



Article

***BRAF* and *MLH1* Analysis Algorithm for the Evaluation of Lynch Syndrome Risk in Colorectal Carcinoma Patients: Evidence-Based Data from the Analysis of 100 Consecutive Cases**

Thais Maloberti ^{1,2,†} , Antonio De Leo ^{1,2,†} , Viviana Sanza ², Lidia Merlo ², Michela Visani ¹ , Giorgia Acquaviva ¹, Sara Coluccelli ^{1,2} , Annalisa Altimari ^{2,3}, Elisa Gruppioni ^{2,3}, Stefano Zagnoni ^{2,3}, Daniela Turchetti ⁴, Sara Miccoli ⁴, Michelangelo Fiorentino ^{5,6} , Antonietta D'Errico ³ , Dario de Biase ^{7,*} and Giovanni Tallini ^{1,2,‡}



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- ¹ Department of Experimental, Diagnostic and Specialty Medicine, Anatomic Pathology Unit-University of Bologna Medical Center, 40138 Bologna, Italy; thais.maloberti2@unibo.it (T.M.); antonio.deleo@unibo.it (A.D.L.); michela.visani@unibo.it (M.V.); giorgiacquaviva85@gmail.com (G.A.); sara.coluccelli2@unibo.it (S.C.); giovanni.tallini@unibo.it (G.T.)
 - ² Solid Tumor Molecular Pathology Laboratory, IRCCS Azienda Ospedaliero-Universitaria di Bologna, 40138 Bologna, Italy; viviana.sanza@ausl.bo.it (V.S.); lidia.merlo@ausl.bo.it (L.M.); annalisa.altimari@aosp.bo.it (A.A.); elisa.gruppioni@aosp.bo.it (E.G.); stefano.zagnoni@aosp.bo.it (S.Z.)
 - ³ Department of Pathology, IRCCS Azienda Ospedaliero-Universitaria di Bologna, 40138 Bologna, Italy; antonietta.derrico@unibo.it
 - ⁴ Unit of Medical Genetics, IRCCS (Istituto di Ricovero e Cura a Carattere Scientifico), Azienda Ospedaliero-Universitaria di Bologna, 40138 Bologna, Italy; daniela.turchetti@unibo.it (D.T.); sara.miccoli@studio.unibo.it (S.M.)
 - ⁵ Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, 40138 Bologna, Italy; michelangelo.fiorentino@unibo.it
 - ⁶ Pathology Department, Maggiore Hospital, 40133 Bologna, Italy
 - ⁷ Department of Pharmacy and Biotechnology, University of Bologna, 40126 Bologna, Italy
- * Correspondence: dario.debiase@unibo.it
† These authors contributed equally to this work.
‡ These authors contributed equally to this work.

Abstract: Several causes may lead to CRC, either extrinsic (sporadic forms) or genetic (hereditary forms), such as Lynch syndrome (LS). Most sporadic deficient mismatch repair (dMMR) CRC cases are characterized by the methylation of the *MLH1* promoter gene and/or *BRAF* gene mutations. Usually, the first test performed is the mismatch repair deficiency analysis. If a tumor shows a dMMR, *BRAF* mutations and then the *MLH1* promoter methylation status have to be assessed, according to the ACG/ASCO screening algorithm. In this study, 100 consecutive formalin-fixed and paraffin-embedded samples of dMMR CRC were analyzed for both *BRAF* mutations and *MLH1* promoter methylation. A total of 47 (47%) samples were *BRAF* p.V600E mutated, while *MLH1* promoter methylation was found in 77 cases (77.0%). The pipeline “*BRAF*-followed-by-*MLH1*-analysis” led to a total of 153 tests, while the sequence “*MLH1*-followed-by-*BRAF*-analysis” resulted in a total of 123 tests. This study highlights the importance of performing *MLH1* analysis in LS screening of *BRAF*-WT specimens before addressing patients to genetic counseling. We show that *MLH1* analysis performs better as a first-line test in the screening of patients with LS risk than first-line *BRAF* analysis. Our data indicate that analyzing *MLH1* methylation as a first-line test is more cost-effective.

