457+1G>T p.(?) in *MSH6* and one gene inversion in the *PMS2* gene in two patients] and five in five different DRG genes in six patients (17.0%) [*BRIP1*: c.2392C>T p.(Arg798Ter) in two patients, *BRC A2*: c.4446\_4451dupAACAGA, p.(Glu1482\_Thr1483dup), *CHEK2*: c.1100del p.Thr367fs *FANCM*: c.2953del p.(Glu985ArgfsTer3) and *ERCC2*: c.1403C>T p.(Pro468Leu), in one patient each]. A large percentage of patients from this group (14/35, 40.0%) had a VUS, of which four in known CRC genes (MMR and *APC*), eight in DRG genes (*BLM*, *CHEK2*, *FANCL* and *PALB2*) and four in genes with different functions (*EZH2*, *CEP57*, *KIT*, *CDH1*). The VUS c.545C>T p.(Thr182Ile) in the *BLM* gene was present in a patient with a *BRC A2* [c.4446\_4451 dup AACAGA p.(Glu1482\_Thr1483dup)] likely pathogenic variant, whereas two patients had two VUS, one of which was in the *PALB2* gene [c.2792T>G p.(Leu931Arg)] and the second variant in the *APC* [c.4073C>T p.(Ala1358Val) and *KIT* c.1688T>A p.(Ile563Lys)] genes, respectively. In 11/35 (31.5%) of the HNPCC patients with MSS tumors we did not detect any

**Table 1.** Summary of clinical data and molecular defects detected in 66 patients with hereditary nonpolyposis colorectal cancer.

n	Sex-Age	History (relatives affected)	Tumor Location	Stage at Dx	Extracolonic Cancers*	Gene	DNA Sequence Change	Amino Acid Change	ACMG Classification [11]	MSI	MLH1	BRCA1
										Status	met	V600E
1	M-42	1: 1 <sup>st</sup> , 2: 2 <sup>nd</sup> degree	transversum	na	G; P; Ur	MLH1	c.896_897insC	p.Pro300SerfsTer7	pathogenic	[+]	[-]	[-]
2	M-55	3: 1 <sup>st</sup> ; 1: 2 <sup>nd</sup> degree	transversum	na	none	MLH1	c.392C>G	p.Ser131Ter	pathogenic	[+]	[-]	[-]
3	F-49	1: 1 <sup>st</sup> ; 1: 2 <sup>nd</sup> degree	transversum <sup>b</sup>	IIA	G; E	MLH1	c.392C>G	p.Ser131Ter	pathogenic	[+]	[-]	[-]
4	M-32	1: 1 <sup>st</sup> ; 2: 2 <sup>nd</sup> degree	transversum	IIA	none	MLH1	c.392C>G	p.Ser131Ter	pathogenic	[+]	[-]	[-]
5	M-33	1: 1 <sup>st</sup> ; 2: 2 <sup>nd</sup> degree	ascendens	IIA	none	MLH1	c.392C>G	p.Ser131Ter	pathogenic	[+]	[-]	[-]
6	F-48	positive family history; NS	ascendens	na	E	MLH1	c.1602del	p.Asn535IlefsTer56	pathogenic	[+]	[-]	[-]
7	F-43	2: 1 <sup>st</sup> ; 3: 2 <sup>nd</sup> degree	descendens	na	G; P; Ur	MLH1	c.896_897insC	p.Thr372ThrfsTer7	pathogenic	[+]	[-]	[-]
8	F-60	2: 1 <sup>st</sup> ; 2: 2 <sup>nd</sup> degree	caecum	IIA	none	MLH1	c.392C>G	p.Ser131Ter	pathogenic	[+]	[-]	[-]
9	F-41	1: 1 <sup>st</sup> ; 2: 2 <sup>nd</sup> degree	caecum	na	E	MLH1	c.1667+1del	p.?	pathogenic	[+]	[-]	[-]
10	F-29	1: 1 <sup>st</sup> ; 2: 2 <sup>nd</sup> ; 2: 3 <sup>rd</sup> degree	descendens	na	none	MLH1	c.392C>G	p.Ser131Ter	pathogenic	[+]	[-]	[-]
11	F-24	1: 1 <sup>st</sup> ; 1: 2 <sup>nd</sup> degree	caecum	IIA	E	MLH1	c.244A>G	p.Thr82Ala	likely pathogenic	[+]	[-]	[-]
