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DIAGNOSIS OF CONSTITUTIONAL MISMATCH REPAIR-DEFICIENCY SYNDROME BASED ON MICROSATELLITE INSTABILITY AND LYMPHOCYTE TOLERANCE TO METHYLATING AGENTS

Short title: Functional diagnosis of CMMRD syndrome

Sahra Bodo[1,2,*], Chrystelle Colas[1,2,3,*], Olivier Buhard[1,2,*], Ada Collura[1,2], Julie Tinat[4], Noémie Lavoine[5], Agathe Guilloux[1,2], Alexandra Chalastanis[1,2], Philippe Lafitte[1,2], Florence Coulet[2,3], Marie-Pierre Buisine[6,7], Denisa Ilencikova[8], Clara Ruiz-Ponte[9], Miriam Kinzel[10], Sophie Grandjouan[11], Hilde Brems[12], Sophie Lejeune[13], Hélène Blanché[14], Qing Wang[15], Olivier Caron[16], Odile Cabaret[25], Magali Svrcek[1,2,17], Dominique Vidaud[18], Béatrice Parfait[18], Alain Verloes[19], Ulrich J Knappe[20], Florent Soubrier[21], Isabelle Mortemousque[22], Alexander Leis[23], Jessie Auclair-Perrossier[15], Thierry Frébourg[4], Jean-François Fléjou[1,2,17], Natacha Entz-Werle[24], Julie Leclerc[6,7], David Malka[26], Odile Cohen-Haguenauer[27], Yael Goldberg[28], Anne-Marie Gerdes[29], Faten Fedhila[30], Michèle Mathieu-Dramard[31], Richard Hamelin[1,2], Badre Wafaa[32], Marion Gauthier-Villars[33], Franck Bourdeaut[34], Eamonn Sheridan[35], Hans Vasen[36], Laurence Brugières[5], Katharina Wimmer[37], Martine Muleris[1,2,#] Alex Duval[1,2,#], on behalf of the European Consortium **"**Care for CMMRD**"** (C4CMMRD)

* equal contribution

[#] Co-leadership and corresponding authors

Centre de Recherche Saint-Antoine (UMRS 938), Equipe "Instabilité des Microsatellites et Cancers", Hôpital Saint-Antoine, Batiment Kourilsky, 184 rue du Faubourg Saint-Antoine, 75012 Paris, France

E-mails: martine.muleris@inserm.fr; alex.duval@inserm.fr

[1]*INSERM, UMR_S 938 Centre de Recherche Saint-Antoine, Equipe « Instabilité des Microsatellites et Cancer », Equipe Labellisée par la Ligue Nationale contre le Cancer, F-75012, Paris, France*

[2]*UPMC Univ Paris 06, F-75005, Paris, France*

[3]*AP-HP, Laboratoire d'Oncogénétique et d'Angiogénétique, GH Pitié-Salpétrière, F-75013, Paris, France*

[4]*Département de génétique, Hôpital universitaire, F-76183, Rouen, France*

[5]*Department of Children and Adolescents Oncology, Gustave Roussy Cancer Institute, F-94805, Villejuif, France*

[6]*Institut de Biochimie et Biologie moléculaire, Oncologie et Génétique Moléculaires, CHRU Lille, F-59037, Lille, France*

[7]*INSERM UMR837 et Université Lille 2, F-59045, Lille, France*

[8]*2nd Pediatric Department of Children University Hospital, Comenius University, Bratislava, Slovakia*

[9]*Fundación Pública Galega de Medicina Xenómica (FPGMX) SERGAS, Grupo de Medicina Xenómica, IDIS, Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERer), Santiago de Compostela, Spain*

[10]*Praxis für Medizinische Genetik, Berlin, Germany*

[11]*CHU Cochin, faculté René-Descartes–Paris-V, F-75014, Paris, France*

[12]*Department of Human Genetics, KU Leuven, Leuven, Belgium*

[13]*CHRU Lille, Service de génétique clinique, F-59037, Lille, France*

[14]*CEPH, Fondation Jean Dausset, Institut de Génétique Moléculaire, F-75010, Paris, France*

[15]*Plateforme de Génétique constitutionnelle HCL-CLB, Laboratoire de recherche translationnelle, Centre Léon Bérard, F-69373, Lyon, France*

[16]*Department of Medical Oncology, Gustave Roussy Cancer Institute, F-94805, Villejuif, France*

[17]*AP-HP, Hôpital Saint-Antoine, Service d'Anatomie et Cytologie Pathologiques, F-75012, Paris, France*

[18]*INSERM UMR745 Université Paris Descartes, Faculté des Sciences Pharmaceutiques et Biologiques, F-75006, Paris, France*

[19]*AP-HP, Département de Génétique and INSERM UMR 1141 « PROTECT », Hôpital Robert Debré, F-75019 Paris, France*

[20]*Department of Neurosurgery, Johannes Wesling Klinikum, Minden, Germany*

[21]*AP-HP, Département de génétique, GH Pitié-Salpêtrière, F-75013, Paris, France*

[22]*CHRU de Tours, Service de Génétique, F-37044, Tours, France*

[23]*French Medical Institute for Children, Kabul, Afghanistan*

[24]*Pédiatrie Onco-Hématologie Pédiatrie CHRU Hautepierre UdS EA 3430, Strasbourg, France*

[25]*Service de Génétique, Département de Biologie et Pathologie Médicales, Institut Gustave Roussy, F-94805, Villejuif, France*

[26]*Department of Cancer Medicine, Gustave Roussy, F-94805, Villejuif, France*

[27]*Service d'Oncologie Médicale, Hôpital Saint-Louis, Paris, France*

[28]*Sharett Institute of Oncology, Hadassah-Hebrew University Medical Center, Jerusalem, Israel*

[29]*Department of Clinical Genetics, Copenhagen University Hospital Rigshospital, Copenhagen, Denmark*

[30]*Service de médecine infantile, hôpital d'enfants de Tunis, Tunis, Tunisia*

[31]*Unit of medical Genetics, Amiens University Hospital, Amiens, France*

[32]*Department of Hepato-Gastro-Enterology, Ibn Rochd, Hospital University Center, Casablanca 20270, Morocco*

[33]*Service de Génétique, Institut Curie, Paris, France*

[34]*Department of Pediatric Oncology & INSERM U830, Institut Curie, Paris, France*

[35]*Department of Molecular Medicine, University of Leeds, LS2 9JT, Leeds, UK*

[36]*Department of Gastroenterology & Hepatology, Leiden University Medical Centre, Leiden, The Netherlands*

[37]*Division of Human Genetics, Medical University Innsbruck, Innsbruck, Austria*

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Abbreviations

CMMRD, constitutional mismatch repair; *ev*MSI, *ex vivo* microsatellite instability; gMSI, germline microsatellite instability; IHC, immunohistochemical; FAP, familial adenomatous polyposis; LCL, lymphoblastoid cell line; LS, Lynch syndrome; MMR, mismatch repair; MNNG, N-methyl-N-nitro-N-nitrosoguanidine; MSI, microsatellite instability; NF1, neurofibromatosis type 1; PBLs, peripheral blood lymphocytes; VUS, variant of unknown functional significance; 6-TG, 6-thioguanine.

Author contributions

study concept and design: M Muleris, A Duval

acquisition of data: S Bodo, O Buhard, C Colas, A Collura, J Tinat, N Lavoine, A Chalastanis, P Lafitte, F Coulet, MP Buisine, M Svrcek

analysis and interpretation of data: S Bodo, O Buhard

drafting of the manuscript: S Bodo, A Duval, M Muleris

critical revision of the manuscript: K Wimmer

statistical analysis: A Guilloux

study supervision: M Muleris, A Duval

biological material suppliers: all other authors

Conflict of interest

The authors disclose no conflicts.

ABSTRACT

BACKGROUND & AIMS: Patients with bi-allelic germline mutations in mismatch repair (MMR) genes (*MLH1*, *MSH2*, *MSH6*, or *PMS2*) develop a rare but severe variant of Lynch syndrome called constitutional MMR deficiency (CMMRD). This syndrome is characterized by early-onset colorectal cancers, lymphomas or leukemias, and brain tumors. There is no satisfactory method for diagnosis of CMMRD because screens for mutations in MMR genes are non-informative for 30% of patients. MMR-deficient cancer cells are resistant to genotoxic agents and have microsatellite instability (MSI), due to accumulation of errors in repetitive DNA sequences. We investigated whether these features could be used to identify patients with CMMRD.

METHODS: We examined MSI by PCR analysis and tolerance to methylating or thiopurine agents (functional characteristics of MMR-deficient tumor cells) in lymphoblastoid cells (LCs) from 3 patients with CMMRD and 5 individuals with MMR-proficient LCs (controls). Using these assays, we defined experimental parameters that allowed discrimination of a series of 14 patients with CMMRD from 52 controls (training set). We then used the same parameters to assess 23 patients with clinical but not genetic features of CMMRD.

RESULTS: In the training set, we identified parameters, based on MSI and LC tolerance to methylation, that detected patients with CMMRD vs controls with 100% sensitivity and 100%. Among 23 patients suspected of having CMMRD, 6 had MSI and LC tolerance to methylation (CMMRD highly probable), 15 had neither MSI nor LC tolerance to methylation (unlikely to have CMMRD), and 2 were considered doubtful for CMMRD based on having only 1 of the 2 features.

CONCLUSION: The presence of MSI and tolerance to methylation in LCs identified patients with CMMRD with 100% sensitivity and specificity. These features could be used in diagnosis of patients.

KEYWORDS: functional tests, colon cancer, tumor, predisposition

INTRODUCTION

Individuals with Lynch syndrome (LS) harbor germline heterozygous mutations affecting one of the four major mismatch repair (MMR) genes (*i.e. MLH1, MSH2, MSH6* or *PMS2*) and are at greatly increased risk of developing colorectal and other epithelial tumors [1]. Typically, individuals with germline *MLH1* or *MSH2* defects develop MMR-deficient cancers during their 4th or 5th decade, whereas those with *MSH6* or *PMS2* mutations are affected less consistently. Patients with bi-allelic germline mutations in MMR genes suffer from Constitutional MMR-Deficiency (CMMRD) [2-5], a distinct inherited cancer syndrome (OMIM #276300) [6]. This syndrome is characterized by the development of childhood tumors such as early-onset colorectal cancers, lymphomas/leukemias, and brain tumors [6-8]. Since CMMRD is mainly due to bi-allelic inheritance of *PMS2* or *MSH6* germline mutations, the family history of patients shows only a low incidence of LS-related cancers in first- and second-degree relatives. To date, CMMRD has been reported in 146 patients from 91 distinct families. Because of variable clinical presentation, lack of unequivocal diagnostic features, and phenotypical overlap with other cancer syndromes (*e.g.* neurofibromatosis type 1 (NF1), Li-Fraumeni, syndrome, familial adenomatous polyposis (FAP)), CMMRD syndrome is frequently unrecognized by clinicians and its incidence is almost certainly underestimated.

Within the European Consortium 'Care for CMMRD' (C4CMMRD), we recently proposed clinical diagnostic criteria that should raise the suspicion of CMMRD when observed in a child or young adult cancer patient, based on the phenotypic presentation [9]. The suspected diagnosis then needs to be either confirmed or refuted. The current diagnosis of CMMRD requires identification of bi-allelic, deleterious germline MMR defects. Unfortunately, mutation analysis leads to non-informative results when variants of unknown functional significance (VUS) are detected, as observed in around 30% of patients. Moreover, the detection of *PMS2* alterations responsible for 60% of CMMRD families is complicated by

the presence of numerous pseudogenes, resulting in a lack of sensitivity when performing mutation analysis only. Hence, although extensive mutation screening that includes comprehensive searches for large genomic rearrangements of MMR genes remains crucial for identification of CMMRD patients and genetic counseling in CMMRD families, tests that can unequivocally confirm or refute a suspected diagnosis are highly desirable.

Since all CMMRD patients share a common and specific functional property, *i.e.* MMR deficiency, we hypothesized that the detection of characteristic functional features of MMR-deficient blood cells from such patients could be used to diagnose this syndrome. Inactivation of MMR is known to increase cellular tolerance to specific genotoxic agents such as methylating and thiopurine drugs [10-15]. Moreover, MMR-deficient cancer cells specifically exhibit a microsatellite instability (MSI) phenotype due to accumulation of replication errors in repetitive DNA sequences [16]. In tissues derived from MMR-deficient neoplastic cells, MSI is easily detected through PCR amplification of microsatellites. However, earlier studies have shown that MSI cannot be detected in the germline DNA of CMMRD patients except by using the laborious technique of “small pool PCR” [6, 17]. The presence of somatic mutations within DNA repeats in MMR-deficient cells is related to cell division. We therefore hypothesized that *in vitro* culture of immortalized lymphoblastoid cells from CMMRD patients would eventually lead to the onset of both an MSI phenotype and tolerance to methylating/thiopurine agents.

In the present work we first validated the proof of concept that MSI and tolerance to methylating/thiopurine agents could be detected in lymphoblastoid cell lines (LCLs) derived from several CMMRD patients, but not in LCLs from MMR-proficient controls including LS patients. In a case-control study, we next determined the experimental conditions that allowed accurate discrimination of a series of CMMRD patients from MMR-proficient controls. Finally, we tested our functional approach using the same experimental conditions in a series of patients who showed clinical characteristics of CMMRD but for whom the standard diagnostic method was non-informative. This was performed within a European

Consortium 'Care for CMMRD' (C4CMMRD) that allowed us to collect a unique series of confirmed CMMRD cases and at-risk individuals for this syndrome.

PATIENTS AND METHODS

Patients

At the 1st workshop of the European Consortium 'Care for CMMRD' (C4CMMRD) held in Paris on June 9, 2013, a call was made to contribute blood samples or LCLs from definite or possible CMMRD patients. Eligible subjects included patients already diagnosed with CMMRD, *i.e.* with bi-allelic deleterious germline mutations in any of the 4 major MMR genes, as well as patients with a strong clinical suspicion of CMMRD, *i.e.* with a clinical score ≥3 according to Wimmer *et al.* [9]. LCLs were available (n=10) or were established (n=27) for 37 of the 42 eligible patients. MMR-proficient LCLs used as controls originated from 47 LS patients and 15 subjects considered free of MMR germline defects including patients with FAP or NF1 syndrome. All patients gave written informed consent. This study was approved by the institutional review boards/ethics committees of the participating centres.

Mutation screening of MMR genes

All analyses were performed in clinically approved laboratories. Analysis of *MLH1*, *MSH2* and *MSH6* genes was performed across different laboratories whereas analysis of *PMS2* was performed in the Rouen, Lille or Innsbruck laboratories. Bi-directional Sanger sequencing from genomic DNA or direct cDNA sequencing [18] was performed to identify point mutations in exonic and flanking intronic regions. Sequencing reactions were performed using the ABI PRISM Kit (Applied Biosystems) and sequences were analyzed on an automated sequencer (ABI 3130XL Genetic Analyzer, Applied Biosystems) using Sequencing Analysis Software v5.2 (Applied Biosystems) [19]. Screening for large rearrangements in the *MLH1*, *MSH2* and *MSH6* genes was performed using Multiplex

Ligation-dependent Probe Amplification and/or Quantitative Multiplex PCR of Short Fluorescent Fragments. Rearrangements of the *PMS2* gene were analyzed by Quantitative Multiplex PCR of Short Fluorescent Fragments for exons 6, 7, 8 and 10, and/or by Multiplex Ligation-dependent Probe Amplification using the SALSA MLPA kit P008 (MRC-Holland, Amsterdam, The Netherlands) together with appropriate reference DNAs that have an equal (2:2) distribution of gene- and pseudogene-derived sequences in exons 13-15 [20]. In patient C26, the *PMS2*-exon 12 deletion escaped detection by Multiplex Ligation-dependent Probe Amplification, but was identified by direct cDNA sequencing. Screening of the *NF1* gene was performed using a variety of methodologies including DNA and RNA sequencing for small lesions, polymorphic microsatellite marker analysis and Multiplex Ligation-dependent Probe Amplification or real-time PCR-based gene dosage analysis to allow the assessment of microdeletions, as previously described [21]. Mutation analysis of the *APC* gene was performed by direct sequencing and Multiplex Ligation-dependent Probe Amplification [22].

Lymphoblastoid cell lines

LCLs obtained following standard Epstein-Barr virus infection were grown in RPMI 1640 with stable glutamine supplemented with 20% fetal calf serum, 100 IU/ml penicillin and 100mg/ml streptomycin (PAA). Only LCLs with comparable growth rates and with viability greater than 85% were included.

Ex vivo microsatellite instability analysis

PCR products following amplification of the NR27, NR21 and BAT26 microsatellites were separated by capillary electrophoresis on an ABI 3100 genetic analyzer and quantified using Gene Mapper software v3.7. In order to confidently detect allelic shifts of as little as 1 base pair in size, DNA from LCL and peripheral blood lymphocytes (PBLs) were analyzed concurrently in octuplicate.

Chemicals

All chemicals were obtained from Sigma unless otherwise indicated. Cells were exposed to 6-Thioguanine (6-TG) and N-Methyl-N'-Nitro-N-Nitrosoguanidine (MNNG) (TCI Europe). To exclude differences in MNNG cytotoxicity due to variations in O^6-methylguanine methyltransferase enzyme activity, the latter was abrogated by exposure to O^6-benzylguanine (20µM final concentration) during the entire experiment. All chemicals were dissolved in DMSO to a concentration of 20mM, protected from light and stored at -20℃ until used.

Methylation tolerance assay

Exponentially growing lymphoblastoid cells were seeded into 96-well round-bottom plates at a density of 0.15-1×10^4 cells/well. After 24-h incubation, extemporaneously reconstituted MNNG solution was added at 1.25, 2.5 and 5µM final concentration. Because of the short half-life of MNNG in aqueous solution (1 hour), the medium was not replaced after drug treatment and 1, 2 or 3 rounds of treatment separated by 24-h were performed. Cell growth was evaluated after a total incubation time of 10 days and all samples were tested in triplicate. Each experiment was conducted at least in duplicate. Cytotoxicity was examined by the WST kit according to the supplier's recommendations (Roche). Absorbance was read at 450nm using a microplate reader (Tecan Infinite F500) and analyzed using Xfluor4GENiosPro software. Percent cell survival was represented as the absorbance of treated sample relative to control.

Statistical analyses

A Metropolis-Hastings algorithm was used to estimate the sensitivity and specificity of the three different diagnostic methods (MMR gene sequencing, functional testing, gMSI testing). This algorithm was applied to results obtained from genetically confirmed CMMRD patients, control patients, and patients with a strong clinical suspicion of CMMRD but without a molecularly confirmed diagnosis.

Further information is available in the Supplementary material.

RESULTS

Proof-of-concept study

We first investigated whether MSI and methylation/thiopurine tolerance could be detected in LCLs from 3 CMMRD patients with bi-allelic deleterious mutations in *MSH6*, *PMS2* or *MLH1*, but not in LCLs from 5 negative controls comprising 4 LS patients (*MSH6*, *MSH2*, *PMS2* or *MLH1* heterozygous mutations) and one individual with wild-type MMR status.

***MSI screening*.** As expected, MSI was not detected in PBLs from CMMRD patients (figure 1A) following the analysis of 3 mononucleotide microsatellite markers (NR27, NR21, BAT26) that are used routinely to assess MSI status in tumor cells. In contrast, a clear MSI phenotype showing characteristic, aberrant alleles was observed in LCLs from all 3 CMMRD patients (figure 1A), whereas the 5 control LCLs displayed stable allelic profiles (figure 1B and supplementary table 2). The MSI phenotype was only demonstrated *ex vivo* in LCLs and was thus termed *ev*MSI to distinguish it from the *in vivo* MSI phenotype detected in MMR-deficient cancer cells.

***Drug tolerance assay*.** We first evaluated the cytotoxic effects of MNNG (methylating agent) and 6-thioguanine (6-TG, thiopurine) in 11 human colorectal cancer cell lines. Cell lines that were *MLH1*-, *MSH2*- or *MSH6*-deficient were on average up to 10-fold more tolerant to 1µM MNNG than MMR-proficient cell lines and 2-fold more tolerant to 15µM 6-TG (supplementary figure 1). We next investigated the response of LCLs to both drugs. All 3 CMMRD-derived LCLs were phenotypically distinguishable from cells with heterozygous or wild-type MMR status. They displayed better cell survival compared to controls and there was no overlap between the two groups under several MNNG experimental conditions (figure 1C), whereas 6-TG was less discriminant (figure 1D).