Keywords: *MLH1*; *BRAF*; colorectal cancer; Lynch syndrome; mismatch repair; microsatellite instability

1. Introduction

Colorectal cancer (CRC) is the third leading cause of cancer death in the world and its incidence is increasing in many countries [1–4] (Figure 1).

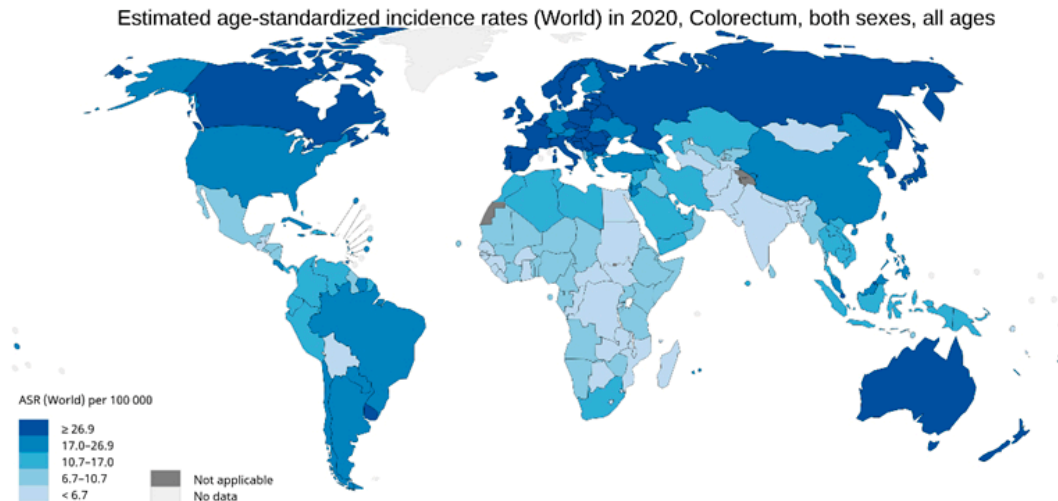


Figure 1. Estimated age-standardized incidence rates (World) in 2020, colorectal carcinoma, both sexes, all ages. Available at: <https://gco.iarc.fr/today> (accessed on 17 December 2021) [2–4].

In the US, 149,500 new cases and 52,980 deaths were estimated in 2021 [5]. Several causes may lead to CRC, both extrinsic (sporadic forms) and genetic (hereditary forms), such as Lynch syndrome—previously called hereditary non-polyposis colorectal cancer (HNPCC). Lynch syndrome is one of the most common hereditary cancer predisposition syndromes and is defined by germline alterations in the DNA mismatch repair (MMR) system [6].

During the replication processes, mutations may occur frequently and some (but not all of them) are corrected by the proofreading function of the DNA polymerases. By decreasing the number of spontaneous mutations, the MMR system increases the overall fidelity of replication [7]. The MMR system is composed of several proteins, including MSH2, MSH6, MLH1, and PMS2.

A heterozygous alteration in one of these genes is insufficient to give a mismatch repair deficiency (dMMR). Inactivating mutations in one of the MMR genes or 3'-deletion of EPCAM (the epithelial-cell adhesion molecule gene) lead to the malfunction of repair mechanisms and then to genomic instability. MMRd is evident mainly in microsatellite regions, short tandem repeats of nucleotides particularly susceptible to DNA polymerase slippage [8–11].

The National Comprehensive Cancer Network (NCCN) 2019 guidelines recommended deficient mismatch repair (dMMR) analysis to be performed in patients with CRC, for clinical management and to identify Lynch syndrome [12]. In fact, dMMR/high-microsatellite instability (MSI-H) in somatic tumor cells is associated with better outcomes in CRC patients. Whether 5-FU adjuvant chemotherapy is appropriate to treat MSI-H/dMMR tumors is still controversial [13–15]. Cases with inactivated *MLH1* gene due to promoter methylation represents approximately 90% of sporadic dMMR/MSI-H CRC [16,17]. *BRAF* mutations rarely occur in the presence of germline mutations in MMR genes (1.6%), while the mutation is present in approximately 50% of sporadic CRC [18,19]. It is then clear that the absence of oncogenic *BRAF* variants in CRC is not sufficient to suspect Lynch syndrome. Moreover, some patients with Lynch syndrome may have a sporadic form of CRC, thus *BRAF* mutation analysis alone is not sufficient to discriminate between hereditary (Lynch syndrome) and sporadic dMMR/MSI-H CRC [6,20].