12	M-40	1: 1 <sup>st</sup> degree	ascendens	IIA	none	MLH1	c.244A>G	p.Thr82Ala	likely pathogenic	[+]	[-]	[-]
13	M-55	1: 2 <sup>nd</sup> degree	caecum	IIA	none	MLH1	c.244A>G	p.Thr82Ala	likely pathogenic	[+]	[-]	[-]
14	M-38	2: 1 <sup>st</sup> ; 2: 2 <sup>nd</sup> ; 2: 3 <sup>rd</sup> degree	ascendens	IIA	P; B	MLH1	c.62C>T	p.Ala21Val	likely pathogenic	[+]	[-]	[-]
15	F-57	1: 1 <sup>st</sup> ; 2: 2 <sup>nd</sup> degree	ascendens	na	B	MLH1	c.683T>C	p.Leu228Pro	likely pathogenic	[+]	[-]	[-]
16	M-15	1: 1 <sup>st</sup> ; 2: 2 <sup>nd</sup> degree	transversum	IIA	E	MSH2	c.2211-2A>C	p.?	pathogenic	[+]	[-]	[-]
17	F-41	2: 1 <sup>st</sup> ; 2: 2 <sup>nd</sup> degree	caecum	IIA	E	MSH2	c.2211-2A>C	p.?	pathogenic	[+]	[-]	[-]
18	M-50	1: 1 <sup>st</sup> ; 2: 2 <sup>nd</sup> ; 2: 3 <sup>rd</sup> degree	transversum	IIA	E	MSH2	c.2211-2A>C	p.?	pathogenic	[+]	[-]	[-]
19	M-41	3: 2 <sup>nd</sup> degree	rectosygma	IIIC	E	MSH2	c.209_211+11del	p.?	pathogenic	[+]	[-]	[-]
20	M-46	1: 1 <sup>st</sup> ; 4: 2 <sup>nd</sup> ; 2: 3 <sup>rd</sup> degree	rectum	na	G	MSH2	c.1786-1788del	p.Asn596del	likely pathogenic	[+]	[-]	[-]
21	F-31	1: 1 <sup>st</sup> ; 2: 2 <sup>nd</sup> degree	ascendens	IVA	none	MSH6	c.(?-152)(260+1_261-1)	p.?	pathogenic	[-]	NA	[-]
22	F-44	1: 1 <sup>st</sup> ; 2: 2 <sup>nd</sup> degree	rectum	IIIB	E	MSH6	c.458+1G>T	p.?	pathogenic	[-]	NA	[-]
23	F-44	2: 1 <sup>st</sup> ; 2: 2 <sup>nd</sup> degree	transversum	IIIA	O	MSH6	c.2384T>C	p.Ile795Thr	VUS	[-]	NA	[-]
24	M-81	1: 1 <sup>st</sup> degree	rectum	IIIC	none	PMS2	gene inversion	-	pathogenic	[-]	NA	[-]
25	M-61	1: 1 <sup>st</sup> degree	sygma	IIIB	none	PMS2	gene inversion	-	pathogenic	[-]	NA	[-]
26	M-31	1: 1 <sup>st</sup> degree	caecum	IIA	none	PMS2	c.(803+1_804-1)*1_?)del	p.?	pathogenic	[+]	[-]	[-]
27	M-39	1: 1 <sup>st</sup> degree	ascendens	IIIC	E	PMS2	c.(803+1_804-1)*1_?)del	p.?	pathogenic	[+]	[-]	[-]
28	M-68	1: 1 <sup>st</sup> ; 1: 2 <sup>nd</sup> degree	rectosygma + caecum	IIA	none	PMS2	c.2192_2196del	p.Leu731CysfsTer3	pathogenic	[+]	[-]	[-]
29	M-65	1: 1 <sup>st</sup> degree	caecum	na	none	PMS2	c.1327del	p.Pro443ThrfsTer16	pathogenic	[+]	[+]	[-]
30	M-40	positive family history; NS	caecum	na	none	PMS2	c.(803+1_804-1)*1_?)del	p.?	pathogenic	[+]	[-]	[-]
31	F-53	2: 2 <sup>nd</sup> degree	caecum + rectum	IIIB	E	PMS2	c.418G>A	p.Ser128Leu	VUS	[+]	[+]	[-]
32	F-59	3: 1 <sup>st</sup> ; 1: 2 <sup>nd</sup> degree	rectum	IIA	E; G; T; L	PMS2	c.934A>G	p.Met312Val	VUS	[-]	NA	[-]
33	F-53	2: 1 <sup>st</sup> ; 3: 2 <sup>nd</sup> ; 3: 3 <sup>rd</sup> degree	sygma	in situ	L	PMS2	c.726G>A	p.Gly207Glu	VUS	[-]	NA	[-]
34	M-53	2: 1 <sup>st</sup> degree	caecum	IIIA	B	CHEK2	c.1100del	p.Thr367fs	pathogenic	[-]	NA	[-]
35	M-52	positive family history; NS	caecum	na	none	CHEK2	c.470T>G	p.Ile157Ser	likely pathogenic	[+]	[+]	[-]