Case-control study

We next sought to identify experimental conditions that would best discriminate CMMRD patients from controls. Among 42 patients collected from several European cancer centers (including the 3 patients previously analyzed in the proof of concept study), 19 had been diagnosed as definite CMMRD cases by molecular analysis, *i.e.* bi-allelic pathogenic MMR gene alterations. Of these, LCLs from 14 cases were available for the present case-control study (table 1, figure 2). Pedigrees for all previously unreported patients are shown in supplementary figure 2. Clinical and tumor data together with detailed results of germline MMR analysis are provided in supplementary table 1.

***evMSI*.** LCLs from the 14 CMMRD patients comprising carriers of bi-allelic mutations in *PMS2* (n=10), *MSH6* (n=3) or *MLH1* (n=1) displayed microsatellite deletions ranging from 1 to 7 base pairs (figure 4A). Detection of the *ev*MSI phenotype in the cell lines was achieved 120 days after immortalization at the latest. The median culture time for a positive *ev*MSI phenotype was 83.6 ± 22.6 days (range 45-120) (supplementary table 2). In contrast, LCLs from all 23 MMR-proficient controls (12 LS patients and 11 MMR wild-type individuals) showed no deletions. For all 23 controls except two, the cell lines were grown for at least 120 days without any evidence of deletions (median culture time = 175 days ± 62.6, range 83-304 days). Five control cell lines were grown for longer than 220 days without any evidence of deletions. Therefore, for subsequent experiments the cut-off value used to define a cell line as positive for *ev*MSI was set as a 1 base pair deletion across all three markers and a maximum culture time of 120 days was used.

***Drug tolerance assays*.** By varying the MNNG concentration and number of treatments, we found the optimal experimental condition that allowed CMMRD patients to be discriminated from controls was two rounds of 2.5 µM MNNG. Using this condition, LCLs from all 14 CMMRD patients displayed a cell survival rate above 60%. In contrast, 51/52 LCLs from controls displayed a cell survival rate lower than 40% (median cell survival rates

of 87.5% and 20.9%, respectively; $P < .0001$; Student's t test) (figures 3 and 4A). At an arbitrary cut-off value of 50% cell survival, the methylation tolerance assay was therefore shown to be 100% sensitive (14/14) and 98% specific (51/52; the positive sample was from an LS patient with an *MSH6* defect). The thiopurine tolerance test was found to be less discriminatory and hence was not continued further (supplementary figure 3). This result concurs with previous findings that MMR-deficient cells are 100-fold more tolerant than MMR-proficient cells to death induced by methylating agents, but only about 10-fold more tolerant to 6-TG treatment [23].

Overall, *ev*MSI and methylation tolerance assays were found to be highly specific and sensitive and gave concordant results for all cases tested with both methods. In subsequent studies we therefore deemed that both assays must show abnormal results in order to conclude a definite diagnosis of CMMRD. To rule out a diagnosis of CMMRD, both assays should display normal results. Diagnosis should be considered as doubtful if results from the two functional tests are discordant.

Application of functional tests for the detection of CMMRD in at-risk individuals

In 23 of the 42 patients from our series, a diagnosis of CMMRD was suspected based on clinical presentation, but the diagnosis could not be confirmed by MMR gene mutation analysis (table 1, figure 2). These comprised 8 patients with bi-allelic MMR mutations that included one or two VUS, 5 patients with a single MMR mutation and 10 patients in which no MMR mutation was detected. We evaluated these patients using the functional assay conditions described above (table 2, figure 4B). Six patients displayed positive results for both the *ev*MSI and methylation tolerance assays, indicating a highly probable diagnosis of CMMRD. They included 5 patients with *MSH6* or *PMS2* bi-allelic MMR alterations comprising VUS. Consistent with our results, *in silico* prediction favored a pathogenic

nature for the *MSH6* and *PMS2* variants in four of these patients (C20.1, C20.2, C18, C22). In the 6th patient (C29.1), no apparent germline MMR mutations were detected. In another 15 patients, *ev*MSI and methylation tolerance assays were both negative, indicating that a diagnosis of CMMRD was very unlikely. These included one compound heterozygote for an *MSH2* variant, 5 patients with a single *MLH1*, *PMS2* or *MSH2* alteration, and 9 patients where no MMR alteration had been detected. In the two remaining patients (C21 and C23, with bi-allelic *MSH6* mutations comprising one or two VUS, respectively), the data showed methylation tolerance but no *ev*MSI phenotype. We therefore concluded a result of "doubtful" for both patients.

Comparison of functional assays with other methodological approaches

We trialed a recently described method that evaluates dinucleotide repeats for the detection of MSI in germline DNA (gMSI) [24]. In the case-control cohort, the gMSI assay yielded interpretable results in 15 of 18 CMMRD patients and in 16 of 19 controls. CMMRD patients with bi-allelic mutations involving *PMS2* (n=11), *MLH1* (n=1) or *MSH2* (n=1) displayed abnormal gMSI values. In agreement with the original report [24], we found however that CMMRD patients with bi-allelic deleterious mutations involving *MSH6* (n=2) displayed normal gMSI ratios, thus reducing the sensitivity of this method (table 2, supplementary table 3, supplementary figure 4). gMSI ratios were normal for all controls. gMSI also yielded interpretable results in 21 of 23 patients suspected of having CMMRD. The five carriers of bi-allelic *MSH6* alterations displayed normal gMSI, as expected. Moreover, gMSI corroborated the results of our functional assays in all patients with *PMS2*, *MLH1* or *MSH2* mutations, with the exception of one (C18). This patient carried one deleterious mutation and one VUS in the *PMS2* gene. He displayed normal gMSI but abnormal *ev*MSI and methylation tolerance results (table 2). The c.2249G>A missense mutation found in the *PMS2* gene of patient C18, together with complete deletion of the other *PMS2* allele, was previously reported in a patient diagnosed with rectal cancer and a brain tumor at 22 and 23

years of age, respectively [25]. This further corroborates a pathogenic role for the VUS in patient C18 and is consistent with the results of our functional assay. The *ev*MSI, methylation tolerance and gMSI assay results were all abnormal in patient C29.1 who lacked apparent MMR germline mutations. This prompted us to conduct additional *PMS2* screening using Multiplex Ligation-dependent Probe Amplification, which led to the identification of a homozygous deletion of exons 14-15. Normal functional test results and gMSI ratio were found in an asymptomatic brother aged 11 years (C29.2) who was later found to be heterozygous for the *PMS2* deletion.

Another tool proposed for CMMRD screening is IHC analysis to detect loss of MMR protein expression in normal tissues. IHC was recently reported to be 100% sensitive when performed on normal colonic or skin tissues from 5 CMMRD patients [26]. However, based on previous observations in LS patients, IHC may lack sensitivity, especially for the detection of some missense and truncating MMR gene mutations [27, 28], resulting in false negative diagnosis for CMMRD. This was demonstrated in the present study where positive MSH6 staining was observed in two patients (C20.2 and C22) with homozygous *MSH6* missense mutations and who are likely to be CMMRD according to the functional assays and *in silico* predictions. Conversely, PMS2 protein was not expressed in the normal colonic mucosa of patient C25, the carrier of a single deleterious *PMS2* mutation in which a diagnosis of CMMRD was ruled out based on normal results for the *ev*MSI, methylation tolerance and gMSI tests (table 2 and supplementary table 1).

Estimation of sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for the functional assays and for other methodological approaches

In our case-control cohort, the functional assay (*i.e. ev*MSI and methylation tolerance) was 100% sensitive and 100% specific whereas gMSI testing was 86.7% (13/15) sensitive and

100% (16/16) specific (see above). It is worth noting that the sensitivity of gMSI is likely to be an overestimate, since this depends on the proportion of CMMRD patients with *MSH6* alterations. In our series this was only 13% (2/15), however *MSH6* alterations are thought to be responsible for a higher proportion (~20%) of all CMMRD patients [9]. The performance of IHC could not be evaluated properly due to the lack of a standardized method for the analysis of MMR gene expression in the normal tissue of controls (*i.e*. MMR-proficient cases).

We next estimated the performance of functional testing compared to the standard method of MMR gene sequencing. This was done for the entire cohort, including patients deemed to be at-risk. Using a Metropolis-Hastings algorithm, the functional assay revealed higher sensitivity (94.2% (95% CI: 79.4% - 99.9%) *vs* 80.1% (54.1% - 99.0%)), higher NPV (97.2% (89.8% - 99.9%) *vs* 91.2% (76.6% - 99.6%)), but lower specificity (90.1% (76.1% - 99.5%) *vs* 97.6% (91.2% - 99.9%)) and lower PPV (80.5% (53.9% - 99%) *vs* 93.6% (77.9% - 99.8%)) for CMMRD diagnosis. In order to include gMSI in the comparison, an estimation of the sensitivity and specificity was made in the smaller series of patients and controls for which results from all tests were available. Functional testing still offered the highest sensitivity (93.3% (76.7% - 99.8%) and a lower specificity (Supplementary table 4), however these differences did not reach statistical significance because of small cohort sizes. As stated above, the lack of a standardized method for IHC analysis of normal tissues meant we were unable to properly evaluate the sensitivity and specificity of this method.

DISCUSSION

In this paper, we propose a new approach for the diagnosis of CMMRD that involves the common and specific functional characteristic of all CMMRD patients, *i.e.* MMR deficiency. Our approach was based on the exploitation of this feature through the evaluation of MSI and methylation tolerance in MMR-deficient, immortalized lymphoblastoid cells. This

method gave unequivocal results in CMMRD patients with known bi-allelic deleterious mutations. If one assumes that abnormal results for both assays indicate a diagnosis of CMMRD, whereas normal results for both assays rule this out, our method was 100% sensitive and 100% specific in this case-control study. When applied to additional patients suspected of having CMMRD syndrome because of evocative clinical criteria but who lacked the confirmatory standard genetic defects, a clear discrimination into two groups was obtained. In the first group showing abnormal results for both tests, we considered that CMMRD was highly probable. In contrast, a diagnosis of CMMRD was highly unlikely in the second group of patients showing normal results for both tests. Our novel functional approach may therefore be especially useful for the confirmation or rejection of CMMRD diagnosis in patients with VUS by providing an assessment of the pathogenicity of MMR variants. It is also useful in cases where the diagnostic method failed to detect bi-allelic MMR mutations despite an evocative CMMRD clinical phenotype (*e.g.* patient C29.1). Furthermore, our approach can rule out that a second mutation has been missed in patients with heterozygous, pathogenic *PMS2* or *MSH2* mutations who nevertheless show an unusually early onset of cancer (*e.g.* colon tumors at 12, 17 and 25 years of age in patients C24, C26 and C25, respectively). The results from our functional approach support the existence of a clinical continuum that spans the less severe CMMRD phenotypes that mimic LS (*e.g.* patient C18), to more severe and early onset LS phenotypes that mimic CMMRD [29]. Overall, our findings highlight that functional tests capable of assessing constitutional MMR-deficiency are highly desirable for the accurate diagnosis of CMMRD patients.

Although we have investigated by far the largest CMMRD series reported to date in the literature, our method requires further confirmation in additional cohorts of CMMRD patients. This will help to refine the criteria for the functional assays in cases with ambiguous results, such as the two patients who harbored VUS in the *MSH6* gene and showed methylation tolerance but not *ev*MSI (C21, C23). One possible explanation for this

observation is that certain MMR gene mutations might uncouple the DNA mismatch repair and DNA damage-induced apoptosis functions, as reported in mice [30, 31]. Overall, we found that functional testing showed better sensitivity than either MMR gene sequencing or gMSI, although it may have a lower specificity. Bearing this in mind, we propose a flow chart for the use of our assay alone or in combination with other tests in routine clinics in the next future (figure 5). IHC could not be evaluated properly in this study due to the lack of a standardized method for assessment of MMR gene expression in the normal tissues of MMR-proficient subjects. The results with IHC are likely to be highly dependent on the type of tissue being studied (*e.g.* colon, brain, skin, lymphoid cells). Moreover, it is well known that IHC can give rise to false negative results for MMR deficiency in cases where inactivating missense mutations nevertheless result in expression of the mutant protein [27, 28]. Further studies should evaluate MMR protein expression using standardized methods in normal and tumor tissues from large cohorts of CMMRD patients, MMR-proficient controls and Lynch syndrome patients, in the same manner as performed here to assess our functional assay.

In summary, the novel functional approach proposed here showed higher sensitivity for CMMRD diagnosis compared to MMR sequencing or gMSI, the two other methods used so far. This approach can be used to determine whether MMR variants of uncertain pathogenicity are responsible for functional inactivation of the MMR system. The ability to classify variants as pathogenic or neutral is a major challenge in clinical genetics, particularly with the advent of next-generation sequencing. Moreover, the diagnosis of CMMRD syndrome based solely on clinical and genetic data is presently inadequate. As an overall diagnostic strategy, we therefore recommend the implementation of our functional assays in combination with IHC and gMSI analysis (figure 5). These tests can be performed in any order upon suggestion of CMMRD syndrome based on an evocative clinical score. This strategy has already been introduced at the Saint-Antoine Hospital in Paris with the

aim of further validating our assay in an independent cohort of CMMRD patients. The service is available upon request. We are confident this assay will provide a functional definition, or “signature”, for CMMRD, similar to the chromosomal breakage test for diagnosis of Fanconi anemia. In the near future, we believe that individuals who are at-risk of CMMRD will be tested solely using functional assays as the initial test.

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Author names in bold designate shared co-first authorship.

LEGENDS

Figure 1. Proof of concept study. **(A)** LCLs but not PBLs from CMMRD patients display *ev*MSI. Electrophoretograms of fluorescent amplification products for NR27, NR21 and BAT26 microsatellites. The length of the predominant allele in base pairs (bp) and the fluorescence intensity are indicated in the box below each profile. Deletions (red arrows) occurred at these loci in lymphoblastoid cell lines (LCLs) derived from CMMRD patients C01.1 (*PMS2* deficient), C14 (*MSH6* deficient) and C15 (*MLH1* deficient) compared with their respective peripheral blood lymphocytes (PBLs). In PBLs, the PCR profiles were similar in CMMRD patients C01.1 or C14 and their respective parents, demonstrating that MSI could only be demonstrated *ex vivo*. **(B).** CMMRD patients but not controls display *ev*MSI. Deletions, expressed as the size of deletion for each marker and the cumulative size of deletion (*i.e.* the sum of the deletions observed in the 3 markers) were observed in LCLs from the 3 CMMRD patients but not in MMR-proficient controls that included 4 LS patients and one individual with wild-type (wt) MMR status. **(C)** LCLs from CMMRD patients displayed methylation tolerance. One, two or three rounds of MNNG treatment at 24-hour intervals were performed. LCLs from the 3 CMMRD patients (red) were phenotypically distinguishable from heterozygous (green) and wild-type (blue) LCLs using several experimental conditions. **(D)** 6-TG response of LCLs from the 3 CMMRD patients (red), 4 LS patients (green) and one individual with wild-type MMR status (blue).

Figure 2. Flow diagram of patient study cohort.

The functional assay, which includes the *ev*MSI and methylation tolerance tests, allowed either the diagnosis or exclusion of CMMRD.

DM, deleterious mutation; VUS, variant of unknown significance; LCL, lymphoblastoid cell line.

Figure 3. Tolerance of immortalized lymphoblasts derived from 14 CMMRD patients and a series of MMR-proficient controls (including LS patients and MMR wild-type individuals) to increasing concentrations of MNNG.

Because of the short half-life of MNNG in aqueous solution, 1, 2 or 3 pulses of treatment were performed. With the exception of *MLH1*$^{+/-}$ LCLs (n=11) that behaved similarly to MMR wild-type lymphoblasts (n=12) in all experimental conditions, *MSH2*$^{+/-}$ (n=12), *PMS2*$^{+/-}$ (n=4) and mainly *MSH6*$^{+/-}$ cell lines (n=13) exhibited increased cell survival under low MNNG concentrations. At higher MNNG concentrations and/or increasing numbers of drug treatments, the survival of *MSH2*$^{+/-}$, *PMS2*$^{+/-}$ and *MSH6*$^{+/-}$ lines decreased towards that of *MLH1*$^{+/-}$ and MMR wild-type cells, whereas CMMRD LCLs remained quite tolerant to the drug. The best experimental condition to discriminate CMMRD patients from controls was two rounds of 2.5µM MNNG (red box). Patients with CMMRD or LS are represented with distinct colors depending on the MMR gene that was mutated (red for *PMS2*, blue for *MSH6*, yellow for *MSH2* and green for *MLH1*).

Figure 4. *ev*MSI and methylation tolerance assays in a case-control study (A) and in patients considered at-risk for this syndrome (B).

A. Case-control study involving 14 CMMRD patients and 23 MMR-proficient controls comprising 12 LS patients with heterozygous mutations affecting MMR genes and 11 patients with no detected germline MMR mutation. *Ev*MSI assay (**left**): Deletion sizes (in

base pair, bp) are expressed as the sum of the deletions for the 3 markers (NR27, NR21 and BAT26). The cut-off value used to define a cell line as positive for *ev*MSI was set at 1 bp deletion for all 3 markers (red dotted line). LCLs from all 14 CMMRD patients showed decreased allele size, regardless of which MMR gene was mutated, whereas no deletions were detected in the 23 MMR-proficient controls tested. Methylation tolerance assay **(right**): Survival (%) of immortalized lymphoid cells derived from the same 14 CMMRD patients and from controls after 2 rounds of 2.5 µM MNNG treatment. Since some LS patients displayed increased tolerance to MNNG compared to MMR wild-type controls, a larger series of LS patients was used for the drug assay. Whereas *MLH1*$^{+/-}$ LCLs behaved similarly to MMR wild-type lymphoblasts, *MSH2*$^{+/-}$ and especially *MSH6*$^{+/-}$ cell lines exhibited significantly increased median cell survival. Overall, all CMMRD-derived LCLs displayed cell survival higher than 60%, whereas cell survival of all MMR-proficient LCLs was lower than 40%, with the exception of one case. The cut-off value was arbitrarily set at 50% cell survival (red dotted line). Student's t test.

B. *ev*MSI (**left**) and methylation tolerance (**right**) tests were applied for the detection of CMMRD syndrome in 23 patients with a clinical presentation suggestive of CMMRD, but for whom the diagnosis could not be confirmed (or excluded) by sequencing of MMR genes. These comprised of 8 patients with bi-allelic MMR alterations involving one or two VUS, 5 patients with a single MMR alteration and 10 patients without germline MMR mutation. One of the latter (patient C29.1) showed abnormal functional assay results for both tests, which prompted us to perform additional *PMS2* genetic screening that led to the identification of a homozygous deletion.

Vertical line=VUS, cross=deleterious mutation.

CMMRD and LS patients are represented using distinct colors depending on the MMR gene that was mutated.

Figure 5. Proposed algorithm for the evaluation of patients suspected of having CMMRD.

In the next future, individuals with a clinical score of ≥3 according to Wimmer *et al.* [9] should be initially tested by functional assays. Since this approach has a high NPV, a normal result obtained with the functional assays would confidently allow the diagnosis of CMMRD to be excluded without the need for additional tests. Alternatively, an abnormal result would be highly suggestive of CMMRD. However, due to the relatively low PPV (80.5%) associated with this assay, we recommend that medical geneticists and pediatricians further investigate these 'at-very-high-risk' cases using other approaches (IHC, gMSI, sequencing of MMR genes) in order to confirm the diagnosis. It is worth noting that IHC results can be used to guide germline mutation analysis to a specific MMR gene, whereas in this context the finding of a normal gMSI ratio would direct genetic analysis to the *MSH6* gene.