A screening algorithm is recommended for all CRC specimens to select patients for germline testing to diagnose Lynch syndrome (American College of Gastroenterology, and

the American Society of Clinical Oncology (2015)) [21–23]. The first step consists of dMMR analysis, usually performed with immunohistochemistry (IHC) for MLH1, MSH2, MSH6, and PMS2 proteins. Positive immunostaining for all proteins is compatible with an intact MMR system, and no additional analyses are needed. Samples with immunohistochemical loss of MMR proteins must then be analyzed for the *BRAF* mutation. The ACG/ASCO screening algorithm suggests performing *BRAF* analysis in CRC with the absence of *MLH1* protein staining, followed by *MLH1* methylation analysis if *BRAF* p.V600E mutation is not detected [21] (Figure 2).

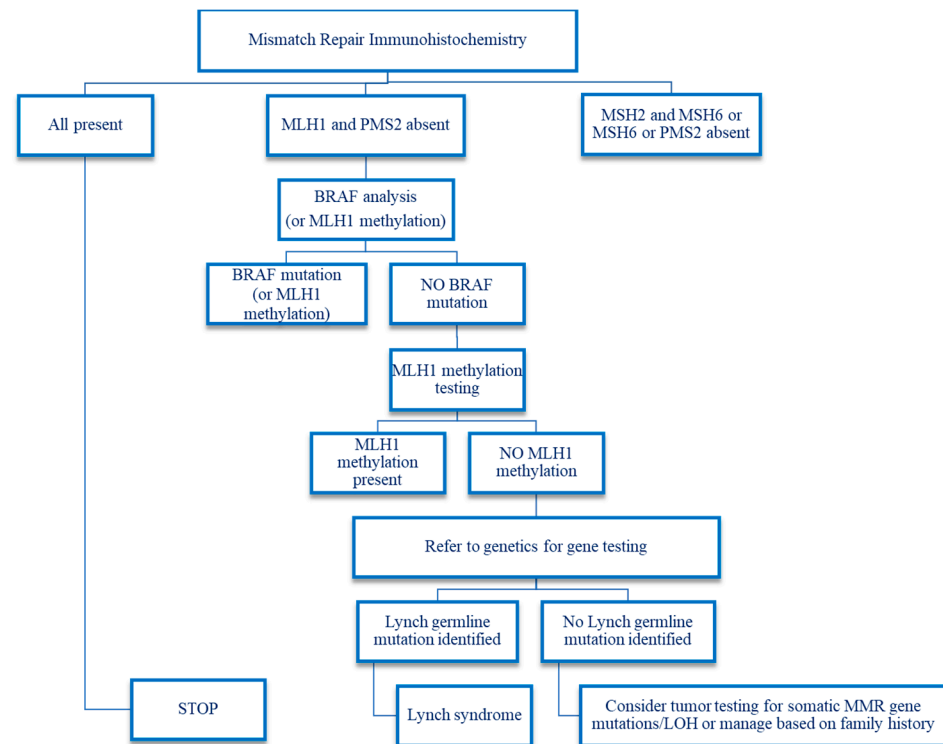


Figure 2. Algorithm for *MLH1* methylation and *BRAF* analyses in dMMR/MSI-H CRC. Modified from Chen W. et al. [24].

If an oncogenic *BRAF* mutation is identified, the tumor is considered sporadic. If no *BRAF* mutations are identified, *MLH1* promoter methylation analysis must be performed to infer whether the lack of *MLH1* protein expression is due to epigenetic causes (a somatic event) pointing to the sporadic origin of the tumor or putative inactivating mutations (a genetic event) pointing to Lynch syndrome [24].

If neither oncogenic *BRAF* mutations nor *MLH1* promoter methylation are found, genetic counseling and germline MMR gene analysis are recommended to diagnose Lynch syndrome.