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36	F-51	1: 1 <sup>st</sup> ; 1: 2 <sup>nd</sup> ; 2: 3 <sup>rd</sup> degree	rectum	na	B; P	<i>CHEK2</i>	c.374T>G	p.Phe125Cys	VUS	[-]	NA	[-]
37	F-59	positive family history; NS	sygma	IV	none	<i>CHEK2</i>	c.1313A>G	p.Asp438Gly	VUS	[-]	NA	[-]
38	M-57	positive family history; NS	ascendens	IIIB	none	<i>FANCL</i>	c.2T>C	p.Met1Thr	pathogenic	[+]	[+]	[+]
39	F-43	3: 2 <sup>nd</sup> ; 1: 3 <sup>rd</sup> degree	transversum	IIA	G; R	<i>FANCL</i>	c.1111_1114dup ATTA	p.Thr372Asnfs	VUS	[-]	NA	[-]
40	M-64	2: 1 <sup>st</sup> degree	rectum	IIIC	none	<i>FANCL</i>	c.1111_1114dup ATTA	p.Thr372Asnfs	VUS	[-]	NA	[-]
41	F-42	1: 1 <sup>st</sup> degree	caecum	na	E; B	<i>FANCM</i>	c.2953del	p.Glu985ArgfsTer3	pathogenic	[-]	NA	[-]
42	M-75	2: 1 <sup>st</sup> ; 1: 2 <sup>nd</sup> degree	rectum	na	G	<i>FANCM</i>	c.643G>A	p.Glu215Lys	VUS	[+]	NA	[-]
43	F-53	1: 1 <sup>st</sup> ; 1: 3 <sup>rd</sup> degree	transversum	I	none	<i>BRIP1</i>	c.2392C>T	p.Arg798Ter	pathogenic	[-]	NA	[-]
44	M-55	1: 1 <sup>st</sup> ; 1: 2 <sup>nd</sup> ; 2: 3 <sup>rd</sup> degree	ascendens	IIIC	L	<i>BRIP1</i>	c.2392C>T	p.Arg798Ter	pathogenic	[-]	NA	[-]
45	M-50	1: 1 <sup>st</sup> ; 3: 2 <sup>nd</sup> degree	rectum	IIB	E; G	<i>ERCC2</i>	c.1403C>T	p.Pro468Leu	pathogenic	[-]	NA	[-]
46	F-38	1: 1 <sup>st</sup> ; 4: 2 <sup>nd</sup> degree	ascendens	na	G; P	<i>BLM</i>	c.481G>A	p.Asp161Asn	VUS	[-]	NA	[-]
47	M-60	2: 1 <sup>st</sup> degree	rectum	IIA	O; P	<i>BRCA2</i> ; <i>BLM</i>	c.4446_4451dup; c.545C>T AACAGA	p.Glu1482_Thr1483dup; p.Thr182Ile	likely pathogenic; VUS	[-]	NA	[-]
48	M-44	1: 1 <sup>st</sup> ; 2: 2 <sup>nd</sup> degree	transversum	IIIB	T	<i>APC</i> ; <i>PALB2</i>	c.4073C>T; c.2792T>G	p.Ala1358Val; p.Leu931Arg	VUS; VUS	[-]	NA	[-]
49	M-55	1: 1 <sup>st</sup> ; 1: 2 <sup>nd</sup> degree	caecum	IIIB	none	<i>KIT</i> ; <i>PALB2</i>	c.1688T>A; c.2792T>G	p.Ile563Lys; p.Leu931Arg	VUS; VUS	[-]	NA	[-]
50	F-70	2: 1 <sup>st</sup> degree	sygma	IIB	none	<i>CDH1</i>	c.1348T>A	p.(Tyr450Asn)	VUS	[-]	NA	[-]
51	M-59	1: 1 <sup>st</sup> degree	ascendens	na	none	<i>CEP57</i>	c.154C>T	p.Arg52Cys	VUS	[-]	NA	[-]
52	F-50	2: 1 <sup>st</sup> ; 1: 2 <sup>nd</sup> degree	transversum	na	B	<i>EZH2</i>	c.821G>A	p.Arg274Lys	VUS	[-]	NA	[-]
53	M-17	1: 2 <sup>nd</sup> degree	rectum	IIIC	R	<i>KIT</i>	c.2484C>T	p.Asn828Asn	VUS	[-]	NA	[-]
54	M-49	2: 2 <sup>nd</sup> degree	caecum	na	E	unknown	[-]	[-]	[-]	[-]	NA	[-]
55	M-37	positive family history; NS	caecum	IIB	none	unknown	[-]	[-]	[-]	[+]	[+]	[-]
56	M-47	2: 1 <sup>st</sup> ; 1: 2 <sup>nd</sup> degree	caecum	IIA	none	unknown	[-]	[-]	[-]	[-]	NA	[-]
57	M-67	3: 1 <sup>st</sup> ; 1: 2 <sup>nd</sup> ; 2: 3 <sup>rd</sup> degree	caecum	IIIB	E; B	unknown	[-]	[-]	[-]	[-]	NA	[-]
58	F-43	1: 2 <sup>nd</sup> degree	transversum	na	none	unknown	[-]	[-]	[-]	[-]	NA	[-]
59	F-30	1: 2 <sup>nd</sup> ; 1: 3 <sup>rd</sup> degree	rectum	IIIC	none	unknown	[-]	[-]	[-]	[-]	NA	[-]
60	M-49	3: 2 <sup>nd</sup> degree	rectum	na	Br	unknown	[-]	[-]	[-]	[-]	NA	[-]
61	F-64	2: 1 <sup>st</sup> ; 2: 2 <sup>nd</sup> degree	rectum	IIB	L; B	unknown	[-]	[-]	[-]	[-]	NA	[-]
62	F-62	2: 1 <sup>st</sup> ; 1: 3 <sup>rd</sup> degree	crassl	na	E	unknown	[-]	[-]	[-]	[-]	NA	[-]
63	M-65	1: 1 <sup>st</sup> ; 3: 2 <sup>nd</sup> degree	rectosygma	IIIC	E	unknown	[-]	[-]	[-]	[-]	NA	[-]
64	F-53	1: 1 <sup>st</sup> degree	crassl	IV	B; E	unknown	[-]	[-]	[-]	[-]	NA	[-]
65	F-38	1: 1 <sup>st</sup> degree	caecum	IIA	E	unknown	[-]	[-]	[-]	[+]	[+]	[-]
66	F-78	2: 1 <sup>st</sup> degree	ascendens <sup>c</sup>	na	R; B; Ur	unknown	[-]	[-]	[-]	[-]	NA	[-]