* LS should be sought in cases with evocative criteria

Patient	Clinical score[a]	Germline MMR analysis				Publication or physician (country)
		Gene	Mutation type	Class [b]	Status	
CMMRD patients with confirmed molecular diagnosis, *i.e.* with bi-allelic pathogenic MMR gene alterations						
C01.1	7	*PMS2*	Frameshift / Missense	DM / DM	Compound heterozygous	Auclair *et al*., 2007 [32]
C01.2	7	*PMS2*	Frameshift / Missense	DM / DM	Compound heterozygous	Auclair et al., 2007 [32]
C02	3	*PMS2*	Frameshift / Frameshift	DM / DM	Homozygous	Ilencikova (Slovakia)
C03.1	8	*PMS2*	Frameshift / Frameshift	DM / DM	Homozygous	patient 1 in Chmara et al., 2013 [33]
C03.2	9	*PMS2*	Frameshift / Frameshift	DM / DM	Homozygous	patient 1.2 in Chmara et al., 2013 [33]
C04	7	*PMS2*	Large deletion / Large deletion	DM / DM	Compound heterozygous	patient 2 in Chmara et al., 2013 [33]
C05	10	*PMS2*	Nonsense / Nonsense / Frameshift	DM / DM / DM	Compound heterozygous	Brugières (France)
C06	8	*PMS2*	Splice / Splice	DM / DM	Homozygous	Brugières (France)
C07	8	*PMS2*	Missense / Missense	DM / DM	Homozygous	Colas (France)
C08	8	*PMS2*	Missense / Missense	DM / DM	Homozygous	Malka (France)
C09.1	5	*PMS2*	Splice / Splice	DM / DM	Homozygous	Brugières (France)
C10	4	*PMS2*	Splice / Splice	DM / DM	Homozygous	Brugières (France)
C11	11	*PMS2*	Large deletion / Large deletion	DM / DM	Homozygous	Fedhila / Colas (Tunisia)
		MSH2	Missense	VUS	Heterozygous	
		MSH6	Missense	VUS	Heterozygous	
C12	5	*MSH6*	Frameshift / Frameshift	DM / DM	Homozygous	patient PIV.5 in Ilencikova et al., 2011 [34]
C13.1	10	*MSH6*	Frameshift / Frameshift	DM / DM	Compound heterozygous	patient P6 in Gardes et al., 2012 [35]
C14	8	*MSH6*	Frameshift / Frameshift	DM / DM	Compound heterozygous	Auclair *et al*., 2007 [32]
C15	10	*MLH1*	Splice / Splice	DM / DM	Homozygous	Entz Werle (France)
C16	9	*MLH1*	Missense / Missense	DM / DM	Homozygous	Raevaara *et al*., 2004 [36]
C17	6	*MSH2*	Large deletion / Large deletion	DM / DM	Homozygous	Verloes (France)
Patients with clinical characteristics of CMMRD syndrome but a lack of confirmatory standard genetic defect						
C18	6	*PMS2*	In frame deletion / Missense	DM / VUS	Compound heterozygous	Lejeune (France)
C19	8	*PMS2*	Missense / Frameshift	VUS / DM	Compound heterozygous	Dramard (France)
C20.1	7	*MSH6*	Missense / Missense	VUS / VUS	Homozygous	Leis (Afghanistan)
C20.2	7	*MSH6*	Missense / Missense	VUS / VUS	Homozygous	Leis (Afghanistan)
C21	14	*MSH6*	Frameshift / In frame deletion	DM / VUS	Compound heterozygous	Bougeard *et al*. 2014 [29]
		MSH2	Missense	VUS	Heterozygous	
C22	8	*MSH6*	Missense / Missense	VUS / VUS	Homozygous	Wafaa / Colas (Marocco)
		PMS2	Missense	VUS	Heterozygous	
C23	13	*MSH6*	In frame duplication / In frame duplication	VUS / VUS	Heterozygous	Gauthier-Villars (France)
C24	6	*MSH2*	Splice / Splice	DM / VUS	Compound heterozygous	Ruiz Ponte (Spain)
C25	5	*PMS2*	Frameshift	DM	Heterozygous	Colas (France)
C26	4	*PMS2*	Large deletion	DM	Heterozygous	Kinzel (Germany)
C27	3	*MLH1*	Frameshift	DM	Heterozygous	Colas (France)

		MSH2	Missense	VUS	Heterozygous	
C28	1	*MLH1*	Splice	DM	Heterozygous	Caron (France)
C29.1	4	-	no MMR mutation identified [c]	-	-	Brugières (France)
C30	4	*MSH2*	Splice	VUS	Heterozygous	Brugières (France)
C31	3	-	no MMR mutation identified	-	-	Mortemousque (France)
C32	3	-	no MMR mutation identified	-	-	Brugières (France)
C33	4	-	no MMR mutation identified	-	-	Wang (France)
C34.1	4	-	no MMR mutation identified	-	-	Grandjouan (France)
C35	6	-	no MMR mutation identified	-	-	Brugières (France)
C36	3	-	no MMR mutation identified	-	-	Grandjouan (France)
C37	4	-	no MMR mutation identified	-	-	Colas (France)
C29.2	NA	-	no MMR mutation identified [c]	-	-	Brugières (France)
C34.2	4	-	no MMR mutation identified	-	-	Brugières (France)

Table 1. Data set for known and putative CMMRD patients

[a] Clinical score according to Wimmer *et al.* [9]; NA, not applicable

[b] DM, deleterious mutation; VUS, variant of unknown significance

[c] Extensive genetic screening was performed *post-hoc* in view of the abnormal functional assay results found in patient C29.1. It led to the identification of a homozygous deletion of exons 14-15 of the *PMS2* gene, c.276-? (*160?) del, while the brother (patient C29.2) was found as heterozygote for the *PMS2* deletion.

Detailed description of the MMR gene alterations is provided in supplementary table 1

A.

Patient	MMR sequencing		Functional assays		Diagnosis according to functional assays	Comparison with other tests	
	status	MMR gene	*ev*MSI	methylation tolerance		gMSI	MMR protein expression in normal tissue (IHC)
CMMRD patients n=14							
C15	hmz DM	*MLH1*			CMMRD		lost
C06, C07, C08, C09.1	hmz DM	*PMS2*					lost
C10	hmz DM	*PMS2*	+	+		+	NA
C04, C05	cpd htz DM	*PMS2*					lost
C01.1	cpd htz DM	*PMS2*					NA
C01.2	cpd htz DM	*PMS2*	+	+		NA	lost
C02	hmz DM	*PMS2*	+	+		NI	NA
C14	cpd htz DM	*MSH6*					lost
C12	hmz DM	*MSH6*	+	+		-	NA
C13.1	cpd htz DM	*MSH6*					NA
Patients at-risk for CMMRD n=23							
C29.1	no mutation[a]		+	+	CMMRD	+	lost
C20.1	hmz VUS	*MSH6*	+	+		-	lost
C20.2, C22	hmz VUS	*MSH6*					conserved
C18	DM + VUS	*PMS2*					lost
C19	DM + VUS	*PMS2*	+	+		NI	lost
C24	DM + VUS	*MSH2*	-	-	not CMMRD	-	conserved
C30	htz VUS	*MSH2*					NA
C25	htz DM	*PMS2*					lost
C26	htz DM	*PMS2*					conserved
C27	htz DM	*MLH1*					NA
C28	htz DM	*MLH1*					conserved
C34.2	no mutation						conserved

C29.2, C31, C32, C34.1, C35, C36, C37	no mutation[a]					NA
C33	no mutation		-	-	NI	NA
C21	DM + VUS	*MSH6*	-	+	Doubtful	-	lost
C23	hmz VUS	*MSH6*					lost

Table 2. *ev*MSI, methylation tolerance, gMSI and IHC data in the series of 14 CMMRD patients with bi-allelic pathogenic MMR gene alterations and in 23 patients at-risk for whom diagnosis could not be confirmed by MMR sequencing.

[a] extensive genetic screening that was performed *post-hoc* led to the identification of a deletion of exons 14-15 in the *PMS2* gene that was found at an homozygous or heterozygous status in patients C29.1 and C29.2, respectively.

Detailed data on the expression of MMR proteins in normal tissue and on gMSI test are provided in supplementary tables 1 to 3.

VUS, variant of unknown significance; DM, deleterious mutation; hmz, homozygous; htz, heterozygous; cpd, compound; +, positive/abnormal; -, negative/normal; NI, not interpretable; NA, not available.

A.

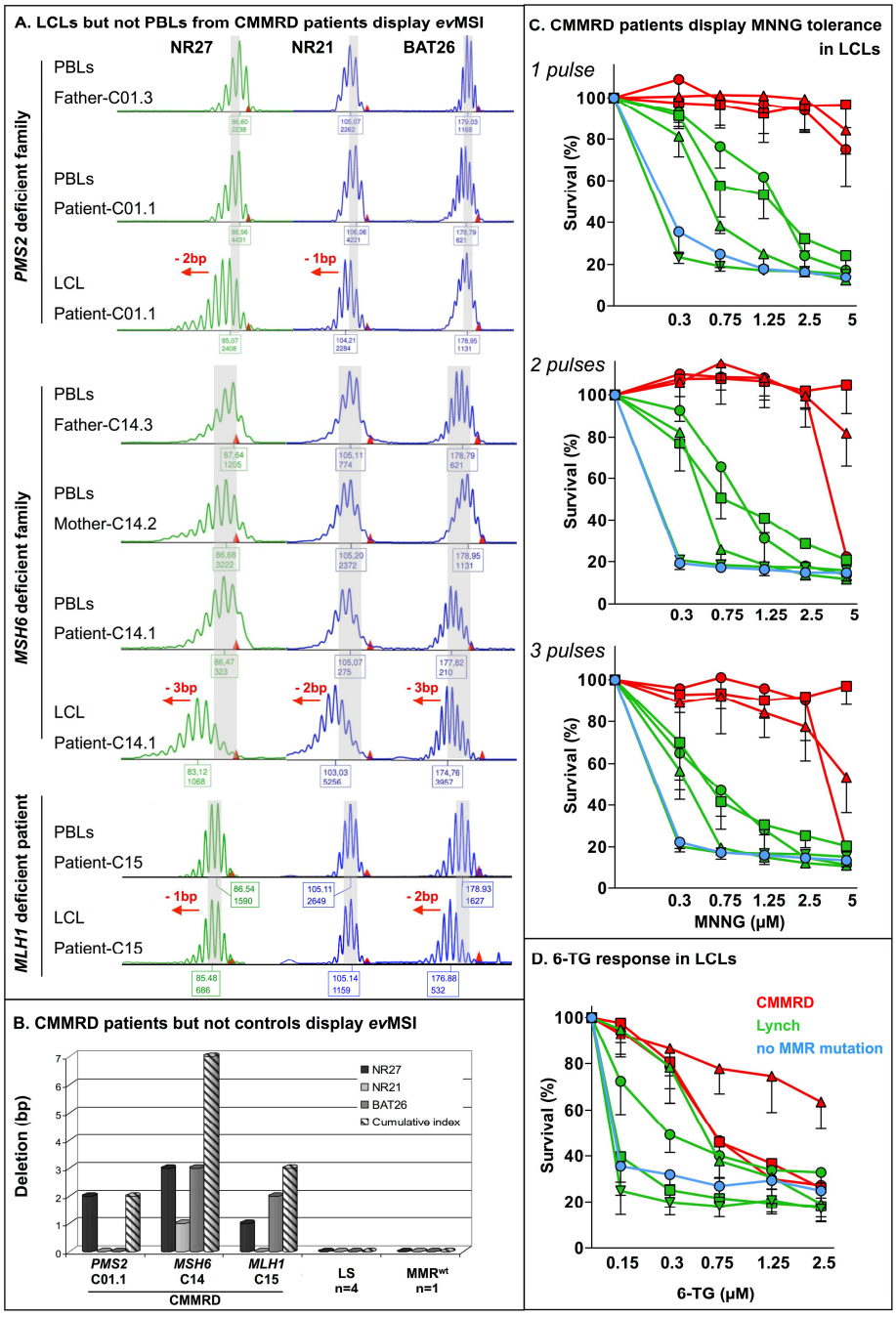
Patient	MMR sequencing		Functional assays		Diagnosis according to functional assays	Comparison with other tests	
	status	MMR gene	*ev* MSI	methylation tolerance		gMSI	MMR protein expression in normal tissue (IHC)
CMMRD patients n=14							
C15	hmz DM	*MLH1*	+	+	CMMRD	+	lost
C06, C07, C08, C09.1	hmz DM	*PMS2*					lost
C10	hmz DM	*PMS2*					NA
C04, C05	cpd htz DM	*PMS2*					lost
C01.1	cpd htz DM	*PMS2*					NA
C01.2	cpd htz DM	*PMS2*	+	+		NA	lost
C02	hmz DM	*PMS2*	+	+		NI	NA
C14	cpd htz DM	*MSH6*					lost
C12	hmz DM	*MSH6*	+	+		-	NA
C13.1	cpd htz DM	*MSH6*					NA
Patients at-risk for CMMRD n=23							
C29.1	no mutation[a]		+	+	CMMRD	+	lost
C20.1	hmz VUS	*MSH6*	+	+		-	lost
C20.2, C22	hmz VUS	*MSH6*					conserved
C18	DM + VUS	*PMS2*					lost
C19	DM + VUS	*PMS2*	+	+		NI	lost
C24	DM + VUS	*MSH2*	-	-	not CMMRD	-	conserved
C30	htz VUS	*MSH2*					NA
C25	htz DM	*PMS2*					lost
C26	htz DM	*PMS2*					conserved
C27	htz DM	*MLH1*					NA
C28	htz DM	*MLH1*					conserved
C34.2	no mutation						conserved
C29.2, C31, C32, C34.1, C35, C36, C37	no mutation[a]						NA
C33	no mutation		-	-		NI	NA
C21	DM + VUS	*MSH6*	-	+	Doubtful	-	lost
C23	hmz VUS	*MSH6*					lost

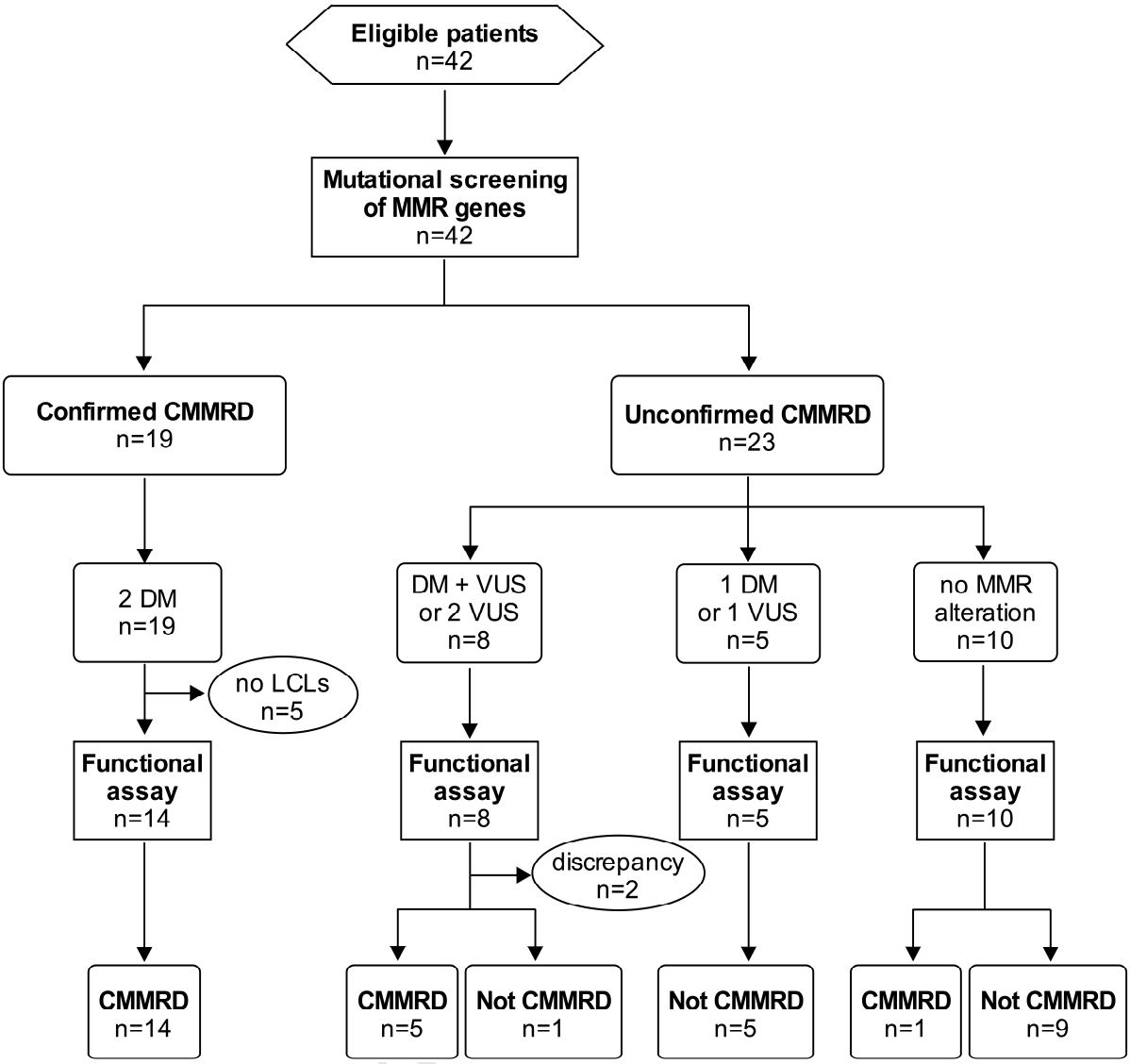
Table 2. *ev* MSI, methylation tolerance, gMSI and IHC data in the series of 14 CMMRD patients with bi-allelic pathogenic MMR gene alterations and in patients at-risk for whom diagnosis could not be confirmed by MMR sequencing.

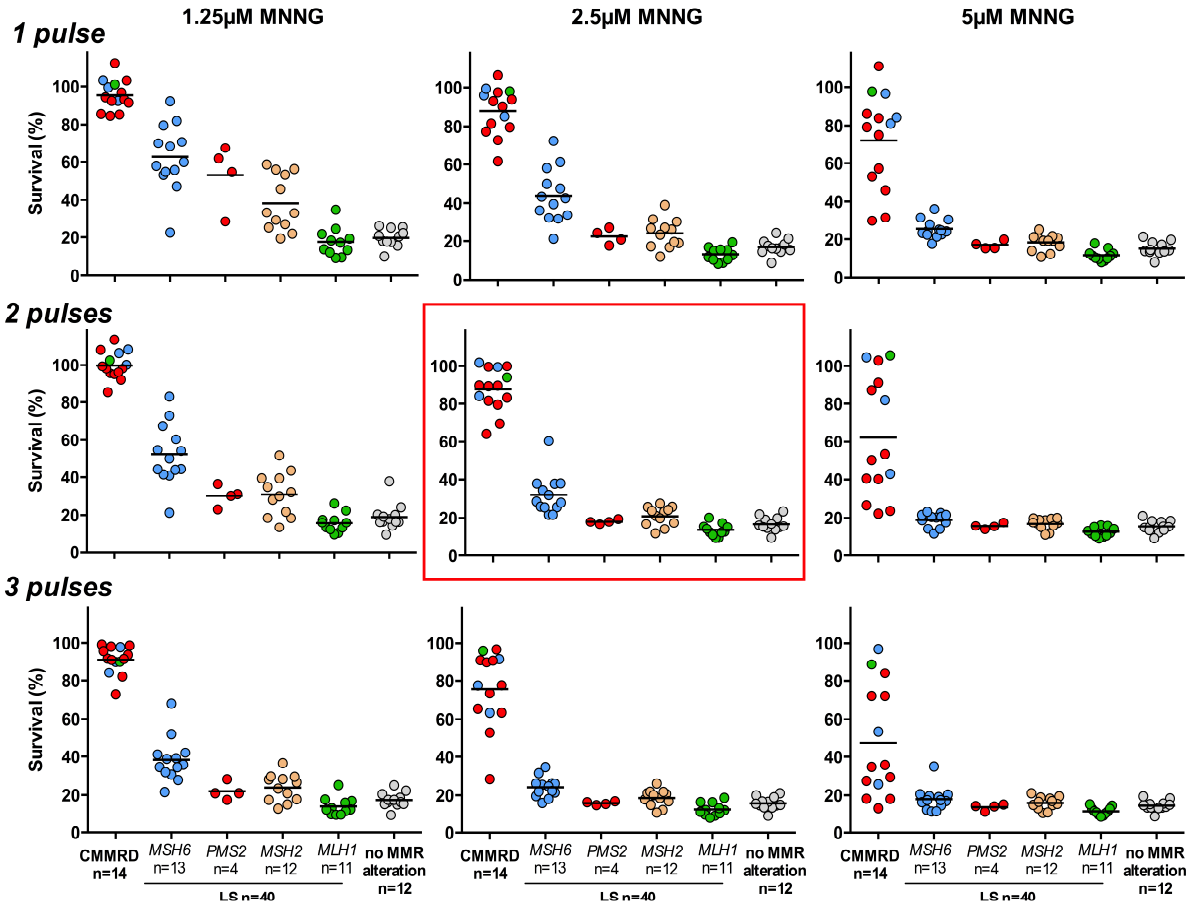
[a] extensive genetic screening that was performed *post-hoc* led to the identification of a deletion of exons 14-15 in the *PMS2* gene that was found at an homozygous or heterozygous status in patients C29.1 and C29.2, respectively.