While the ACG/ASCO screening algorithm is widely accepted, it is not clear whether the order in which *BRAF* and *MLH1* promoter methylation are tested makes any difference. The aim of this study was to define whether it is more cost-effective to analyze dMMR/MSI-H CRC first for *BRAF* and then for *MLH1* promoter methylation or vice versa, first for *MLH1* promoter methylation and then for *BRAF* [21].

2. Materials and Methods

2.1. Case Selection

A total of 100 consecutive formalin-fixed and paraffin-embedded (FFPE) samples of colorectal carcinoma (CRC) with a deficient mismatch repair (dMMR) system, with loss of *MLH1*/*PMS2*, were analyzed for *BRAF* and *MLH1* molecular status during the period 2018–2021 at the solid tumor molecular pathology laboratory of the University of Bologna

medical center. The MMR status of all cases had been previously evaluated by IHC for MSH2, MSH6, MLH1, and PMS2 using an automatic immunostainer (BenchMark ULTRA System—Roche Diagnostics, Mannheim, Germany), following established guidelines [25].

2.2. DNA Extraction and Quantification

DNA was extracted from 2–4 unstained 10 µm thick sections. The area of interest was selected by a pathologist on a final hematoxylin and eosin (H&E) section. DNA extraction was performed using the QuickExtract FFPE DNA Extraction Kit (Epicentre, Madison, WI, USA), according to the manufacturer's instructions. DNA concentration was calculated using the Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. BRAF Analysis

BRAF analysis was performed using a laboratory-developed next-generation sequencing (NGS) panel designed to analyze common hot-spot mutations in solid tumors, commercially available real-time PCR, or pyrosequencing kits for *BRAF* analysis, as follows.

NGS was performed using approximately 50 ng of input DNA, and libraries were set up using a laboratory-developed next-generation multi-gene panel as previously described [26]. The entire *BRAF* exons 11 and 15 were sequenced using this multi-gene panel. The analysis was performed with the Gene Studio S5 sequencer (Thermo Fisher Scientific). The sequences were evaluated using the Ion Reporter tools (v5.16, Thermo Fisher Scientific) and the Integrative Genome Viewer tool (v5.11.2, <https://software.broadinstitute.org/software/igv/>, accessed on 7 January 2021).

Real-time PCR was performed using the EasyPGX *BRAF* kit (Diatech pharmacogenetics, Jesi, AN, Italy), according to the manufacturer's protocol and results analyzed using the EasyPGX Analysis Software (v4.0.5, Diatech pharmacogenetics, Jesi, Italy). Briefly, approximately 20 ng of input DNA was used in each one of the 4 reactions, allowing the detection of 4 different mutations in *BRAF* codon 600: p.V600E (c.1799T>A, c.1799_1800delinsAA), p.V600K (c.1798_1799delinsAA), p.V600D/p.V600R (c.1799_1800delinsAT, c.1798_1799delinsAG—not distinguishable between them).

Pyrosequencing was performed using a *BRAF* pyro kit, allowing the detection of mutations in codons 599, 600, and 601 of the *BRAF* gene. A minimum input of 10ng was used to perform the analysis. The results were analyzed by the PyroMark Q24 software. The pyrosequencing analysis was run on the PyroMark Q24 system. The analytical sensitivity was 2–11% of mutated DNA on wild-type (WT) genomic DNA, according to the detected mutation.

2.4. MLH1 Promoter Methylation Analysis

MLH1 promoter methylation was evaluated with the PyroMark[®] Q24 CpG *MLH1* kit (Qiagen). DNA was converted using the EZ DNA Methylation-Lightning kit (Zymo Research Corporation). Pyrosequencing was run on the PyroMark Q24 system. Results were analyzed by the PyroMark Q24 software. The PyroMark[®] Q24 CpG *MLH1* kit allows the detection of the methylation status of 5 CpG islands in the *MLH1* promoter region, from nucleotide c.1-209 to c.1-181. A sample was considered methylated when the percentage of methylation exceeded 10%, according to the manufacturer's protocol.

3. Results

All 100 samples were successfully analyzed for *BRAF* and *MLH1* promoter methylation.