D<sub>x</sub>: diagnosis; ACMG: classification according to the American Collage of Medical Genetics [11]; MSI: microsatellite instability; MLH1 met: methylation of the promoter of the *MLH1* gene; BRAF V600E; V600E somatic mutation in the *BRAF* gene; VUS: variant of unknown significance; NS: not specified; NA: not analyzed.

<sup>a</sup> G: gastric cancer; P: prostate cancer; Ur: ureteral cancer; E: endometrial cancer; B: breast cancer; O: ovarian cancer; T: thyroid cancer; L: leukemia; R: renal cancer; Br: brain tumor: no mutation detected; [-]: absent; na: not available

<sup>b</sup> Synchronous: colon + gastric cancer, 5 years before endometrial cancer.

<sup>c</sup> Synchronous: colon + gastric cancer.

pathogenic or VUS in the 114 genes tested. The general clinical and pathohistological characteristics of these patients were similar to those of the other patients from the HNPCC MSS subgroup.

**Molecular Analysis of Patients with Polyposis.** On the whole, deleterious variants were detected in 28/41 (68.0%) patients with polyposis (Figure 3, Table 2). In the FAP group, deleterious variants were detected in 15/16

(93.7%) patients, most of which (13/16; 81.2%) were located in the *APC* gene. Two novel large deletions removing the entire *APC* gene were detected in four unrelated families [c.-19+5016\_\*2113+20168del p.(?) (123,466 bp deletion) and c.-19+2475\_\*2113+34050del p.(?) (139,889 bp deletion), in two families each]. Four previously described small out-of-frame deletions in exon 15 [c.3927\_3931del p.(Glu1309AspfsTer4) and c.3183\_3187del p.(Gln1062Terfs), found in two patients each, c.3404\_3405del p.(Tyr1135fsTer) and c.3199\_3202del p.(Ser1068 GlyfsTer57)] and three nonsense mutations [c.1269G>A p.(Trp423Ter), c.904C>T p.(Arg302Ter) and c.1660C>T p.(Arg554Ter)] were also detected. In one patient with juvenile polyposis (presence of hamartomatous polyps), we detected a novel mutation in the 5' untranslated region (5'UTR) exon of the *BMPRIA* gene [c.-152-2A>G p.(?)]. Surprisingly, a pathogenic variant was found in the *FLCN* gene [c.1285insC: p.(His429ProfsTer27)] in one family with classical FAP presentation. A VUS in the *KIT* gene [c.2484C>T p.(Asn828=)] was detected in one patient with deleterious mutation in the *BMPRIA* gene. Only one of the FAP patients did not have a pathogenic/VUS in any of the 114 genes tested.

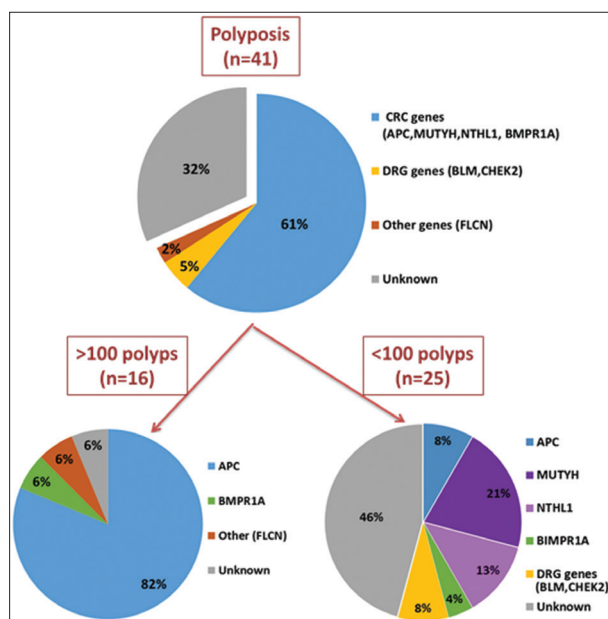
The molecular defect in patients with oligopolyposis was extremely heterogeneous (Figure 3, Table 2). Deleterious variants were detected in 13/25 (52.0%) of the patients. In 11/25 (44.0%), the variants were located in genes associated with polyposis syndromes, including *APC* [c.256A>T p.(Lys86Ter) and c.3920T>A p.(Ile1307Lys)],

*MUTYH* ([c.734G>A];[c.734G>A] [p.(Arg245His)]; [p.(Arg245His)]) in two patients and ([c.536A>G]; [c.536A=] [p.(Tyr179Cys)];[p.(Tyr179=)] in three patients), *NTHL1* ([c.268G>A];[c.806C>T] [p.(Gln90 Ter)]; [p.(Trp269Ter)]) in one patient and [c.268G>A]; [c.268=] [p.(Gln90Ter)]; [p.(Gln90=)] in two patients) and *BMPRIA* [c.1A>G p.(Met1Val)] in one patient) genes. The other three deleterious variants were detected in three different genes, all in the DRG pathway [*BLM* c.1642C>T p.(Gln548Ter), *CHEK2* c.902del p.(Leu301TrpfsTer3) and *FANCL* c.2T>C p.(Met1?)]. The *FANCL* mutation was detected in the patient that was heterozygous for the *MUTYH* c.536A>G p.(Tyr179Cys) mutation. A total of eight different VUS were detected in 8/25 (32.0%) patients. Six of these variants were found as individual genetic changes, two in *ATM* [c.2149C>T p.(Arg717Trp) and c.9016G>C p.(Ala3006Pro)], one in *PALB2* [c.1846G>C p.(Asp616His)], one in *MRE11* [c.1462C>T p.(Arg488 Cys) in two patients], one in *POLE* [c.2527A>G p.(Ile843 Val)] and in one *FH* [c.1431\_1433 dupAAA p.(Lys477 dup)] gene. One patient was detected with three VUS in different genes [*BLM* c.3416G>C p.(Arg1139Pro), *MRE11* c.1462C>T p.(Arg488Cys) and *DIS3L2* c.1447 C>G p.(Arg483 Gly)], while one novel VUS in the *RUNX1* gene [c.711 G>C p.(Gln237His)] was found in the patient with a mono-allelic deleterious mutation in the *NTHL1* gene. Only four patients from this group did not have any variant in the 114 genes tested. It is worth noting that extracolonic cancers (breast, pancreatic, endometrial) were present in family members of patients with mutations in *NTHL1*, *CHEK2* and *BLM* genes, in 1/4 patients without any genetic variant (breast cancer) and were absent in family members of patients with VUS.