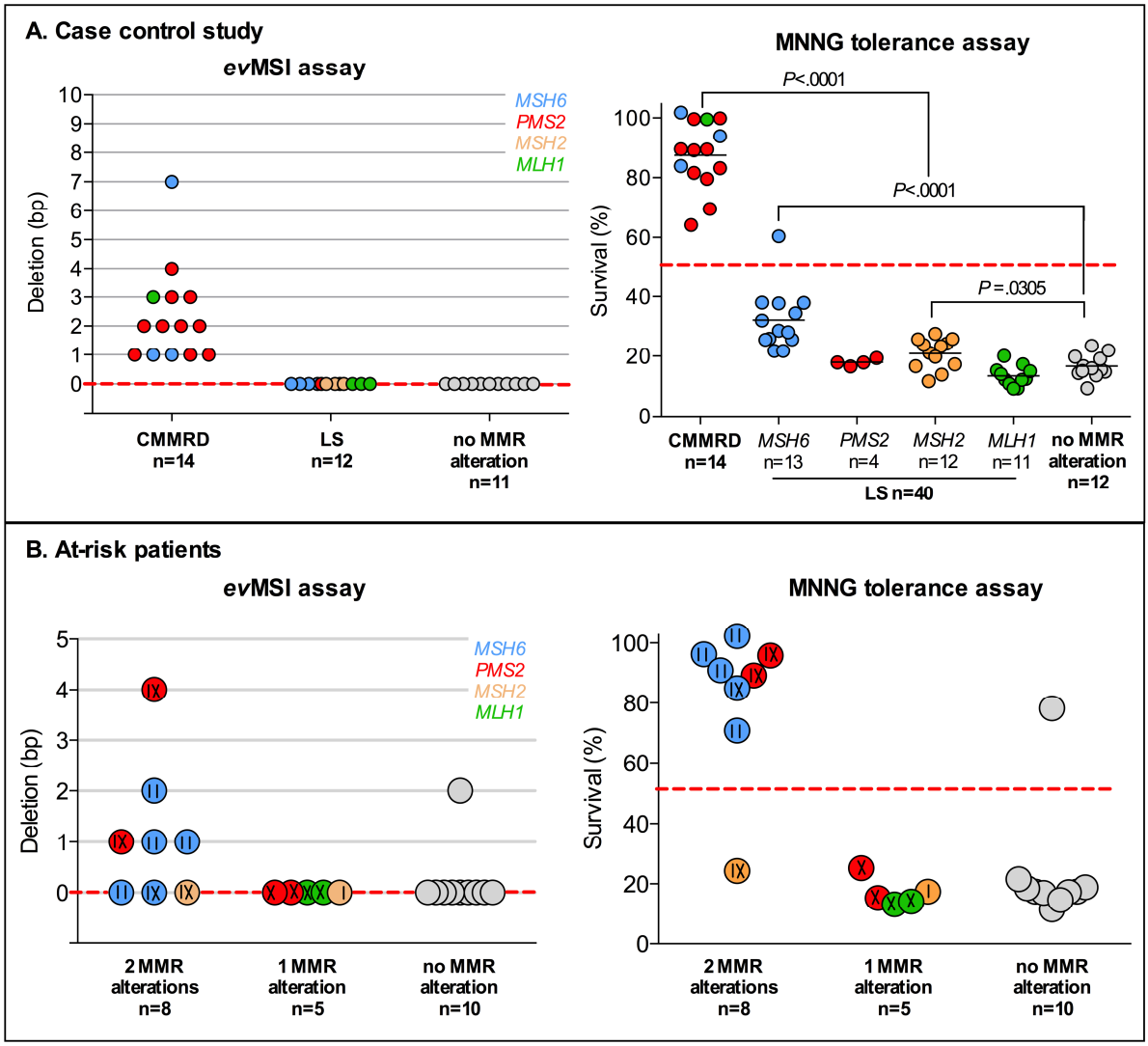
Detailed data on the expression of MMR proteins in normal tissue and on gMSI test are provided in supplementary tables 1 to 3.

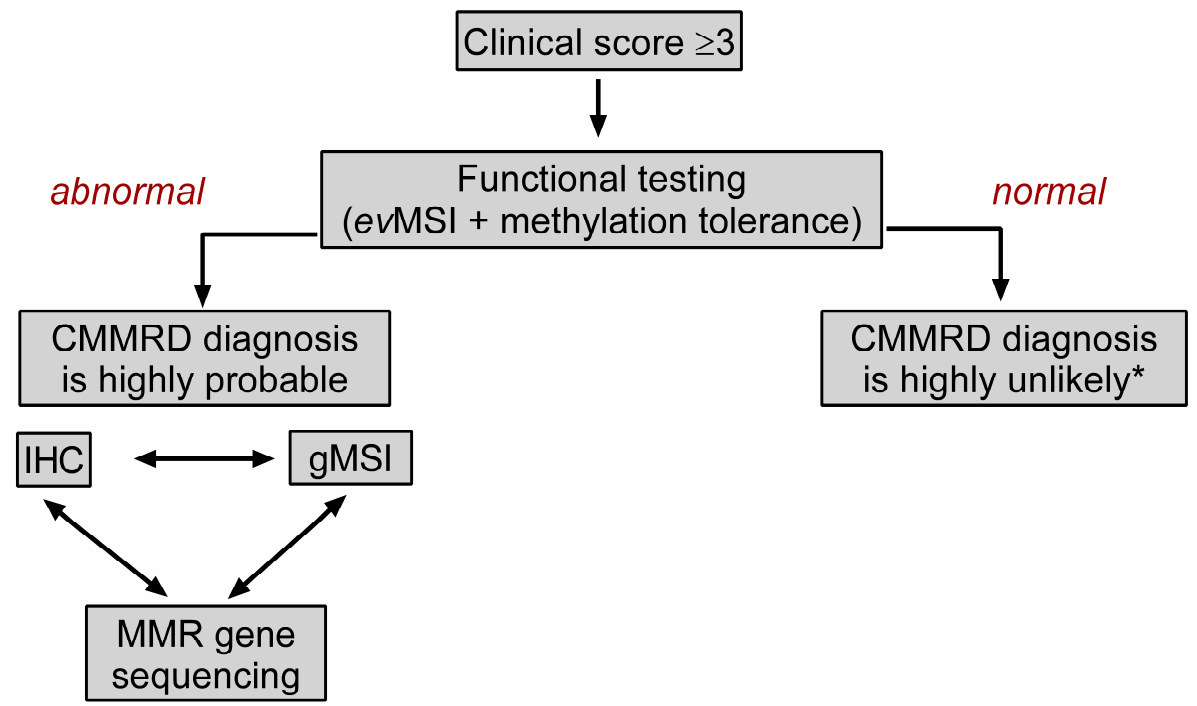
VUS, variant of unknown significance; DM, deleterious mutation; hmz, homozygous; htz, heterozygous; cpd, compound; +, positive/abnormal; -, negative/normal; NI, not interpretable; NA, not available.


A. LCLs but not PBLs from CMMRD patients display evMSI
NR27
NR21
BAT26
PMS2 deficient family
PBLs
Father-C01.3
PBLs
Patient-C01.1
- 2bp
- 1bp
LCL
Patient-C01.1
MSH6 deficient family
PBLs
Father-C14.3
PBLs
Mother-C14.2
PBLs
Patient-C14.1
- 3bp
- 2bp
- 3bp
LCL
Patient-C14.1
MLH1 deficient patient
PBLs
Patient-C15
- 1bp
- 2bp
LCL
Patient-C15
B. CMMRD patients but not controls display evMSI
Deletion (bp)
NR27
NR21
BAT26
Cumulative index
PMS2 C01.1
MSH6 C14
MLH1 C15
CMMRD
LS n=4
MMRwt n=1
C. CMMRD patients display MNNG tolerance in LCLs
1 pulse
2 pulses
3 pulses
Survival (%)
MNNG (µM)
D. 6-TG response in LCLs
CMMRD
Lynch
no MMR mutation
Survival (%)
6-TG (µM)









SUPPLEMENTARY MATERIAL AND METHODS

Patients. All of the 19 genetically confirmed and 19 of the 23 suspected CMMRD patients included in this study had a score ≥3 points according to the recently published clinical criteria for the suspected diagnosis of CMMRD. Additionally, four patients were included in this study. One patient (C28) was included because he displayed a very severe clinical history with four LS-related tumors from 32 to 36 year and osteosarcoma at the age of 11 years old (osteosarcoma was found in the CMMRD patient C05 at the age of 24 years old). Equally, one patient (C27) was included since, compared to other members of this LS family, he had a very early onset (30 years) of two synchronous colon cancers with an adenoma and a brother who had a malignant brain tumour at the age of 18 years. Another patient (C33) had a cerebral tumor at the age of 27 years old as well as CALMs and her sister displayed a cerebral tumor (22 years). Finally, one was an asymptomatic sibling with CALMs of a possible CMMRD patient (C29.2). Altogether, the study included 42 patients (37 families) from several European cancer centers. Control subjects considered free of MMR germline defects included five FAP and two NF1 individuals with identified germline *APC* or *NF1* mutations, respectively (FAP and NF1 were chosen because they represent cancer predisposition syndromes showing clinical overlap with CMMRD) and eight control patients diagnosed with sporadic colorectal cancer without familial cancer history. These patients had developed microsatellite stable tumors (6 cases) or MSI tumors due to epigenetic silencing of *MLH1* because of somatic methylation (2 cases) and thus were not suspected of having CMMRD syndrome.

Colorectal cell lines. Human colorectal cancer cell lines were grown in DMEM with glutamax supplemented with 10% FCS, 100 IU/ml penicillin and 100mg/ml

streptomycin (PAA). They included 6 microsatellite unstable (HCT116, LIM2405, LS174T, KM12 (all *MLH1*-deficient), HCT15 (*MSH6* mutated) and LoVo (homozygous deletion of exons 2-8 of *MSH2*)) and 5 microsatellite stable (LS513, SW620, Caco-2, FET and HCT116 mlh1-2 (HCT116 transfected with an MLH1-expression vector [1]) cell lines.

Treatment of colorectal cell lines. Cells in the exponential growth phase were counted by trypan blue exclusion and seeded into 24-well plates (Falcon) at a density of 0.2-5×10^5 cells/well in complete medium. After 24-h incubation, 6-TG (1, 5, 15, 20µM final concentrations) or extemporaneously reconstituted MNNG (0.1, 1, 5, 20µM final concentrations) was added. Medium was removed and replaced with fresh medium after 24-h or 1-h incubation, respectively. Cell growth was evaluated after a total incubation period of 7 to 9 days. To exclude differences in MNNG cytotoxicity due to variations in O^6-methylguanine methyltransferase enzyme activity, the latter was abrogated by exposure to O^6-benzylguanine (20µM final concentration) during the entire experiment. All samples were tested in quadruplicate.

6-TG treatment of lymphoblastoid cell lines. Cells suspended in complete medium (3x10^5 cell/mL) were distributed into 6 microtubes with increasing concentrations of 6-TG (0.15, 0.3, 0.6, 1.25 and 2.5µM final concentrations) into 5 of them. After 24-h incubation, all microtubes were centrifuged, the cells were rinsed with fresh medium and then seeded in 100µL aliquots into 96-well round-bottom plates (0.6×10^4 cells/well). Cell growth was evaluated after a total incubation time of 7 days and all samples were tested in sextuplicate.

DNA extraction for *ev*MSI and gMSI assays. Ficoll-Plaque PLUS was used to isolate human lymphocytes from blood patients, according to the supplier's recommendations (GE Healthcare). DNA extraction from lymphocytes or LCL was

performed using QIAmp DNA kit according to the supplier's recommendations (Qiagen).

Determining the gMSI ratio. Multiplex PCR amplification in triplicate (denaturation of 95℃ for 2 min, followed by 35 cycles of 95℃ fo r 30sec, 55℃ for 30sec, and 72℃ for 60sec, with a final extension at 72℃ for 10min) of the dinucleotide microsatellite markers D17S791, D2S123 and D17S250 was developed using the primers previously described [2], and using 10ng of patient germinal DNA. PCR products were separated by capillary electrophoresis on an ABI3100 genetic analyzer and quantified using Gene Mapper software v3.7. Briefly, the gMSI ratio was determined by dividing the height of an allele's trailing "stutter" peak (n+1) by the height of the allele's major peak (n). Interpretation required that the size difference between alleles in heterozygous individuals was ≥6bp [2].

Statistical analysis. We developed a Bayesian approach to conduct inference for the unknown prevalence, sensitivity and specificity of the three diagnostic methods as performed in Joseph *et al.* [3]. Our setting was however different from theirs, in particular we knew the true disease status for controls and genetically confirmed CMMRD patients, which removes the lack of identifiability of Joseph *et al.* approach pointed out in Johnson *et al.* [4].

In the saturated model, the joint distribution of the tests or combination of tests was assumed to be multinomial with 16 categories, corresponding to all possible observations. The multinomial parameters were expressed as the true proportion of confirmed CMMRD patients, sensitivity and specificity of the tests. We assumed conditional independence of the tests to ensure identifiability in the unsaturated model. A Metropolis-Hastings algorithm was run on the data to estimate the seven parameters and two-sided confidence intervals[3, 5]. Let D be the true CMMRD status

(+/-), T_1 (+/-), T_2 (+/-) and T_3 (+/-) be the result of MMR gene sequencing, functional testing and gMSI testing, respectively. The true proportion of CMMRD patients, sensitivity and specificity of the three tests or combination of tests are defined as:

$$\begin{aligned}
\pi &= \mathbb{P}(D=+) \\
s_1 &= \mathbb{P}(T_1=+|D=+) \text{ and } c_1=\mathbb{P}(T_1=-|D=-) \\
s_2 &= \mathbb{P}(T_2=+|D=+) \text{ and } c_2=\mathbb{P}(T_2=-|D=-) \\
s_3 &= \mathbb{P}(T_3=+|D=+) \text{ and } c_3=\mathbb{P}(T_3=-|D=-)
\end{aligned}$$

The observed data are summarized in the table below (the rows with no observations are not reported), the usual latent variables are denoted by X, Y, Z.

Genetic testing	**Methylation tolerance and *ev*MSI**	**gMSI**	**True status**	**Data**
+	+	+	CMMRD+	9
+	+	-	CMMRD+	2
-	-	-	CMMRD+	Y
-	-	-	CMMRD-	9+15-Y
-	+	+	CMMRD+	X
-	+	+	CMMRD-	1-X
-	+	-	CMMRD+	Z
-	+	-	CMMRD-	4-Z

We particularized *MSH6* cases, since gMSI is not relevant to identify CMMRD patients with *MSH6* defects. As a consequence, we rewrite

$$\begin{aligned}
Y &= Y_{\text{MSH6}} + Y_{\text{noMSH6}} \\
&\quad \text{with } \; Y_{\text{MSH6}} \sim \mathcal{B}(2, \frac{\pi(1-s_1)(1-s_2)}{\pi(1-s_1)(1-s_2)+(1-\pi)c_1c_2}) \\
&\quad \text{and } \; Y_{\text{noMSH6}} \sim \mathcal{B}(13, \frac{\pi(1-s_1)(1-s_2)(1-s_3)}{\pi(1-s_1)(1-s_2)(1-s_3)+(1-\pi)c_1c_2c_3}) \\
Z &= Z_{\text{MSH6}} + Z_{\text{noMSH6}} \\
&\quad \text{with } \; Z_{\text{MSH6}} \sim \mathcal{B}(1, \frac{\pi(1-s_1)s_2}{\pi(1-s_1)s_2+(1-\pi)c_1(1-c_2)}) \\
&\quad \text{and } \; Z_{\text{noMSH6}} \sim \mathcal{B}(3, \frac{\pi(1-s_1)s_2(1-s_3)}{\pi(1-s_1)s_2(1-s_3)+(1-\pi)c_1(1-c_2)c_3})
\end{aligned}$$

conditionally to the parameters.

This formulation allowed us to fit the model through the Metropolis-Hastings algorithm [6]. In the latter, we considered a Dirichlet prior for the joint distribution of the seven parameters. The parameters of the marginal prior distributions were chosen as (1,1) for the true proportion of CMMRD patients, the sensitivities and the specificities. The Metropolis-Hastings algorithm was run on 50000 iterations and the last 25000 iterations were used to derive estimations and confidence intervals for the sensitivities, specificities, positive and negative predictive values of MMR gene sequencing, functional testing and gMSI testing. The same procedure has been applied for the comparison between MMR gene sequencing and functional testing.

References

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Legends

Supplementary figure 1. Tolerance of human colon cancer cell lines to increasing concentrations of MNNG (A) and 6-TG (B).

Regardless of the defective MMR gene, MMR-deficient colon cancer cell lines (red symbols) showed statistically significant increases (10-fold using 1µM MNNG and 2-fold using 15µM 6-TG) in mean cell survival compared with MMR-proficient cell lines (blue symbols). Mean ± SD. * for P<0.05, ** for P<0.01, *** for P<0.001; ns, not significant ; Student's t test.

Supplementary figure 2. Pedigrees of all previously unreported patients with indications for LS- or CMMRD-related (filled symbols) and other (striped symbols) malignancies / pre-malignancies and age at diagnosis (in years).

Arrows indicated the patients included in the study and their concise MMR genotype is shown (bold characters). Ad, adenoma ; AML, acute myeloid leukemia; C, cancer ; CALMs, café-au-lait macules; CRC, colorectal cancer; CT, cerebral tumor; DM, deleterious mutation; EC, endometrial cancer; hmz, homozygous; htz, heterozygous; mut, mutation; VUS, variant of unknown significance.

Supplementary figure 3. Tolerance of immortalized lymphoblasts derived from 14 CMMRD patients and a series of MMR-proficient controls (including LS patients and

MMR wild-type individuals) to increasing concentrations of 6-TG.

Patients with CMMRD or LS are represented with distinct colors depending on which MMR gene was mutated (red for *PMS2*, blue for *MSH6*, yellow for *MSH2* and green for *MLH1*).

Supplementary figure 4. Histogram showing gMSI ratios at each marker (D17S791, D2S123 and D17S250) for the 18 CMMRD patients tested (the deficient MMR gene is indicated) and for a series of 19 LS patients and 220 controls from the Human Genome Diversity Panel.

Error bars represent the standard error of the mean. The horizontal blue, red and green lines indicate the gMSI cut-off values for markers D17S791, D2S123 and D17S250, respectively. Test result is positive (*i.e.* abnormal) when the gMSI ratios of at least 2 markers are above the cut-off value, and negative (*i.e*. normal) when the gMSI ratios of at least 2 markers are below the cut-off value. Otherwise, the result is considered as not interpretable which made the test non-informative in 3/18 (16.7%) CMMRD patients (labeled with *), 3/19 (15.8%) LS patients and 39/220 (17.7%) controls. The two CMMRD patients with *MSH6* deficiency (C12 and C13.1) were not detected by this method whereas CMMRD patients with *PMS2*, *MLH1* or *MSH2* deficiency displayed abnormal gMSI values. All controls were negative.