## DISCUSSION

Using the approach described above, we were able to detect clear pathogenic variants (deleterious mutations) in 65/107 (60.7%) patients with hereditary CRCs in our population, of which 54/107 (50.5%) in known well-established CRC susceptibility genes and 11/107 (10.2%) in other genes [Figure 4(A) and Figure 5(A)]. Similar results were obtained in several large studies of patients with a hereditary cancer using whole exome sequencing [13,14], supporting the role of our approach for cascade testing of this disorder.

The majority of these deleterious variants were detected in HNPCC families with MSI+ tumors and families expressing the FAP phenotype [Figure 4(B)]. In the HNPCC group, the Lynch syndrome was confirmed in 25/31 (80.6%) of the MSI+ patients and in 4/35 (11.4%) patients with MSS tumors, indicating the need for Lynch syndrome



**Figure 3.** Distribution of deleterious variants in 41 patients with polyposis syndrome. The distribution of the mutations in different genes in the two subgroups of patients divided by the number of polyps at diagnosis is also shown.

**Table 2.** Summary of clinical data and molecular defects detected in 41 patients with polyposis syndromes.

n	Sex-Age	Clinical D <sub>x</sub>	Number of Polyps	Type of Polyps <sup>a</sup>	Family History (relatives affected)	Extracolonic Cancers <sup>b</sup>	Affected Gene	DNA Sequence Change	Amino Acid Change	ACMG Classification [11]
1	M-40	FAP	>100	AD	2: 1 <sup>st</sup> ; 1: 2 <sup>nd</sup> ; 4: 3 <sup>rd</sup> degree	none	APC	c.-19+2475_*2113+34050del	whole gene deletion	pathogenic
2	M-38	FAP	>100	AD	positive family history; NS	G	APC	c.-19+5016_*2113+20168del	whole gene deletion	pathogenic
3	F-29	FAP	>100	AD	2: 1 <sup>st</sup> ; 1: 2 <sup>nd</sup> degree	none	APC	c.-19+5016_*2113+20168del	whole gene deletion	pathogenic
4	F-33	FAP	>100	AD	2: 1 <sup>st</sup> ; 2: 2 <sup>nd</sup> degree	none	APC	c.-19+2475_*2113+34050del	whole gene deletion	pathogenic
5	F-29	FAP	>100	AD	1: 1 <sup>st</sup> ; 1: 2 <sup>nd</sup> degree	none	APC	c.1269G>A	p.Trp423Ter	pathogenic
6	M-35	FAP	>100	AD	1: 1 <sup>st</sup> ; 5: 2 <sup>nd</sup> degree	none	APC	c.1660C>T	p.Arg554Ter	pathogenic
7	M-32	FAP	>100	AD	2: 1 <sup>st</sup> degree	G	APC	c.3183_3187del	p.Gln1062Terfs	pathogenic
8	F-59	FAP	>100	AD	1: 1 <sup>st</sup> ; 1: 2 <sup>nd</sup> degree	none	APC	c.3183_3187del	p.Gln1062Terfs	pathogenic
9	M-38	FAP	>100	AD	1: 1 <sup>st</sup> ; 1: 2 <sup>nd</sup> degree	none	APC	c.3199_3202del	p.Ser1068GlyfsTer57	pathogenic
10	M-52	FAP	>100	AD	no family history	none	APC	c.3404_3405del	p.Tyr1135fsTer	pathogenic
11	F-38	FAP	>100	AD	2: 1 <sup>st</sup> ; 1: 3 <sup>rd</sup> degree	none	APC	c.3927_3931del	p.Glu1309AspfsTer4	pathogenic
12	M-44	FAP	>100	AD	1: 1 <sup>st</sup> degree	none	APC	c.3927_3931del	p.Glu1309AspfsTer4	pathogenic
13	F-39	FAP	>100	AD	no family history	none	APC	c.904C>T	p.Arg302Ter	pathogenic
14	M-9	FAP	>100	JP	1: 1 <sup>st</sup> degree	none	<i>BMPRIA</i> ; <i>KIT</i>	c.-152-2A>G; c.2484C>T	p.?; p.Asn828Asn	pathogenic; VUS
15	M-47	FAP	>100	AD	1: 1 <sup>st</sup> ; 3: 3 <sup>rd</sup> degree	none	<i>FLCN</i>	c.1285dupC	p.His429ProfsTer27	pathogenic
16	M-38	FAP	>100	AD	no family history	none	unknown	[-]	[-]	[-]
17	M-39	oligopolyposis	~30	AD	1: 1 <sup>st</sup> degree	none	APC	c.256A>T	p.Lys86Ter	pathogenic
18	M-38	oligopolyposis	~10	AD	no family history	none	APC	c.3920T>A	p.Ile1307Lys	pathogenic
19	F-44	oligopolyposis	>10	AD/HP	2: 1 <sup>st</sup> ; 4: 2 <sup>nd</sup> degree	none	<i>BIMPRIA</i>	c.1A>G	p.Met1Val	pathogenic
20	F-40	oligopolyposis	~30	AD	no family history	none	<i>MUTYH</i>	c.734G>A/c.734G>A	p.Arg245His/p.Arg245His	pathogenic
21	M-47	oligopolyposis	~10	AD	2: 1 <sup>st</sup> degree	none	<i>MUTYH</i>	c.734G>A/c.734G>A	p.Arg245His/p.Arg245His	pathogenic
22	M-48	oligopolyposis	>10	AD	1: 1 <sup>st</sup> degree	none	<i>MUTYH</i>	c.536A>G/=	p.Tyr179Cys/=	pathogenic
23	M-55	oligopolyposis	>10	AD	1: 1 <sup>st</sup> degree	none	<i>MUTYH</i>	c.536A>G/=	p.Tyr179Cys/=	pathogenic
24	F-54	oligopolyposis	~50	AD	positive family history; NS	none	<i>MUTYH</i> ; <i>FANCL</i>	c.536A>G/=; c.2T>C	p.Tyr179Cys/=; p.Met1Thr	pathogenic
25	M-67	oligopolyposis	50-100	AD	1: 1 <sup>st</sup> degree	Pa	<i>NTHL1</i>	c.268C>T/c.806G>A	p.Gln90Ter/p.Trp269Ter	pathogenic
26	F-71	oligopolyposis	7	AD	2: 1 <sup>st</sup> degree	E; Pa	<i>NTHL1</i>	c.268C>T/=	p.Gln90Ter/=	pathogenic
27	M-58	oligopolyposis	~10	AD	1: 1 <sup>st</sup> ; 2: 3 <sup>rd</sup> degree	none	<i>NTHL1</i> ; <i>RUNXI</i>	c.268C>T/=; c.711G>C	p.Gln90Ter/=; p.Gln237His	VUS
28	M-39	oligopolyposis	>10	AD	1: 1 <sup>st</sup> degree	E	<i>BLM</i>	c.1642C>T	p.Gln548Ter	pathogenic
29	F-53	oligopolyposis	>10	AD/SE	1: 1 <sup>st</sup> degree	B	<i>CHEK2</i>	c.902delT	p.Leu301TrpfsTer3	pathogenic
30	M-53	oligopolyposis	21	AD	no family history	none	<i>ATM</i>	c.2149C>T	p.Arg717Trp	VUS
31	M-63	oligopolyposis	>10	AD	no family history	none	<i>ATM</i>	c.9016G>C	p.Ala3006Pro	VUS
32	F-56	oligopolyposis	NA	AD	3: 1 <sup>st</sup> degree	none	<i>MRE11A</i>	c.1462C>T	p.Arg488Cys	VUS
33	M-45	oligopolyposis	~10	AD	no family history	none	<i>MRE11A</i>	c.1462C>T	p.Arg488Cys	VUS
34	M-46	oligopolyposis	NA	no data	no family history	none	<i>MRE11A</i> ; <i>BLM</i> ; <i>DIS3L2</i>	c.1462C>T; c.3416G>C; c.1447C>G	p.Arg488Gly; p.Arg1139Pro; p.Arg483Gly	VUS; VUS; VUS
35	M-57	oligopolyposis	>10	AD	1: 1 <sup>st</sup> degree	none	<i>PALB2</i>	c.1846G>C	p.Asp616His	VUS
36	F-74	oligopolyposis	10	AD	2: 1 <sup>st</sup> degree	none	<i>POLE</i>	c.2527A>G	p.Ile843Val	VUS
37	M-38	oligopolyposis	~10	AD/HP	2: 1 <sup>st</sup> ; 2: 2 <sup>nd</sup> degree	Br; L; P; R	<i>FH</i>	c.1431_1433dupAAA	p.Lys477dup	VUS
38	M-54	oligopolyposis	>30	AD	1: 1 <sup>st</sup> degree	none	unknown	[-]	[-]	[-]
39	M-58	oligopolyposis	~10	AD	2: 1 <sup>st</sup> ; 7: 2 <sup>nd</sup> degree	none	unknown	[-]	[-]	[-]
40	M-67	oligopolyposis	20-30	AD	2: 1 <sup>st</sup> degree	none	unknown	[-]	[-]	[-]
41	F-50	oligopolyposis	NA	AD	1: 1 <sup>st</sup> degree	none	unknown	[-]	[-]	[-]