Patient	Clinical and tumor data[a]	Clinical score[b]	Familial history[c]	Germline MMR analysis				
				Gene	Exon	Mutation (Amino-acid change)	Type	Class[e]
CMMRD patients with confirmed molecular diagnosis, *i.e.* with bi-allelic pathogenic MMR gene alterations								
C01.1	oligodendroglioma (19); two colorectal cancers (MSI) (24)	7	S, R	*PMS2*	11	c.1730dup ; p.Arg578Alafs*3	Frameshift	DM
					4	c.137G>T ; p.Ser46Ile	Missense	DM
C01.2	CALMs; colorectal cancer (MSI / PMS2 normal at 1rst analysis; lost in N and T at 2nd look) with 12 adenomas (20); endometrial cancer (24)	7	S, R	*PMS2*	11	c.1730dup ; p.Arg578Alafs*3	Frameshift	DM
					4	c.137G>T ; p.Ser46Ile	Missense	DM
C02	glioblastoma (4)	3	Co, R	*PMS2*	15	c.2521del ; p.Trp841Glyfs*10	Frameshift	DM
					15	c.2521del ; p.Trp841Glyfs*10	Frameshift	DM
C03.1	pilomatricomas (PMS2 lost in N and T) (2), oligodendroglioma (11)	8	S, Co	*PMS2*	11	c.1164del ; p.His388Glnfs*10	Frameshift	DM
					11	c.1164del ; p.His388Glnfs*10	Frameshift	DM
C03.2	CALMs; pilomatricomas (2), pre B-cell non Hodgkin lymphoma (3); glioblastoma (9)	9	S, Co	*PMS2*	11	c.1164del ; p.His388Glnfs*10	Frameshift	DM
					11	c.1164del ; p.His388Glnfs*10	Frameshift	DM
C04	CALMs; glioblastoma (4); B-cell non Hodgkin lymphoma (5); pilomatricomas (PMS2 lost in N and T)	7	-	*PMS2*	7-9	c.706-?_903+?del ; p.?	Large deletion	DM
					9-15	c.904-?_*+?del ; p.?	Large deletion	DM
C05	glioblastoma (22); colorectal cancer (MSI / PMS2 lost in N and T) with three adenomas (24); osteosarcoma (24); acute myeloblastics leukemia (30)	10	Co	*PMS2*	5	c.400C>T ; p.Arg134*	Nonsense	DM
					5	c.400C>T ; p.Arg134*	Nonsense	DM
					11	c.1579del ; p.Arg527Glyfs*68	Frameshift	DM
C06	CALMs; testicular T-lymphoblastic lymphoma (5 and 14); rectal cancer (MSI/PMS2 lost in N and T) (16)	8	Co	*PMS2*	12	c.2007-2A>G ; p.?	Splice	DM
					12	c.2007-2A>G ; p.?	Splice	DM
C07	no CALMs; colorectal cancer (22); colorectal cancer (MSI/PMS2 lost in N and T) (25); glioblastoma (34); endometrial cancer (PMS2 lost in N and T) (36); duodenal cancer (MSS/PMS2 lost in N and T) (37); benin sebaceous cyst (37); multiple colorectal adenomas (>15) (since 22)	8	P, Co	*PMS2*	2	c.137G>T ; p.Ser46Ile	Missense	DM
					2	c.137G>T ; p.Ser46Ile	Missense	DM
C08	CALMs; colorectal cancer (19); colorectal cancer (MSI / PMS2 lost in N and weak in T) (20); lymphoblastic lymphoma (27)	8	-	*PMS2*	2	c.137G>T ; p.Ser46Ile	Missense	DM
					2	c.137G>T ; p.Ser46Ile	Missense	DM
C09.1	CALMs; lymphoblastic lymphoma (4); PMS2 lost in normal skin	5	Co	*PMS2*	12	c.2007-2A>G ; p.?	Splice	DM
					12	c.2007-2A>G ; p.?	Splice	DM
C10	CALMs; glioblastoma (6)	4	Co	*PMS2*	12	c.2007-2A>G ; p.?	Splice	DM
					12	c.2007-2A>G ; p.?	Splice	DM
C11	T-cell lymphoblatic mediastinal lymphoma (14), colorectal cancer with polyposis (16)	11	S	*PMS2*	14	c.2275+210_2446-1356del ; p.Ala759Glyfs*8	Large deletion	DM
					14	c.2275+210_2446-1356del ; p.Ala759Glyfs*8	Large deletion	DM
				MSH2	4	c.728G>A ; p.Arg243Gln	Missense	VUS
				MSH6	i3	c.627+25_627+27del ; p.?	Missense	VUS

C12	CALMs; T-non Hodgkin lymphoma (2) since publication	5	Co, R	*MSH6*	5	c.3261dupC ; p.Phe1088Leufs*5	Frameshift	DM
					5	c.3261dupC ; p.Phe1088Leufs*5	Frameshift	DM
C13.1	CALMs; colon adenomas (10), glioblastoma (12.5); MSH6 lost in tumor of the sister	10	S, R	*MSH6*	9	c.3984_3987dup ; p.Leu1330Valfs*12	Frameshift	DM
					9	c.3959_3962del ; p.Ala1320Glufs*6	Frameshift	DM
C14	CALMs; multiple adenomas (MSS / MSH6 lost in N and T) with high grade dysplasia (9)	8	S, R	*MSH6*	4	c.1596_1597dup ; p.Glu533Valfs*39	Frameshift	DM
					5	c.3261del ; p.Phe1088Serfs*2	Frameshift	DM
C15	CALMs; lymphoblastic lymphoma (MSS/ MLH1 lost in N and T) (5); glioblastoma (MLH1 lost in N and T) (6)	10	Co, S, R	*MLH1*	9	c.678-7_686del ; p.?	Splice	DM
					9	c.678-7_686del ; p.?	Splice	DM
C16	CALMs; neurofibroma (6); several adenomas and rectal cancer (15) since publication	9	Co	*MLH1*	17	c.1942C>T ; p.Pro648Ser	Missense	DM
					17	c.1942C>T ; p.Pro648Ser	Missense	DM
C17	CALMs; cavernoma (3), T-cell lymphoblastic lymphoma (3)	6	P, Co	*MSH2*	8	c.1277-?_c.1386+? ; p.?	Large deletion	DM
					8	c.1277-?_c.1386+? ; p.?	Large deletion	DM
Patients with clinical characteristics of CMMRD syndrome but a lack of confirmatory standard genetic defect								
C18	CALMs; colorectal cancer (22); colorectal cancer (MSI/PMS2 lost in N and T) with adenoma (32); multiple adenomas with high grade dysplasia (38); glioblastoma (40)	6	R	*PMS2*	10	c.989?_1144+?del ; p.Glu330_Glu381del	In frame deletion	DM
					13	c.2249G>A ; p.Gly750Asp	Missense	VUS
C19	Colorectal cancer (MSI / PMS2 lost in N and T) (21); glioblastoma (22)	8	S, R	*PMS2*	2	c.161T>C ; p.Ile54Thr	Missense	VUS
					11	c.1831dup ; p.Ile611Asnfs2*	Frameshift	DM
C20.1	CALMs; gliomatosis (MSS /MSH6 lost in N and T) (9)	7	Co, S	*MSH6*	4	c.2216C>A ; p.Thr739Lys	Missense	VUS
					4	c.2216C>A ; p.Thr739Lys	Missense	VUS
C20.2	CALMs; glioblastoma (MSS / MSH6 weak in N and T) (6)	7	Co, S	*MSH6*	4	c.2216C>A ; p.Thr739Lys	Missense	VUS
					4	c.2216C>A ; p.Thr739Lys	Missense	VUS
C21	CALMs; adenomas (14); colorectal cancer (17 and 19); urinary tract carcinoma (MSS / MSH6 lost in N and T) (24)	14	S, R	*MSH6*	5	c.3261dupC ; p.Phe1088Leufs*5	Frameshift	DM
					4	c.2561_2563del ; p.Lys854del	In frame deletion	VUS
				MSH2	5	c.832G>A ; p.Glu278Lys	Missense	VUS
C22	CALMs; colorectal cancer (MLH1, MSH2, PMS2, MSH6 normal in N) (16)	8	Co, S	*MSH6*	5	c.3184T>C ; p.Cys1062Arg	Missense	VUS
					5	c.3184T>C ; p.Cys1062Arg	Missense	VUS
				PMS2	11	c.1688G>T ; p.Arg563Leu	Missense	VUS
C23	CALMs; T-cell lymphoblastic lymphoma (6 and 11); glioblastoma (14); colorectal cancer (MSS/MLH1, MSH6, MSH2 normal at 1rst analysis; MSH2 and MSH6 lost in N and T at 2nd look) with polyposis (14)	13	Co	*MSH6*	4	c.1763_1771dup ; p.His588_Pro590dup	In frame duplication	VUS
					4	c.1763_1771dup ; p.His588_Pro590dup	In frame duplication	VUS
C24	no CALMs; colorectal cancer (MSH2 normal in N, lost in T) (12); 1 skin nodule (neurofibroma histologically not confirmed)	6	R	*MSH2*	i6	c.1076+1G>A ; p.Gly315Ilefs*29	Splice	DM
					i6	c.1077-11A>G ; p.?	Splice	VUS
C25	CALMs; colorectal cancer (MSI, MLH1 lost in T; PMS2 lost in N and T) (25); breast cancer (36)	5	R	*PMS2*	11-14	large genomic conversion with PMS2CL ; p.?	Frameshift	DM

C26	no CALMs; rectal cancer (17); colon cancer (MSI in N and T / PMS2 normal in N, lost in T) (27)	4	S, R	*PMS2*	i11-i12	c.2007-786_2174+493del1447 ; p.Ser669_Ala725delinsArg	Large deletion	DM
C27	two colorectal cancers with one adenoma >1cm (30)	3	S, P, R	*MLH1* *MSH2*	9 5	c.769del ; p.Ile257Serfs*11 c.832G>A ; p.Glu278Lys	Frameshift Missense	DM VUS
C28	osteosarcoma (11); urothelial carcinoma (32 and 33); cholangiosarcoma (MLH1normal in N, lost in T) (36); colorectal cancer (MLH1 lost in T) (36); bladder carcinoma (37)	1	-	*MLH1*	15	c.1731G>A ; p.Ser577Ser	Splice	DM
C29.1	CALMs; glioblastoma (PMS2 lost in N and T) (6)	4	R	-	-	no MMR mutation identified [d]	-	-
C30	CALMs; T-cell lymphoblastic lymphoma (8)	4	-	*MSH2*	i4	c.792+16A>G ; p.?	Splice	VUS
C31	lymphosarcoma (5); oligodendroglioma (MSS / MLH1, MSH2 normal in T) (21); thyroid cancer (29)	3	-	-	-	no MMR mutation identified	-	-
C32	Hodgkin lymphoma (11)	3	R	-	-	no MMR mutation identified	-	-
C33	CALMs; oligodendroglioma (27)	4	S	-	-	no MMR mutation identified	-	-
C34.1	colorectal tumor (MSI / MLH1 lost in T) (18)	4	S	-	-	no MMR mutation identified	-	-
C35	CALMs; glioblastoma (18)	6	R, Co	-	-	no MMR mutation identified	-	-
C36	colorectal tumor (MSI / MLH1 lost in T) (17)	3	-	-	-	no MMR mutation identified	-	-
C37	CALMs; adenomatous polyposis (n>50) with duodenal adenomas (APC, MUTYH negative) (24); bilateral breast cancer (BRCA negative) (35 and 37); diffuse gastric cancer (CDH1 negative) (39); duodenal adenoma with high grade dysplasia (40)	4	R	-	-	no MMR mutation identified	-	-
C29.2	CALMs	NA	S, R	-	-	no MMR mutation identified [d]	-	-
C34.2	pinealoblastoma (MLH1, MSH6, MSH2, PMS2 normal in N and T) (12)	4	S	-	-	no MMR mutation identified	-	-

Supplementary Table 1. Data set relative to proved and putative CMMRD patients

[a] When available, data relative to the microsatellite status of the tumor, *i. e.* stable (MSS) or unstable (MSI), and to immunohistochemistry for MMR proteins in normal (N) and tumoral (T) tissues are indicated. Age at diagnosis is indicated in brackets. CALMs, café-au-lait macules

[b] Clinical score according to [9]; NA, not applicable

[c] Co, consanguinity; S, sibling affected with CMMRD-associated cancer; P, parent affected with Lynch syndrome-associated cancer; R, relative affected with Lynch syndrome- or CMMRD-associated cancer

[d] Extensive genetic screening that was performed *post-hoc* in view of the abnormal functional assay results found in patient C29.1, led to the identification of a homozygous deletion of exons 14-15 of the *PMS2* gene, c.276-? (*160?) del, while the brother (patient C29.2) was found as heterozygote for the *PMS2* deletion

[e] DM, deleterious mutation; VUS, variant of unknown significance

Patient	Mutated gene	methylation tolerance[a]									*ev*MSI[b]			Culture time
		1 pulse MNNG			2 pulses MNNG			3 pulses MNNG						
		1.25µM	2.5µM	5µM	1.25µM	2.5µM	5µM	1.25µM	2.5µM	5µM	BAT26	NR21	NR27	
CMMRD patients														
C01.1	*PMS2*	96.8 ± 14.1	93.9 ± 10.1	75.1 ± 17.7	108 ± 8.5	99.9 ± 6	22.3 ± 10.7	95.8 ± 12.1	90.1 ± 19.2	18.1 ± 4.8	178 / 178 / 0	106 / 106 / 0	86 / 84 / -2	X
C01.2	*PMS2*	85.6 ± 13.8	77.4 ± 16.4	29.7 ± 11.2	85.3 ± 9.6	69.6 ± 15.8	26.6 ± 11.1	72.8 ± 15.4	24.8 ± 10.8	13.2 ± 4.7	178 / 178 / 0	105 / 105 / 0	86 / 84 / -2	X
C02	*PMS2*	91.6 ± 18.6	97.5 ± 12.6	83.9 ± 12.2	96 ± 11.8	89.1 ± 9	90.9 ± 17.9	91.2 ± 15.8	91 ± 16.1	35.7 ± 8.1	179 / 178 / -1	105 / 105 / 0	87 / 86 / -1	78
C04	*PMS2*	92.5 ± 15	93 ± 17.8	46 ± 13.3	97.9 ± 12.4	89.4 ± 12	50.1 ± 18.6	99.1 ± 12.6	91.2 ± 14.5	72.1 ± 15.1	179 / 179 / 0	105 / 104 / -1	86 / 85 / -1	X
C05	*PMS2*	94.4 ± 13.6	79.6 ± 15.2	31.4 ± 10.7	94.9 ± 10.6	79.6 ± 15.6	40.5 ± 17.3	98.1 ± 7.3	77.7 ± 15.1	27.5 ± 12.5	180 / 179 / -1	105 / 103 / -2	85 / 84 / -1	46
C06	*PMS2*	85.3 ± 16.6	61.6 ± 10	53.1 ± 14.1	98 ± 11.6	64.2 ± 17.6	23.8 ± 5.5	98.6 ± 12.7	53.1 ± 11	18.2 ± 1.5	180 / 178 / -2	106 / 105 / -1	86 / 86 / 0	81
C07	*PMS2*	84.6 ± 7.4	72.9 ± 12.6	57.3 ± 10.4	95.3 ± 17.8	83.2 ± 17.7	87 ± 16.3	82.2 ± 15.7	63.5 ± 13.5	72.1 ± 14.3	182 / 182 / 0	105 / 105 / 0	86 / 85 / -1	100
C08	*PMS2*	93.3 ± 8	90.3 ± 13.1	79.3 ± 15.7	99.3 ± 8.4	89.5 ± 10.9	53.2 ± 15.4	91.7 ± 11.8	73.5 ± 12	29.4 ± 6.7	178 / 177 / -1	105 / 104 / -1	86 / 85 / -1	82
C09.1	*PMS2*	112.4 ± 15	106.4 ± 10.9	111 ± 11.6	113.4 ± 14.8	99.6 ± 14.2	102.9 ± 12.9	93.9 ± 16.8	96.8 ± 14.5	84.1 ± 8.7	181 / 180 / -1	105 / 105 / 0	86 / 86 / 0	120
C10	*PMS2*	103.2 ± 12.4	81.4 ± 14.3	86.2 ± 17.6	91.9 ± 14.4	81.6 ± 11.3	40.2 ± 16	91.9 ± 8.2	65.5 ± 18.8	34.8 ± 16.8	180 / 180 / 0	105 / 105 / 0	86 / 85 / -1	115
C12	*MSH6*	103.3 ± 13.3	98 ± 18.5	97.7 ± 14.9	102.5 ± 11	93.8 ± 20.1	105.6 ± 8.7	90.2 ± 15.7	96.1 ± 13.7	89 ± 8.1	181 / 181 / 0	105 / 105 / 0	87 / 86 / -1	80
C13.1	*MSH6*	99.5 ± 9.3	85.2 ± 15.3	81.1 ± 13	100.1 ± 17.7	83.9 ± 17.9	43 ± 9.9	97.8 ± 14.5	63.4 ± 18.1	25.7 ± 6.3	179 / 178 / -1	106 / 106 / 0	86 / 86 / 0	70
C14	*MSH6*	92.5 ± 14.1	96.1 ± 11.6	96.7 ± 11.2	106.4 ± 8.8	101.8 ± 8.6	104.6 ± 13.4	90.1 ± 8.6	91.8 ± 15.9	97 ± 8.9	178 / 175 / -3	105 / 104 / -1	87 / 84 / -3	X
C15	*MLH1*	101.1 ± 8.5	99.4 ± 16.3	84.3 ± 11.5	108.2 ± 14.2	99.5 ± 14.7	81.9 ± 16.1	84.2 ± 11.8	77.6 ± 16.3	53.6 ± 17.2	179 / 177 / -2	105 / 105 / 0	87 / 86 / -1	64
Patients at-risk for CMMRD														
C20.1	*MSH6*	85.6 ± 19.2	92 ± 18.8	67.8 ± 16.6	96.4 ± 14.5	96.2 ± 13.3	42.3 ± 6.7	85.6 ± 12.5	56.3 ± 9.7	27.9 ± 7.1	180 / 179 / -1	105 / 105 / 0	87 / 87 / 0	45
C20.2	*MSH6*	89.2 ± 15.3	80.3 ± 11.3	33.9 ± 7.7	77 ± 13.6	70.8 ± 16.5	17.1 ± 2.1	72.4 ± 13.6	47.4 ± 8.8	18.6 ± 5.1	180 / 179 / -1	105 / 105 / 0	87 / 86 / -1	45
C18	*PMS2*	92 ± 7.7	89.2 ± 3.1	79.9 ± 15.9	94.8 ± 8.4	89.2 ± 9.7	85 ± 16.4	90.8 ± 6	80.9 ± 6.8	59.3 ± 6.8	180 / 180 / 0	106 / 105 / -1	87 / 87 / 0	X
C27	*MLH1*	23.9 ± 11.3	14.2 ± 2	11.4 ± 1.6	18 ± 1.7	13.1 ± 1.2	11.3 ± 1	14.1 ± 2.5	11.4 ± 1.7	10.6 ± 1	180 / 180 / 0	105 / 105 / 0	86 / 86 / 0	182
C25	*PMS2*	16.7 ± 1.8	14.5 ± 2	14.8 ± 2.9	17.1 ± 2.1	15.3 ± 2.6	15.5 ± 2.3	18.9 ± 5.9	16.8 ± 2.7	16.3 ± 2.6	180 / 180 / 0	105 / 105 / 0	87 / 87 / 0	344
C22	*MSH6*	101.7 ± 5.3	93.8 ± 8	84.5 ± 7.6	108.2 ± 9.2	102.3 ± 12.2	83.1 ± 11.6	89.7 ± 16.2	78.2 ± 11.7	67.1 ± 12.5	180 / 179 / -1	105 / 105 / 0	87 / 87 / 0	68
C24	*MSH2*	90.1 ± 13.2	50.2 ± 17.1	22.1 ± 3.9	67.9 ± 12	24.3 ± 6.6	14.3 ± 1.3	50.7 ± 19.9	22 ± 5.6	13.4 ± 2	181 / 181 / 0	106 / 106 / 0	87 / 87 / 0	140
C30	*MSH2*	23.4 ± 7.2	17.1 ± 2.7	15.7 ± 2.4	20.9 ± 4.6	17.4 ± 2.5	15.1 ± 2.9	16.5 ± 5.4	16.1 ± 2.8	13.7 ± 3	180 / 180 / 0	106 / 106 / 0	87 / 87 / 0	125
C19	*PMS2*	97.4 ± 11.1	81.4 ± 9.6	64.1 ± 17.3	105.2 ± 9.5	95.8 ± 4.7	55.3 ± 17.9	94.4 ± 14.8	62.6 ± 11.8	29.1 ± 8.1	181 / 178 / -2	106 / 105 / -1	87 / 86 / -1	70
C21	*MSH6*	106 ± 10.8	88.1 ± 19.4	83.3 ± 18.4	105 ± 15	84.9 ± 18.7	45.9 ± 19.5	79.3 ± 19.7	72.6 ± 16.3	15.8 ± 1.3	180 / 180 / 0	105 / 105 / 0	86 / 86 / 0	270
C28	*MLH1*	17.2 ± 2.4	14.3 ± 3.1	13.3 ± 1.5	17.2 ± 3.4	14.1 ± 1.5	14 ± 1.6	18 ± 5.4	13.3 ± 1.2	13.6 ± 1.6	180 / 180 / 0	106 / 106 / 0	88 / 88 / 0	203
C26	*PMS2*	23.3 ± 6.5	19.3 ± 1.6	19.5 ± 3.9	27.1 ± 3.8	25.2 ± 7.4	21.1 ± 4.1	24.2 ± 3.6	21.4 ± 3.2	21.6 ± 3.9	179 / 179 / 0	105 / 105 / 0	86 / 86 / 0	139
C23	*MSH6*	98.2 ± 11.4	93.4 ± 8.4	91.5 ± 15.4	103.7 ± 17.3	90.9 ± 10.4	69.5 ± 15.9	86.3 ± 19.3	84.1 ± 17.7	28.9 ± 13.7	181 / 181 / 0	104 / 104 / 0	87 / 87 / 0	200
C29.1	*PMS2*	121.5 ± 17.2	99.4 ± 13.8	67.8 ± 13.7	122.1 ± 10.5	78.2 ± 20.6	32.1 ± 5.7	51.5 ± 8.6	39.1 ± 15.8	20.9 ± 2.4	181 / 180 / -1	101 / 100 / -1	86 / 86 / 0	66
C29.2	-	20.6 ± 5	15 ± 1.5	14.8 ± 1.2	16.7 ± 1.5	14.6 ± 1	14.3 ± 0.7	15.2 ± 1.8	14.3 ± 1.1	13.6 ± 0.9	180 / 180 / 0	101 / 101 / 0	86 / 86 / 0	105
C31	-	25 ± 13	13.4 ± 3	10 ± 1.6	13.7 ± 2.7	11.4 ± 1.6	9.5 ± 0.7	12.4 ± 1.3	10 ± 0.7	9.2 ± 0.6	180 / 180 / 0	105 / 105 / 0	86 / 86 / 0	70
C32	-	18.7 ± 1.4	16.8 ± 2.2	14.9 ± 1.1	18.8 ± 3.6	17.4 ± 3	15.3 ± 1.3	17.7 ± 1.4	16.4 ± 1.5	15.1 ± 1.1	180 / 180 / 0	106 / 106 / 0	87 / 87 / 0	131
C33	-	32 ± 15	21.2 ± 5.2	17 ± 5.5	24.7 ± 6.7	18.8 ± 5.6	17.1 ± 5.3	21.8 ± 5.2	19.1 ± 6.3	17.1 ± 6.1	180 / 180 / 0	105 / 105 / 0	87 / 87 / 0	80
C34.1	-	28.5 ± 5.9	17.4 ± 3.4	15.8 ± 2.7	22.9 ± 6.3	17.4 ± 2.3	15.2 ± 2	18.7 ± 5.1	14.5 ± 1.9	12.7 ± 1.2	181 / 181 / 0	105 / 105 / 0	87 / 87 / 0	150
C34.2	-	28.1 ± 2.6	21.5 ± 3.2	21.6 ± 3.9	24.3 ± 3.9	21.6 ± 3.9	21.7 ± 4.7	23 ± 4.9	21.9 ± 4.1	20.2 ± 4.8	180 / 180 / 0	105 / 105 / 0	86 / 86 / 0	140
C35	-	24.2 ± 9.1	15.3 ± 2.1	14.1 ± 2.4	22.5 ± 7.9	17 ± 3.4	15.2 ± 3.9	20.3 ± 3.7	18.2 ± 6.5	14.7 ± 2.5	180 / 180 / 0	106 / 106 / 0	87 / 87 / 0	144
C36	-	20.2 ± 6.7	18.2 ± 5.9	17.7 ± 7.3	21.4 ± 9.3	18.5 ± 7	18 ± 7.1	19.3 ± 7	17.6 ± 6.1	17.5 ± 6.3	180 / 180 / 0	105 / 105 / 0	87 / 87 / 0	175
C37	-	32.5 ± 11.2	18.7 ± 4.3	15.6 ± 2	24.4 ± 7.3	17.2 ± 3.4	15.2 ± 2.4	19.9 ± 5.1	15.8 ± 3.7	14.2 ± 3.1	180 / 180 / 0	106 / 106 / 0	87 / 87 / 0	135

MMR wild-type individuals														
A3	*APC*	ND	ND	ND	ND	ND	ND	ND	ND	ND	180 / 180 / 0	106 / 106 / 0	87 / 87 / 0	> 120
A2	*APC*	ND	ND	ND	ND	ND	ND	ND	ND	ND	180 / 180 / 0	105 / 105 / 0	87 / 87 / 0	> 120
A1	*APC*	17.9 ± 2.2	16.4 ± 2.2	13.8 ± 2.1	16.3 ± 2.9	14.8 ± 2.3	14.7 ± 3.9	16.1 ± 1.7	14.7 ± 1.5	13.6 ± 2.9	180 / 180 / 0	105 / 105 / 0	86 / 86 / 0	> 120
A5	*APC*	15.6 ± 3.5	15.5 ± 3.3	14.6 ± 2.9	16.1 ± 3.6	15.5 ± 4	15.3 ± 2.5	15.3 ± 3.3	14.8 ± 3.5	13.6 ± 2.9	ND	ND	ND	ND
A8	*APC*	18 ± 3.2	14.6 ± 1	13 ± 1	14 ± 1.6	13.6 ± 1	12 ± 0.9	15.1 ± 1.3	13.6 ± 0.9	14 ± 1.2	180 / 180 / 0	105 / 105 / 0	87 / 87 / 0	223
N1	*NF1*	ND	ND	ND	ND	ND	ND	ND	ND	ND	180 / 180 / 0	106 / 106 / 0	87 / 87 / 0	122
N3	*NF1*	20.8 ± 1.2	19.7 ± 1.3	18.3 ± 1.4	20.3 ± 1	19.8 ± 1.4	18.5 ± 1.2	20.5 ± 1.4	20 ± 1.2	18.4 ± 1	181 / 181 / 0	105 / 105 / 0	87 / 87 / 0	83
X1	-	25.3 ± 4.8	16.2 ± 3	13.4 ± 3.1	17.9 ± 3.6	14.4 ± 4.1	13.2 ± 3	18.6 ± 2.9	15 ± 2.3	13.6 ± 2.6	ND	ND	ND	ND
X2	-	20.5 ± 4	18.3 ± 3.9	17.1 ± 4.5	19.2 ± 4.4	19.1 ± 4.7	18 ± 5.3	17.5 ± 2.8	16.6 ± 2.7	15.7 ± 2	180 / 180 / 0	105 / 105 / 0	87 / 87 / 0	135
X5	-	22.2 ± 3.9	17.5 ± 2.3	14.4 ± 1.9	20.1 ± 1.8	16.7 ± 1.1	15.4 ± 1.6	17.5 ± 2.4	16.8 ± 2.6	15.4 ± 2.3	180 / 180 / 0	106 / 106 / 0	86 / 86 / 0	138
X7	-	17.5 ± 2.3	16.3 ± 1.9	14.2 ± 2.1	16.2 ± 2.1	16 ± 2.8	15.1 ± 3.2	16.3 ± 2.1	15.6 ± 2.4	14.9 ± 2.2	180 / 180 / 0	105 / 105 / 0	86 / 86 / 0	142
X12	-	10 ± 1.6	8.9 ± 1	8.1 ± 0.7	9.4 ± 1.1	9.3 ± 0.8	9 ± 1	9.6 ± 0.8	9.3 ± 0.8	9 ± 1.4	180 / 180 / 0	105 / 105 / 0	87 / 87 / 0	173
X14	-	25 ± 2.4	21.4 ± 2	19.7 ± 0.7	24.1 ± 4.4	21.9 ± 3.2	21 ± 2.1	22.3 ± 3	21.1 ± 2.5	19.5 ± 2.1	ND	ND	ND	ND
X13	-	17.7 ± 3.5	14.6 ± 2.6	13.6 ± 2.2	16.3 ± 2.8	15.1 ± 2.6	13.4 ± 2.3	15.4 ± 3.4	13.7 ± 2.4	13.3 ± 1.8	ND	ND	ND	ND
X17	-	26 ± 5.4	24.2 ± 5.3	21.3 ± 5.7	37.8 ± 15.3	23.5 ± 6.7	18.4 ± 4.5	25 ± 8.4	19 ± 5.8	15.1 ± 2.5	181 / 181 / 0	105 / 105 / 0	85 / 85 / 0	237
Patients with Lynch syndrome														
C20.3	*MSH6*	ND	ND	ND	ND	ND	ND	ND	ND	ND	180 / 180 / 0	105 / 105 / 0	86 / 86 / 0	122
L13	*MLH1*	13.3 ± 1.8	12.8 ± 1.2	11.4 ± 1.2	13.1 ± 2.1	12.2 ± 1.4	11.5 ± 1.4	13.2 ± 1.8	12.3 ± 2	11.5 ± 1.9	180 / 180 / 0	106 / 106 / 0	87 / 87 / 0	> 120
L15	*MLH1*	17.2 ± 1.9	16.8 ± 1.7	15.4 ± 1.3	17.5 ± 1.3	17.3 ± 0.7	15.8 ± 0.6	16.9 ± 2.4	16.5 ± 2.2	15.3 ± 1.6	180 / 180 / 0	105 / 105 / 0	87 / 87 / 0	252
L20	*MLH1*	24.4 ± 11.5	15 ± 3.8	12.4 ± 1.9	22.5 ± 11.5	15.3 ± 3.9	12.9 ± 1.2	17.7 ± 1.8	16.4 ± 0.9	14.6 ± 0.9	180 / 180 / 0	106 / 106 / 0	86 / 86 / 0	126
L12	*MSH2*	45.8 ± 7	39 ± 10.6	20.6 ± 4.1	39.4 ± 9.5	24.2 ± 5.6	19.6 ± 4.3	28.1 ± 7.7	20.7 ± 3.2	18.9 ± 3.3	178 / 178 / 0	106 / 106 / 0	87 / 87 / 0	249
L14	*MSH2*	29.2 ± 5.5	23.3 ± 3.3	19.2 ± 3.4	28.1 ± 3.7	21.1 ± 2.2	19 ± 2.2	23.4 ± 2.6	21.1 ± 2.6	18.3 ± 3	180 / 180 / 0	105 / 105 / 0	87 / 87 / 0	> 120
L16	*MSH2*	25.1 ± 6.9	16.8 ± 3	12.2 ± 2.3	18.4 ± 1.9	13.9 ± 2.5	11.7 ± 2	15.1 ± 3.5	12.3 ± 1.2	10.8 ± 1.1	180 / 180 / 0	106 / 106 / 0	86 / 86 / 0	207
L18	*MSH2*	33.4 ± 12.5	19 ± 2.3	13.9 ± 1.9	21.8 ± 2.1	17.3 ± 1.8	14.8 ± 2.1	17.5 ± 2.9	15 ± 1.7	13.2 ± 1	179 / 179 / 0	105 / 105 / 0	85 / 85 / 0	214
L17	*MSH6*	53.2 ± 11.5	32.2 ± 5.8	24.2 ± 6.9	40.9 ± 7.3	28.6 ± 1.9	20.8 ± 5.8	30.6 ± 4.8	25.4 ± 5.7	20.4 ± 8	179 / 179 / 0	105 / 105 / 0	86 / 86 / 0	304
L19	*MSH6*	58 ± 16.8	42.6 ± 19	22.6 ± 9.7	49.8 ± 17	25.6 ± 6.9	14 ± 3.2	38.8 ± 10.6	26.2 ± 13.8	12.4 ± 2	179 / 179 / 0	106 / 106 / 0	85 / 85 / 0	203
C13.2	*MSH6*	47.2 ± 12.2	36.2 ± 9.9	24.9 ± 8.5	41.5 ± 16.4	28 ± 8	20.5 ± 2.8	34.6 ± 10.3	21.9 ± 4.3	19 ± 2.3	180 / 180 / 0	105 / 105 / 0	87 / 87 / 0	100
C09.2	*PMS2*	54.7 ± 16.9	20.9 ± 6.7	15.3 ± 2.2	30.2 ± 12.6	17.9 ± 5.1	15.3 ± 3.3	21 ± 5.2	15.4 ± 2.3	14.2 ± 3.2	181 / 181 / 0	105 / 105 / 0	87 / 87 / 0	120
L42	*PMS2*	28.4 ± 11.8	17.7 ± 3.5	15.4 ± 2.7	23.1 ± 6.5	16.6 ± 3.1	15.6 ± 2.7	17.5 ± 3.7	16.4 ± 3.3	15.2 ± 2.7	ND	ND	ND	ND
L4	*MLH1*	34.9 ± 16.2	19.4 ± 3.2	17.8 ± 2.7	26.1 ± 5.3	20.1 ± 3	16.1 ± 2.8	25.1 ± 11.5	18.7 ± 3.8	12.9 ± 1	ND	ND	ND	ND
L5	*MSH2*	33.1 ± 6.5	26 ± 4.8	20.4 ± 0.6	32.1 ± 5.5	25.8 ± 6.4	17.1 ± 0.9	36.5 ± 14.4	26.1 ± 5.5	19.5 ± 1.7	ND	ND	ND	ND
L7	*MSH2*	56.3 ± 16	27 ± 9.5	25 ± 7.8	51.4 ± 19.7	27.5 ± 13.6	18.8 ± 5.4	28.3 ± 11.4	18.6 ± 5.4	15.5 ± 3.6	ND	ND	ND	ND
L3	*MSH2*	53.3 ± 18.3	26.6 ± 8.8	19.2 ± 4.1	39.4 ± 10.2	23.3 ± 3.4	16.9 ± 4.1	26.6 ± 5.8	17.9 ± 4.8	14.9 ± 3.7	ND	ND	ND	ND
L34	*MSH6*	59.9 ± 15.3	43.3 ± 6.1	17.6 ± 3.2	53.9 ± 9.5	25.9 ± 9	11.6 ± 0.4	42.1 ± 9.9	16 ± 3.1	11.6 ± 0.7	ND	ND	ND	ND
L33	*MSH2*	26.9 ± 9	19.6 ± 6.4	19.2 ± 3.6	30 ± 13.1	19.8 ± 4.4	17.5 ± 2.7	21 ± 4	20.1 ± 4.7	16.6 ± 2	ND	ND	ND	ND
L29	*MSH2*	56 ± 9.4	30.1 ± 5	21.6 ± 3.1	34.9 ± 7.9	23.8 ± 2.1	20 ± 2	29.6 ± 6	21.8 ± 2.1	21.1 ± 1.5	ND	ND	ND	ND
L24	*MSH2*	21.9 ± 4.6	17.3 ± 2.7	16.4 ± 3.7	18.5 ± 3.3	16.7 ± 2.8	15.7 ± 3	17.8 ± 2.8	16.9 ± 2.7	15 ± 1.7	ND	ND	ND	ND
L23	*MSH2*	58.7 ± 13	31.4 ± 10.6	21.1 ± 4.3	43.5 ± 15.9	25.7 ± 5.8	19.8 ± 2.6	29.5 ± 8.1	21.9 ± 4.1	17 ± 1.1	ND	ND	ND	ND
L40	*PMS2*	67.5 ± 9	27 ± 7.6	19.8 ± 3.8	36.4 ± 8.7	19.4 ± 2.9	17.5 ± 2.9	21 ± 5.7	16.9 ± 3.6	14.4 ± 0.6	ND	ND	ND	ND
L41	*PMS2*	61.8 ± 7	24.2 ± 8.7	17.4 ± 3.7	31.1 ± 13.6	17.9 ± 4.2	13.8 ± 2.3	28.2 ± 12.1	14.9 ± 1.8	11.5 ± 2.3	ND	ND	ND	ND
L47	*MSH6*	70.9 ± 12.6	47.6 ± 15.5	30.3 ± 12.6	60.3 ± 15.8	34.4 ± 10.1	22.6 ± 5.5	39.2 ± 17.6	26 ± 8.3	17 ± 1.3	ND	ND	ND	ND
L21	*MSH2*	19.4 ± 7.6	12 ± 2.7	10.9 ± 1.2	13.2 ± 2.1	11.7 ± 0.9	11 ± 0.7	12.9 ± 2.2	11 ± 1	11.1 ± 0.7	ND	ND	ND	ND
L27	*MLH1*	12 ± 4.8	10.8 ± 4.5	10.1 ± 4.2	15.9 ± 6.4	14 ± 5	15.2 ± 5.5	9.8 ± 1.7	8.2 ± 1.7	8.8 ± 0.6	ND	ND	ND	ND

L50	*MSH6*	79.6 ± 14.5	58.2 ± 12.9	27.7 ± 5.1	72.9 ± 14.8	37.9 ± 10.6	18.7 ± 3.8	52.2 ± 18.4	24.6 ± 8.5	16.4 ± 3.7	ND	ND	ND	ND
L52	*MSH6*	68.5 ± 8.4	49.9 ± 12.4	31.4 ± 7	54.4 ± 10	38 ± 8.8	21.6 ± 3.7	41.2 ± 10.8	26.1 ± 6.8	17.5 ± 1.6	ND	ND	ND	ND
L53	*MSH6*	92.3 ± 9.5	72.5 ± 9.2	36 ± 13.5	83 ± 12.3	60.4 ± 10.1	23.3 ± 7.4	68 ± 10.1	34.7 ± 11.6	17.5 ± 5.6	ND	ND	ND	ND
L54	*MSH6*	82 ± 17.2	61.2 ± 14.2	24.5 ± 13.2	67.5 ± 12.1	32 ± 9.8	13.9 ± 3.8	31.9 ± 2.5	18.1 ± 5	11.7 ± 2	ND	ND	ND	ND
L55	*MSH6*	55.8 ± 16	39.5 ± 14.4	23.3 ± 2.8	44.2 ± 11.8	37.8 ± 14.8	20.6 ± 1.3	35.7 ± 8.7	31.5 ± 12.9	35 ± 17.2	ND	ND	ND	ND
L56	*MLH1*	21.7 ± 7.9	15.7 ± 2.7	12.5 ± 3.2	15.9 ± 2	15 ± 1.5	14 ± 0.5	16 ± 1.9	13.3 ± 1.4	12.9 ± 0.6	ND	ND	ND	ND
L28	*MLH1*	9.5 ± 0.9	9 ± 0.5	8.9 ± 1	10.3 ± 1	9.3 ± 1.1	9.6 ± 1.2	10 ± 0.9	9.3 ± 1.1	9.8 ± 1.2	ND	ND	ND	ND
L43	*MLH1*	13.7 ± 2.6	11.8 ± 1.5	11.3 ± 1.5	13 ± 1.5	12.5 ± 1.6	11.9 ± 1.5	12.5 ± 1.3	12.2 ± 1.3	11.8 ± 1.7	ND	ND	ND	ND
L44	*MLH1*	19.3 ± 15.6	10.6 ± 3.2	9.9 ± 2.1	12.1 ± 3.6	10.8 ± 2.3	10.3 ± 1.8	11.7 ± 2.8	10.6 ± 2	11.1 ± 2.9	ND	ND	ND	ND
L45	*MLH1*	18.3 ± 5.2	15.4 ± 6.7	10.4 ± 3.5	17.2 ± 6.7	12.1 ± 3.7	10.5 ± 3.1	12.4 ± 3.1	11.4 ± 3.9	10.6 ± 2.8	ND	ND	ND	ND
L46	*MLH1*	9.2 ± 2.8	8.5 ± 2.1	8.2 ± 1.7	9.5 ± 2.3	9.2 ± 1.8	8.9 ± 1.8	9.7 ± 2.5	9.9 ± 3.3	9.3 ± 1.6	ND	ND	ND	ND
L48	*MSH6*	70.2 ± 14.7	31.7 ± 7.9	21.4 ± 2	44.3 ± 14.9	21.7 ± 2.8	17.5 ± 2.6	27.8 ± 7.3	19.6 ± 4.8	14.7 ± 1.9	ND	ND	ND	ND
L49	*MSH6*	22.4 ± 1.8	21.2 ± 2	24.1 ± 10.4	21.3 ± 1.7	21.7 ± 2.2	20.1 ± 1.4	21.6 ± 1	21.5 ± 1.2	20.6 ± 2	ND	ND	ND	ND
L51	*MSH6*	54.8 ± 11	33.7 ± 9.3	22.4 ± 4.3	43.9 ± 14.8	25.5 ± 6.1	21.6 ± 5.2	34.5 ± 10	22.3 ± 5.4	19.6 ± 5.2	ND	ND	ND	ND

Supplementary Table 2. Raw data relative to methylation tolerance and *ev*MSI tests in all patients analyzed in the study

[a] For each MNNG condition, mean cell survival (%) ± standard deviation are indicated.

[b] The size (in base pairs) of each marker is indicated in peripheral blood lymphocytes, in immortalized lymphocytes, along with the difference between the two (*i.e*. deletion size) at the indicated culture time (in days). For the cell lines displaying a shift in allele size, the shortest culture time showing *ev*MSI is indicated, whereas for the cell lines displaying stable allele profiles, the longest culture time is indicated. Culture time was calculated from the day of lymphoblast immortalization. Since peripheral blood lymphocytes were not available for CMMRD patient C01.2, comparison of the allele size was performed with primary blood lymphocytes from the father.

ND, not done; X, unknown.