D<sub>x</sub>: diagnosis; ACMG: classification according to the American Collage of Medical Genetics [11]; FAP: familial adenomatous polyposis; NA: not available but <100; VUS: variant of unknown significance; [-]: no variant detected.

<sup>a</sup> AD: adenomatous; JP: juvenile polyps; HP: hyperplastic; SE: sessile.

<sup>b</sup> G: gastric cancer; Pa: pancreatic cancer; E: endometrial cancer; B: breast cancer; Br: brain tumor; L: leukemia; P: prostate cancer; R: renal cancer.



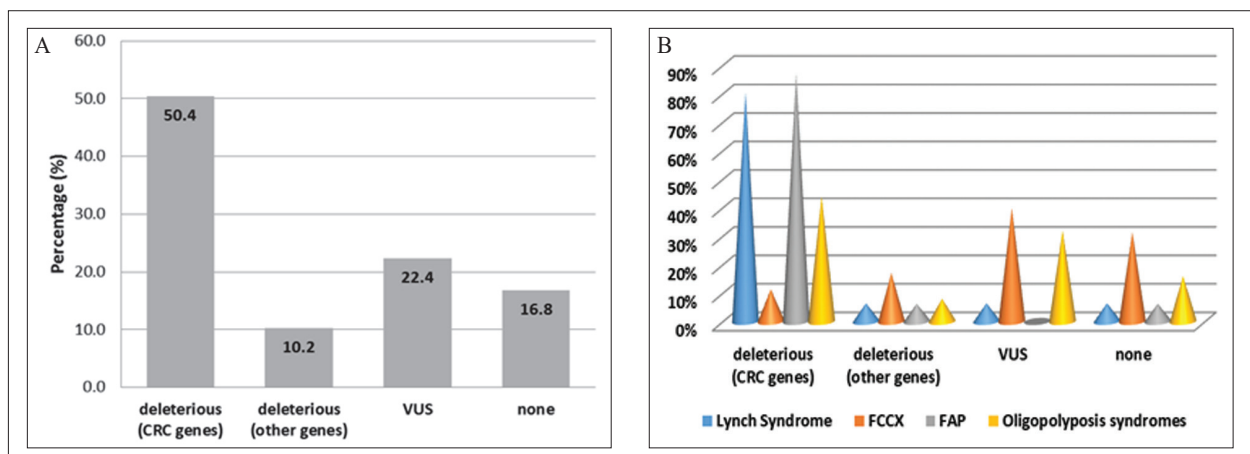
testing even in MSI- negative cases who fulfill the Amsterdam criteria. Half of the detected MMR mutations in the Lynch syndrome patients were located in the *MLH1* gene and the other half were equally distributed in *PMS2*, *MSH2* and *MSH6* genes. Others have observed a higher frequency of *MSH2* mutations in this syndrome, most probably due to the fact that the recruitment of probands for our study was limited to CRC, and we might have missed some probands with endometrial cancer with this syndrome in which the *MSH2* mutations have a higher frequency [15,16]. All six patients with MSI+ tumors, in whom we did not detect a pathogenic variant in the MMR genes, had a somatic inactivation of this system, either by promoter methylation of the *MLH1* gene (four patients) or by bi-allelic somatic mutation in the *MSH2* gene (two patients). These data indicate that the molecular basis of the hereditary CRC in these families is located in genes associated with other syndromes, which is supported by the fact that two of these patients had pathogenic variants in *CHEK2* and *FANCL* genes.

Deleterious mutations in the *APC* gene were present in 13/16 (80.0%) patients with classical polyposis, confirming the diagnosis of the FAP syndrome. In the remaining three patients with classical polyposis, we found a known deleterious mutation in the *BMPRIA* gene in one patient with a hamartomatous component in his polyps, which confirms the diagnosis of juvenile polyposis, a known deleterious mutation in the *FLCN* gene in one patient, and in one patient we did not detect any pathogenic variants. Deleterious mutations in the *FLCN* gene were found in patients with Birt-Hogg-Dubé syndrome (BHDS), an autosomal dominant predisposition to multisystem disorders including a higher risk for colorectal neoplasia in c.1285 insC: p.(His429ProfsTer27) carriers [17-20]. Our index patient with this *FLCN* mutation exhibited skin fibrofolliculomas characteristic for this syndrome.

We do not yet have complete data on the segregation of the mutation with the FAP phenotype in our family and we cannot exclude the presence of *APC* mutation in this patient in the regions not tested by our assay. However, our results further support the notion that certain *FLCN* gene mutations are involved in colorectal cancerogenesis. The absence of any mutation in the only patient with classical polyposis might be explained by either the presence of a mutation in the non coding/regulatory regions, large rearrangements or mosaicism for a mutation in the *APC* gene, or by the presence of a mutation in other gene(s) not analyzed with our approach.

It is worth noting that four mutations, each in the Lynch syndrome and FAP subgroups, were present in >50.0 and >60.0% of patients, respectively, which allowed for the development of specific assays for initial screening and rationale cascade testing for these syndromes in our population (Staninova-Stojovska *et al.*; manuscript in preparation).

In the oligopolyposis group, we detected deleterious mutations in 8/25 (32.0%) patients. In six of these patients, the variants were present in known highly penetrant genes associated with either autosomal dominant (two in *APC* and one in *BMPRIA*) or recessive (two in *MUTYH* and one in *NTHL1*) polyposis syndromes, and two in genes with moderate penetrance (one in *CHEK2* and *BLM* each) [21,22]. In addition, 5/25 (20.0%) patients were carriers of monoallelic deleterious variants in known genes associated with *MUTYH* associated polyposis (MAP), and *NTHL1* associated polyposis (NAP), autosomal recessive polyposis syndromes (three in *MUTYH* and two in *NTHL1*, respectively). Monoallelic carriers of *MUTYH* mutations with first degree relatives with CRC are considered at increased risk for the disease and should be offered an early screening program for disease prevention, whereas, as yet, there are no data for risk in monoallelic carriers of *NTHL1*



**Figure 4.** The overall frequency of different types of variants detected in 107 patients with hereditary CRC (A), and their distribution in different clinical subtypes of patients (B).

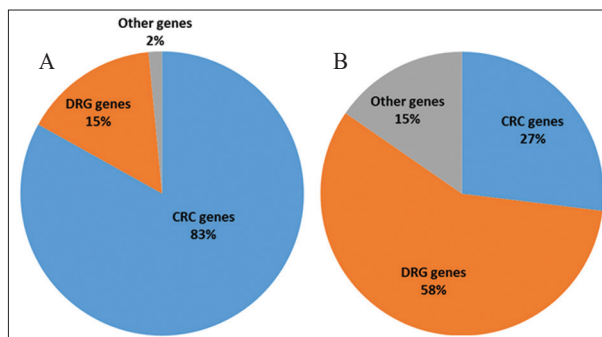
pathogenic variants [22,23]. In 12/25 (48%) patients we did not detect any pathogenic variant, further supporting the notion that the molecular basis of this condition is highly heterogenous and probably involves defect(s) in other gene(s) not tested in our assay [24].