Patient	Mutated gene	gMSI ratio			
		D17S791	D2S123	D17S250	Result
CMMRD patients					
C01.1	*PMS2*	0.58 ± 0.012	0.37 ± 0.037	0.10 ± 0.006	positive
C02	*PMS2*	NA	NA	NA	NI
C04	*PMS2*	0.85 ± 0.007	0.48 ± 0.008	NA	positive
C05	*PMS2*	NA	0.21 ± 0.071	0.77 ± 0.086	positive
C06	*PMS2*	0.40 ± 0.040	0.25 ± 0.015	0.23 ± 0.012	positive
C07	*PMS2*	0.45 ± 0.016	0.14 ± 0.005	0.13 ± 0.009	positive
C08	*PMS2*	0.45 ± 0.013	0.26 ± 0.021	0.08 ± 0.009	positive
C09.1	*PMS2*	NA	0.14 ± 0.015	0.11 ± 0.007	positive
C10	*PMS2*	0.58 ± 0.034	NA	0.33 ± 0.023	positive
C12	*MSH6*	NA	0.00 ± 0.000	0.04 ± 0.002	negative
C13.1	*MSH6*	0.04 ± 0.034	0.03 ± 0.023	0.02 ± 0.014	negative
C14	*MSH6*	0.12 ± 0.005	NA	NA	NI
C15	*MLH1*	0.94 ± 0.012	NA	0.29 ± 0.011	positive
C03.1	*PMS2*	0.44 ± 0.016	0.35 ± 0.023	0.17 ± 0.016	positive
C03.2	*PMS2*	0.36 ± 0.018	0.00 ± 0.000	0.28 ± 0.004	positive
C11	*PMS2*	0.38 ± 0.018	0.19 ± 0.007	0.14 ± 0.029	positive
C16	*MLH1*	0.28 ± 0.025	0.09 ± 0.006	NA	NI
C17	*MSH2*	0.21 ± 0.016	0.16 ± 0.006	0.07 ± 0.008	positive
Patients at-risk for CMMRD					
C20.1	*MSH6*	0.09 ± 0.005	0.03 ± 0.002	NA	negative
C20.2	*MSH6*	0.09 ± 0.005	0.04 ± 0.005	NA	negative
C18	*PMS2*	NA	0.05 ± 0.003	0.04 ± 0.002	negative
C27	*MLH1*	0.08 ± 0.001	0.00 ± 0.000	0.02 ± 0.001	negative
C25	*PMS2*	0.06 ± 0.004	0.03 ± 0.001	0.01 ± 0.013	negative
C22	*MSH6*	0.09 ± 0.009	0.03 ± 0.027	0.04 ± 0.005	negative
C24	*MSH2*	0.07 ± 0.002	0.03 ± 0.002	0.01 ± 0.014	negative
C30	*MSH2*	0.05 ± 0.002	NA	0.04 ± 0.010	negative
C19	*PMS2*	0.24 ± 0.015	0.02 ± 0.028	NA	NI
C21	*MSH6*	0.02 ± 0.034	0.00 ± 0.000	0.02 ± 0.017	negative
C28	*MLH1*	0.08 ± 0.002	0.01 ± 0.018	0.04 ± 0.006	negative
C26	*PMS2*	NA	0.00 ± 0.000	0.02 ± 0.002	negative
C23	*MSH6*	0.09 ± 0.006	0.06 ± 0.007	0.04 ± 0.005	negative
C29.1	*PMS2*	0.41 ± 0.049	0.18 ± 0.021	0.29 ± 0.008	positive
C29.2	-	0.02 ± 0.041	0.01 ± 0.019	0.02 ± 0.001	negative
C31	-	0.08 ± 0.003	0.00 ± 0.000	0.04 ± 0.001	negative
C32	-	0.07 ± 0.005	0.03 ± 0.003	NA	negative
C33	-	NA	NA	NA	NI
C34.1	-	0.05 ± 0.043	0.03 ± 0.001	0.00 ± 0.000	negative
C34.2	-	0.05 ± 0.027	0.00 ± 0.000	0.03 ± 0.046	negative
C35	-	0.00 ± 0.000	0.00 ± 0.000	0.02 ± 0.015	negative
C36	-	0.05 ± 0.046	0.01 ± 0.016	NA	negative
C37	-	0.06 ± 0.011	0.01 ± 0.017	NA	negative

Supplementary Table 3. Data set relative to gMSI testing in patients analyzed in the study

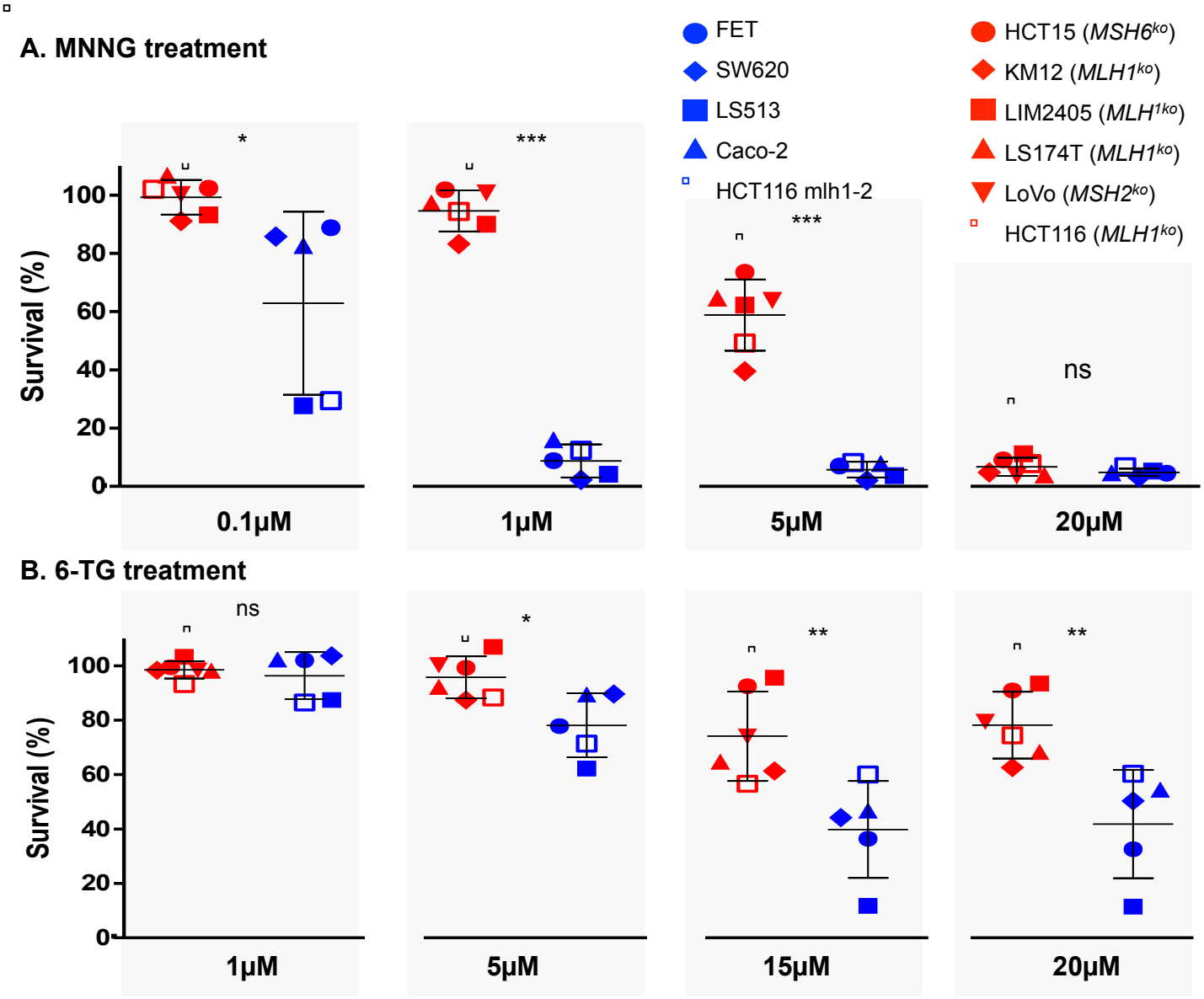
Test result is positive (*i.e.* abnormal) when the gMSI ratios of at least 2 markers are above the cut-off value, and negative (*i.e*. normal) when the gMSI ratios of at least 2 markers are below the cut-off value.
NI, not interpretable; NA, not applicable because of heterozygous markers with alleles closer than 6 base pairs.

	sensitivity % (95% CI)	specificity % (95% CI)	NPV % (95% CI)	PPV % (95% CI)
Patients and controls with available data n=56				
Sequencing of MMR genes	80.1 (54.1 - 99.0)	97.6 (91.2 - 99.9)	91.2 (76.6 - 99.6)	93.6 (77.9 - 99.8)
ev MSI and methylation tolerance	94.2 (79.4 - 99.9)	90.1 (76.1 - 99.5)	97.2 (89.8 - 99.9)	80.5 (53.9 - 99.0)
Patients and controls with available data n=40				
Sequencing of MMR genes	75.5 (49.3 - 96.5)	96.4 (87.0 - 99.9)	87.3 (70.2 - 98.5)	92.1 (72.8 - 99.9)
ev MSI and methylation tolerance	93.3 (76.7 - 99.8)	89.3 (72.5 - 99.5)	96.0 (85.6 - 99.9)	82.6 (55.3 - 99.2)
gMSI test	68.7 (42.6 - 91.0)	96.2 (86.8 - 99.9)	84.4 (67.5 - 96.4)	90.8 (69.0 - 99.8)

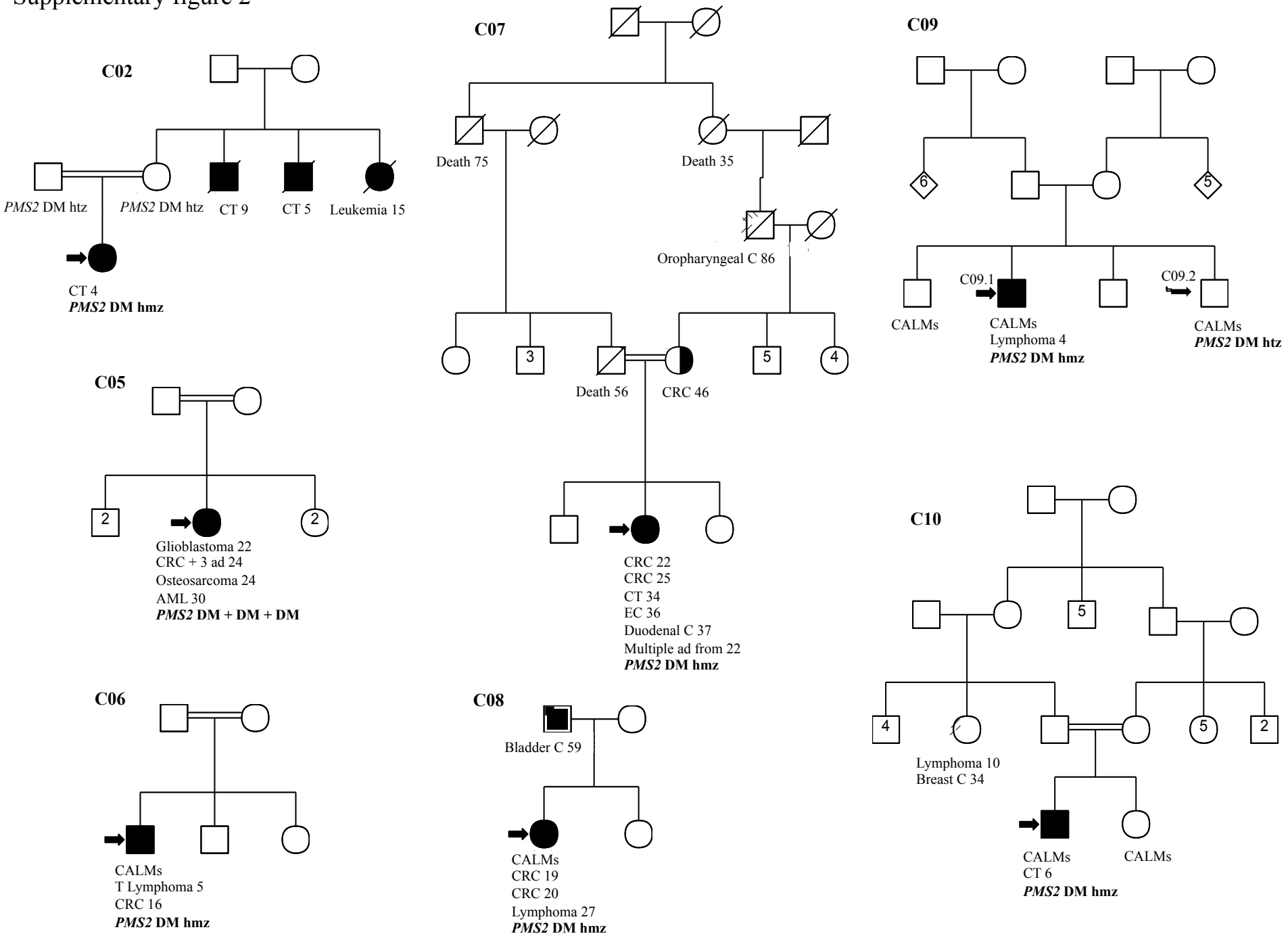
Supplementary table 4. Estimate of the sensitivity, specificity, negative and positive predictive values of the different tests for CMMRD diagnosis. *Ev* MSI and methylation tolerance assays were first compared to the standard diagnostic method, *i. e* . sequencing of the MMR genes (56 patients with available data), then the 3 tests under investigation were further compared (40 patients with available data).

CI, confidence interval; NPV, negative predictive value; PPV; positive predictive value.

Supplementary Figure 1



Supplementary figure 2



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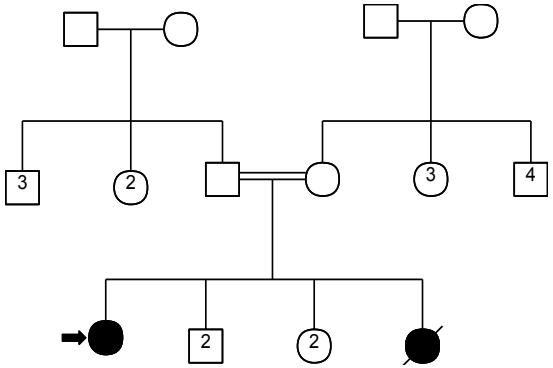

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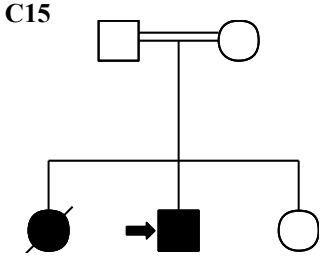

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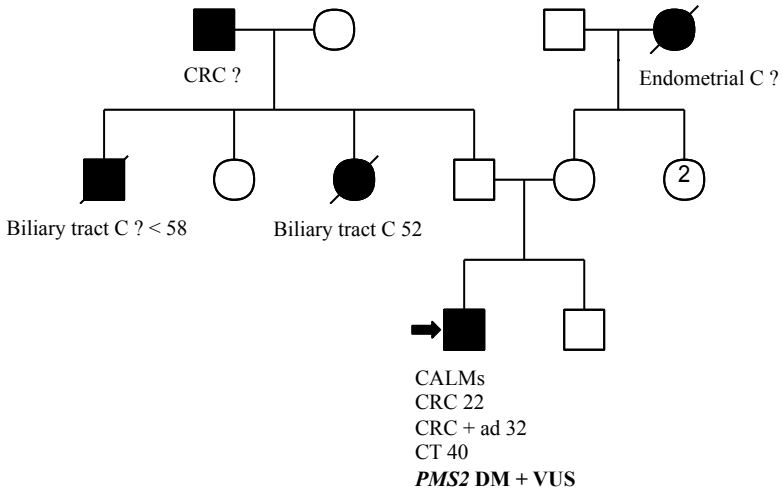

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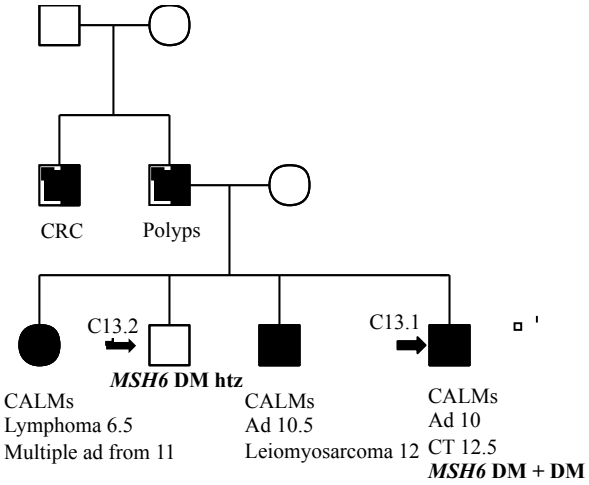

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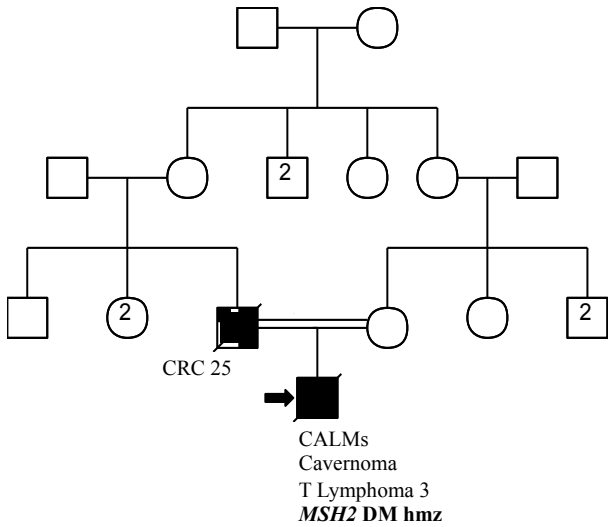

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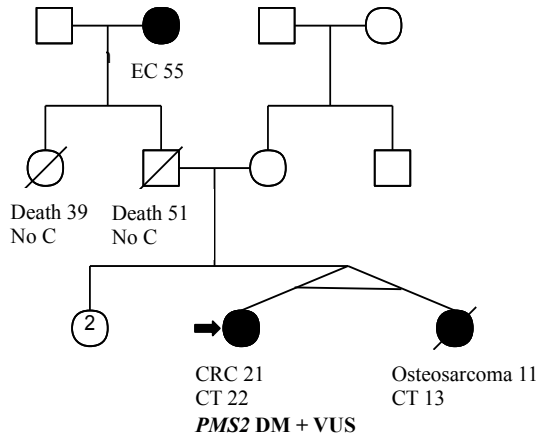

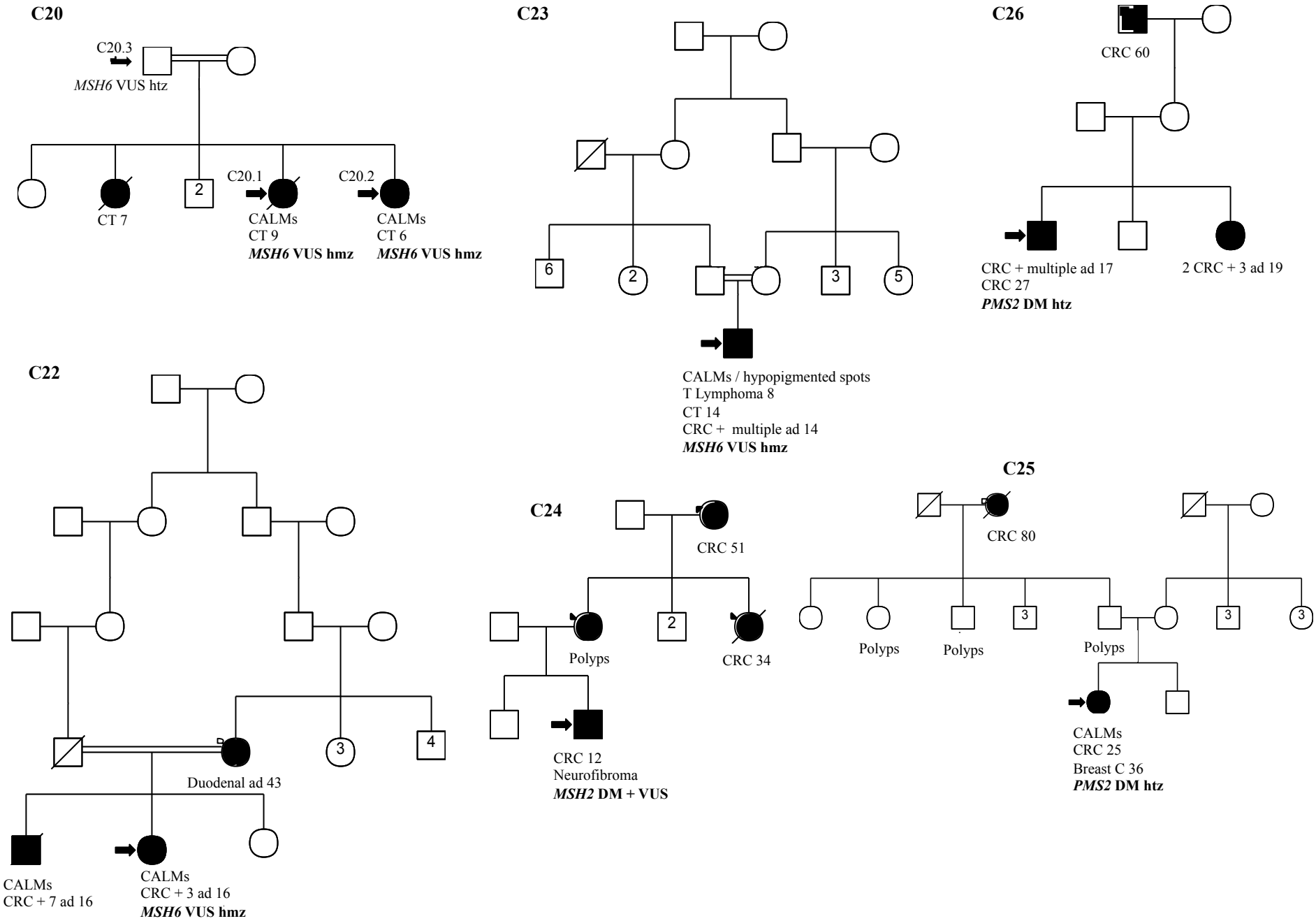
C20
C20.3
MSH6 VUS htz
2
C20.1
C20.2
CT 7
CALMs
CT 9
MSH6 VUS hmz
CALMs
CT 6
MSH6 VUS hmz
C23
6
2
3
5
CALMs / hypopigmented spots
T Lymphoma 8
CT 14
CRC + multiple ad 14
MSH6 VUS hmz
C26
CRC 60
CRC + multiple ad 17
CRC 27
PMS2 DM htz
2 CRC + 3 ad 19
C22
3
4
Duodenal ad 43
CALMs
CRC + 7 ad 16
CALMs
CRC + 3 ad 16
MSH6 VUS hmz
C24
CRC 51
2
Polyps
CRC 34
CRC 12
Neurofibroma
MSH2 DM + VUS
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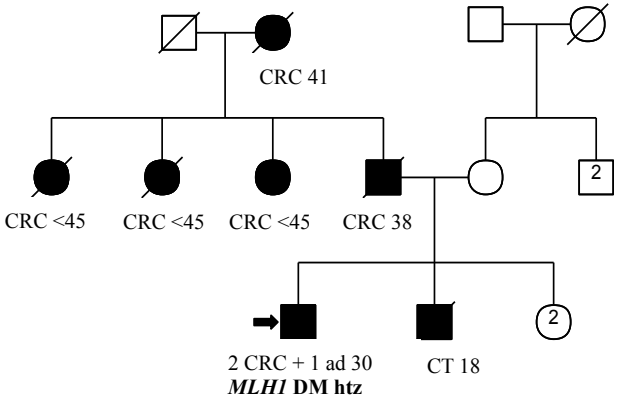
Polyps
Polyps
Polyps
3
3
CALMs
CRC 25
Breast C 36
PMS2 DM htz

C27



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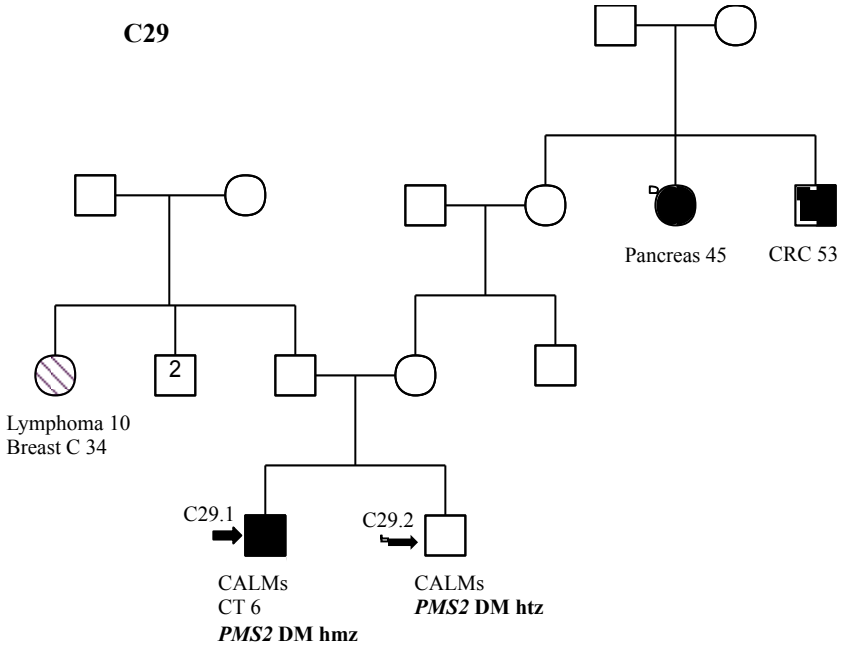

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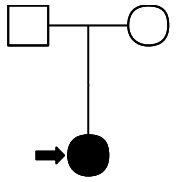

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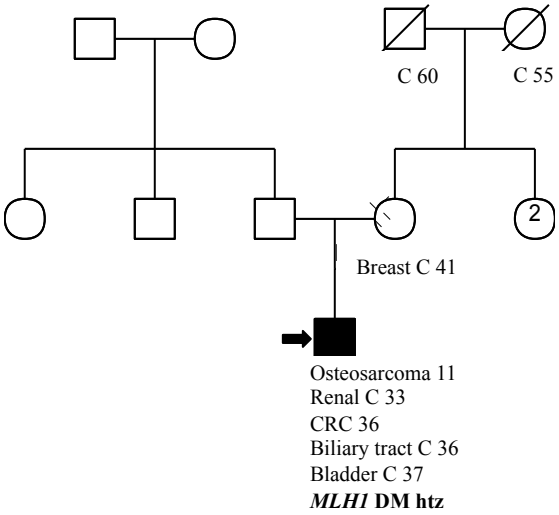

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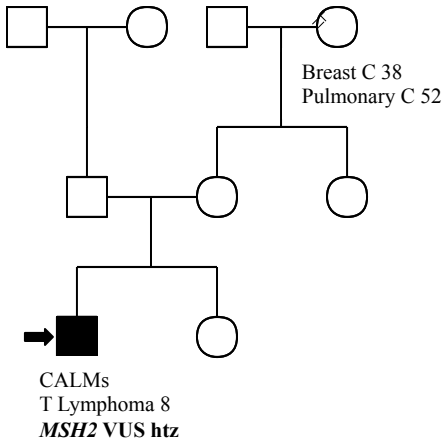

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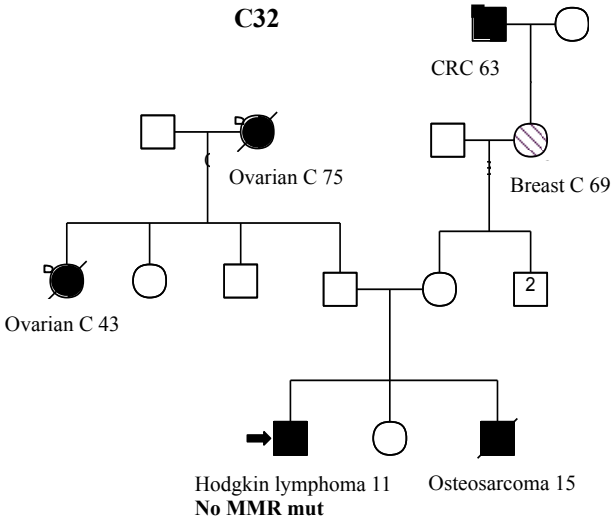

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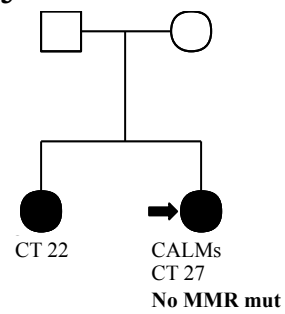

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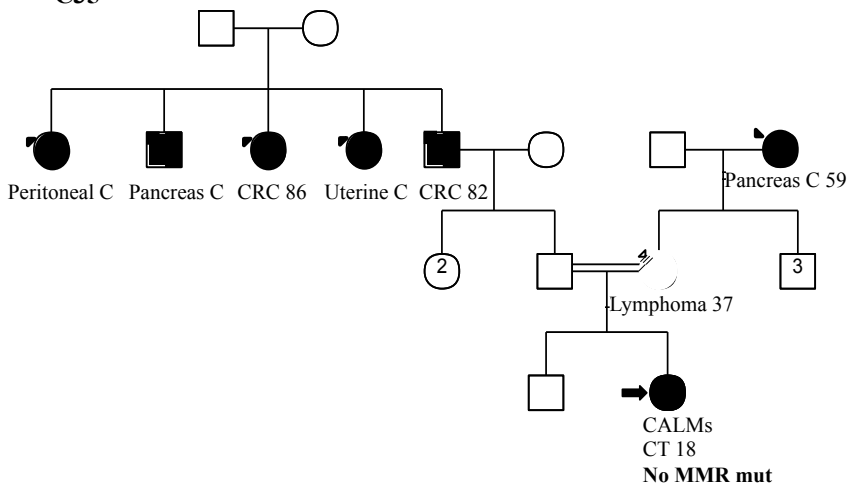

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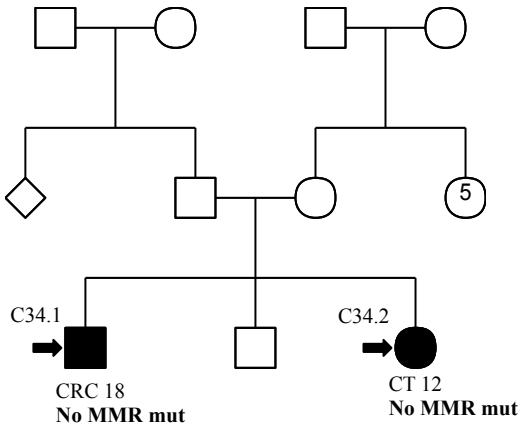

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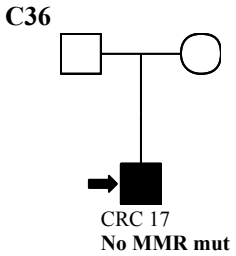

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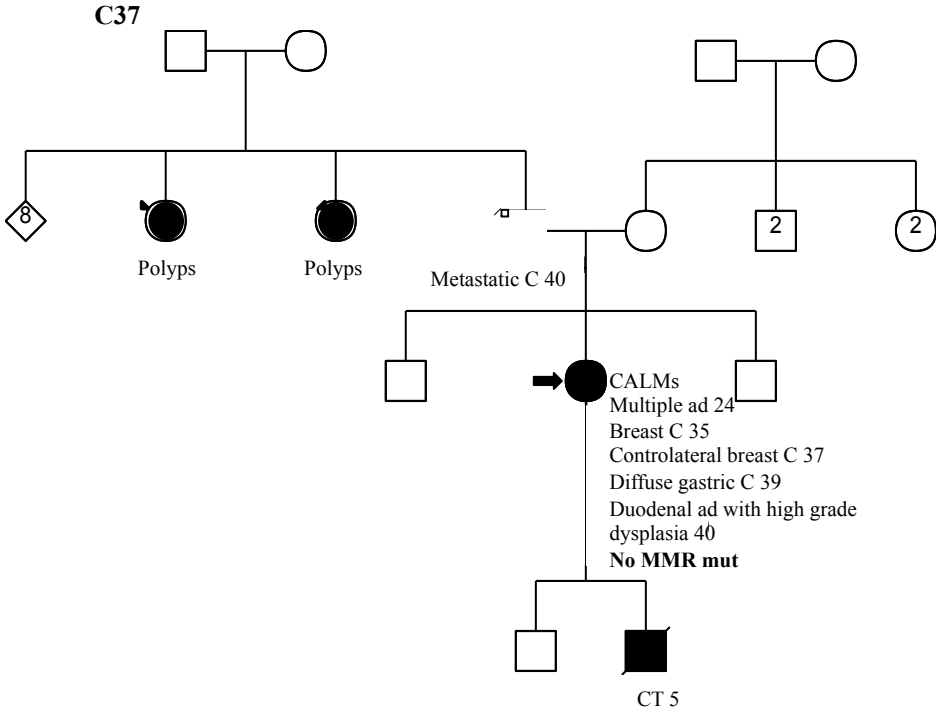
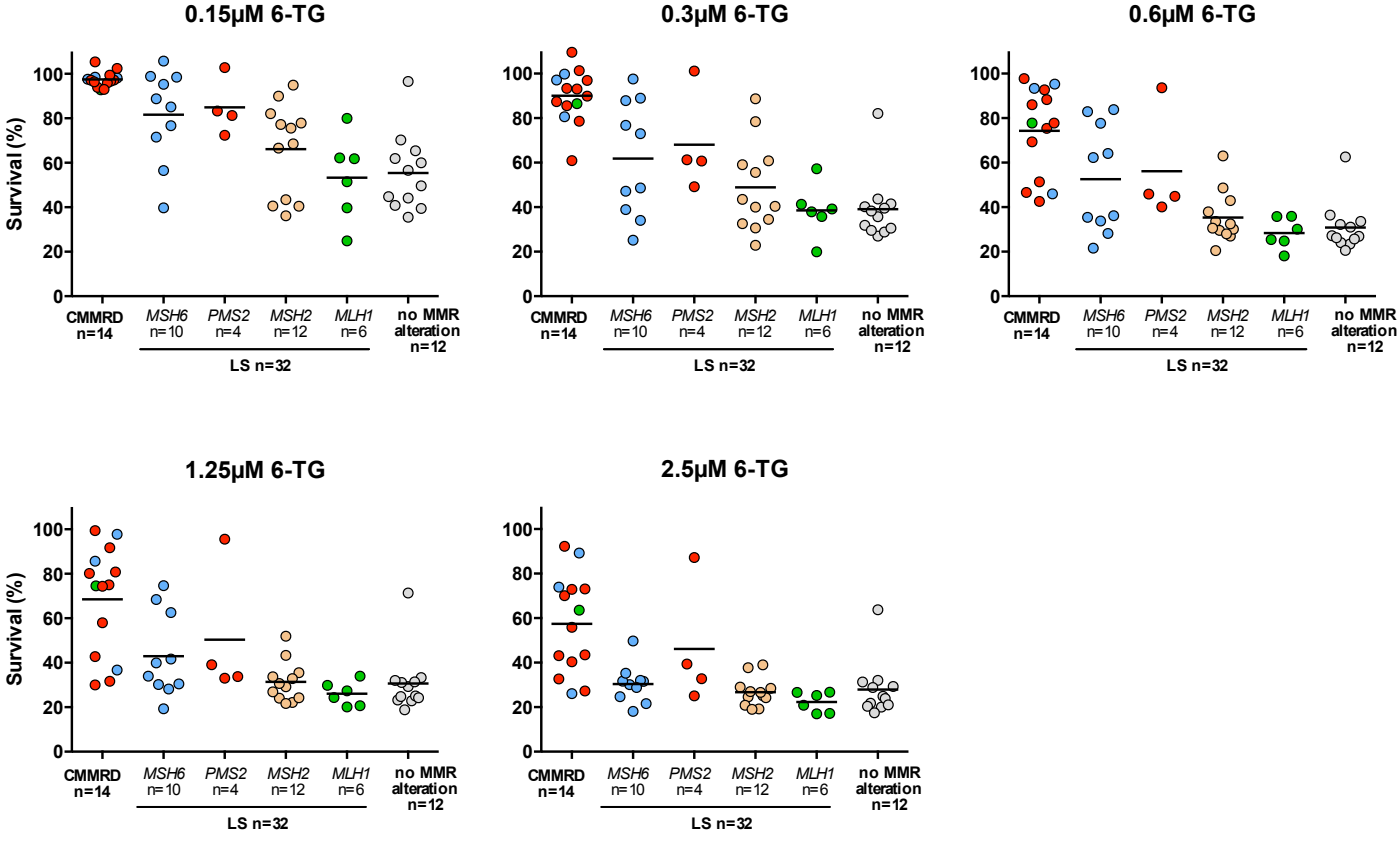
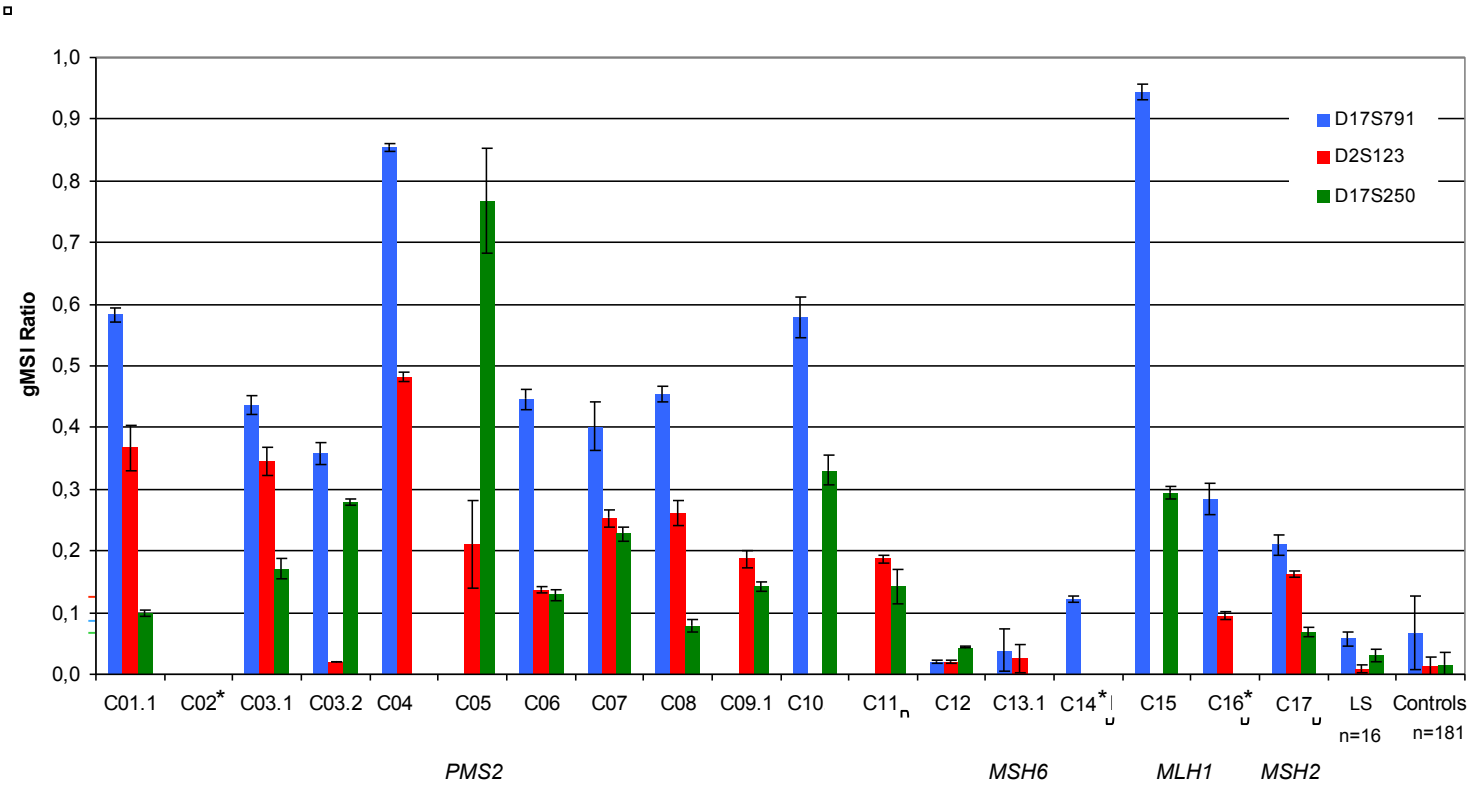


Supplementary Figure 3





Supplementary figure 4