The lowest frequency of pathogenic variants was present in the group of HNPCC patients with MSS tumors (10/35 or 28.5%). As mentioned above, four of these patients had Lynch syndrome due to mutations in the MMR genes (two in *MSH6* and two in *PMS2*), which are known to be associated with a lower degree of MSI that might have been undetected by our assay [25-27]. In the other six patients, the variants were present in five DRG, of which four in genes from DNA double-strand break repair pathway (*BRIP1* in two patients, *BRCA2*, *FANCM* and *CHEK2* in one patient each) and one in nucleotide excision repair pathway (*ERCC2*). Germline mutations in DRG genes have recently been described in individuals with hereditary CRC, though their contribution to a CRC risk is still unknown [14,28-30]. Recent data indicate that DRG defects are also relatively frequent somatic events in CRC, further supporting the notion that the inherited DRG variants are important in CRC carcinogenesis [29,30]. In line with this, some of these variants are described in other hereditary syndromes, predominantly in families with hereditary breast and ovarian cancers (HBOC) as variants with low/moderate penetrance [31]. The observed high prevalence of extracolonic cancers in our families with a DNA damage repair defect is also supporting the premise that mutations in these genes are likely to develop a multitumor phenotype with or without the presence of polyps [32]. The finding of the importance of DRG driven cancerogenesis, particularly in genes involved in homologous recombination, in a significant subset of CRC patients provides a unique opportunity for stratification of patients for platinum-based or target therapy [33-35].

In 42/107 (39.2%) patients, we did not detect any deleterious variants in the analyzed genes. Nevertheless, 25 different variants of unknown significance were detected in the majority of these patients (24/42 or 57.1%). Most of these variants had a moderate to low relative risk, as calculated by comparison of their frequency in cancer cases to controls from the Macedonian population or the controls from the GnomAD database (Supplementary Table 2). The VUSs were present predominantly in HNPCC families with MSS tumors (14/42 or 33.3%) and in families with oligopolyposis (8/42 or 19.0%), whereas only two variants were detected in individual patients with the Lynch syndrome [Figure 4(B)]. Most of these variants were present as single variants in individual patients (20/24 or 83.3%), whereas three patients with HNPCC with MSS tumors had two different variants and only one patient

with oligopolyposis had three different variants (Tables 1 and 2). In addition, only three patients with deleterious mutations also had a VUS. These data strongly suggest that the polygenic inheritance of low/moderate penetrance variants in the 114 analyzed genes is not a major mechanism responsible for the familial CRC type X (FCCX) and oligopolyposis phenotype in our population. It is worth nothing that the majority of these variants, particularly in patients without deleterious mutations, were located in the DRG genes, further supporting their importance in CRC cancerogenesis [Figure 5(B)]. However, additional evidence from functional analysis is needed in order to accurately classify these variants, which might significantly contribute to the current knowledge on the CRC genetic susceptibility.

We could not detect any variants using this approach in 18/107 (16.8%) patients [Figure 4(A)]. As mentioned above, the majority of these patients (13/18 or 72.2%) were HNPCC patients (11 with MSS tumors and two with MSI due to somatic inactivation of the MMR genes), 4/18 (22%) were patients with oligopolyposis and only one patient had a FAP phenotype. Although we cannot exclude the possibility of the presence of a mutation in deep intronic/regulatory regions of the analyzed genes that could be missed by our approach, we provide further evidence for the genetic heterogeneity of hereditary CRC, particularly in HNPCC families with MSS tumors and in families with oligopolyposis. However, it is interesting to note that endometrial cancer was present in family members of 5/11 HNPCC patients with MSS tumors, which indicates that these are Lynch syndrome families with undetected mutations in *MSH6* or *PMS2* genes. Further analysis with a whole exome/genome sequencing should clarify whether the increased CRC risk in these families is due to the presence of different high penetrant variants or due to a polygenic risk caused by multiple rare variants in genes not covered



**Figure 5.** Distribution of deleterious (A) and VUS (B) in known CRC genes (*MLH1*, *MSH2*, *MSH6*, *PMS2*, *APC*, *MUTYH*, *NTHL1*, *BMPRIA*, *POLE*), DRG genes (*BRCA2*, *BLM*, *CHEK2*, *BRIP1*, *PALP2*, *FANCM*, *ATM*, *MRE11*, *FANCL*, *ERCC2*) and other genes (*FLCN*, *FH*, *KIT*, *CDH1*, *EZH2*, *CEP57*, *RUNX1*).

by our assay. The role of gene-environment interactions, as well as epigenetic modifications that are insufficiently explored, should also be considered in understanding the mechanism of the disease development in these families.

## ACKNOWLEDGMENTS

The authors are indebted to Professor Dr. Zoran Kradzov, Professor Dr. Aleksandar Karagjozov, Professor Dr. Nikola Jankulovski, Dr. Toni Josifovski and Dr. Darko Dzambaz from the University Clinic for Abdominal Surgery (Skopje, RN Macedonia) and Professor Dr. Aco Kostovski from the University Clinic for Pediatrics (Skopje, RN Macedonia), for their help in the recruitment of patients, and last but not least, to Professor Dr. Dijana Plaseska-Karanfilska from the Research Center for Genetic Engineering and Biotechnology, Macedonian Academy of Sciences and Arts "Georgi D. Efremov", Skopje, RN Macedonia for providing the unpublished data on the frequencies of certain variants in normal controls from the Macedonian population.

**Declaration of Interest.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

**Funding.** This study was supported, in part, by a grant CRP/MAC14-01 from the International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste, Italy, Cooperation in the Field of Science and Technology Research (Europe) Projects BM1206 and CA17118, grant 07-356/1 from the Macedonian Academy of Sciences and Arts and the funds for science of the Centre for Biomolecular Farmaceutical Analysis (CBFA), UKIM Faculty of Pharmacy, Skopje, NM Macedonia (all to AJD).

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