3.1. BRAF Analysis

BRAF mutations were found in 47 of 100 cases (47.0%), all were *BRAF* p.V600E substitutions (Table 1). No *BRAF* mutations other than p.V600E were identified. Interestingly, all *BRAF*-mutated specimens (47/47, 100%) were also *MLH1* methylated (MET), while no samples were *BRAF* mutated (MUT)/*MLH1* unmethylated (UMET) (Table 1, Figure 3). Of the 53 cases without the *BRAF* mutation, 30 of them (56.6%) were *MLH1* promoter

methylated, while the remaining 23 cases (43.4%) were *BRAF* WT/*MLH1* UMET (Table 1, Figure 3).

Table 1. Molecular profile according to *BRAF* mutational status.

dMMR/MSI-H Samples	N° (Frequency)
<i>BRAF</i> MUT	47 (47.0%)
<i>BRAF</i> MUT & <i>MLH1</i> MET	47 (100.0%)
<i>BRAF</i> MUT & <i>MLH1</i> UMET	0 (/)
<i>BRAF</i> WT	53 (53.0%)
<i>BRAF</i> WT & <i>MLH1</i> MET	30 (56.6%)
<i>BRAF</i> WT & <i>MLH1</i> UMET	23 (43.4%)
TOTAL	100

MUT: Mutated; WT: Wild-type; MET: Methylated; UMET: Unmethylated; *BRAF* MUT: *BRAF* mutated—all these cases were *BRAF* p.V600E.

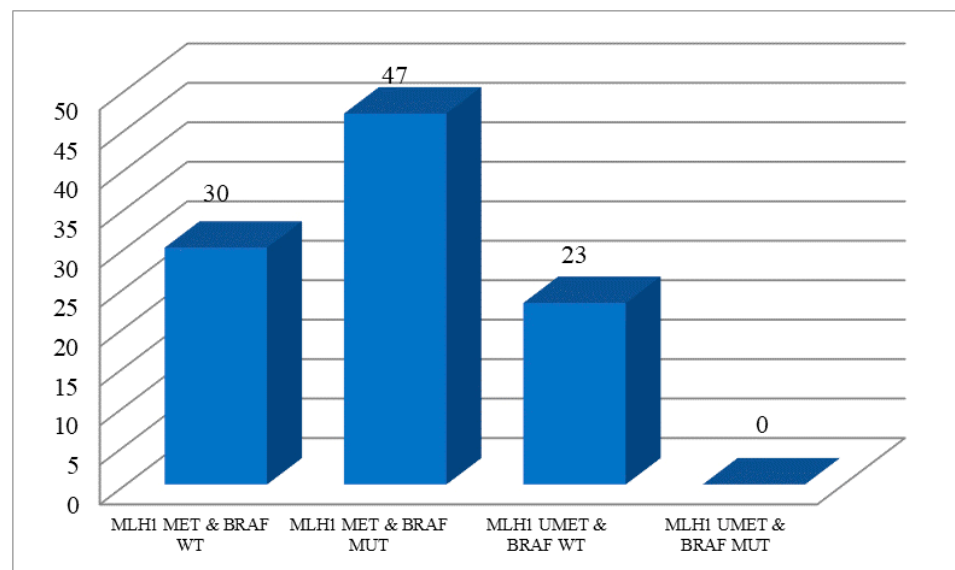


Figure 3. Number of samples according to *BRAF* and *MLH1* molecular status. MET: Methylated; UMET: Unmethylated; WT: Wild-type; MUT: Mutated.

3.2. *MLH1* Methylation Analysis

The *MLH1* promoter region was found methylated in 77 of 100 samples (77.0%) (Table 2). Of these 77 methylated specimens, 30 (39.0%) also harbored a *BRAF* p.V600E mutation (*MLH1* MET/*BRAF* MUT), while the remaining 47 specimens (61.0%) were *MLH1* MET/*BRAF* WT (Table 2). All the 23 *MLH1* unmethylated specimens were also *BRAF* WT (*MLH1* UMET/*BRAF* WT), thus no *MLH1* UMET/*BRAF* MUT specimens were identified (Table 2, Figure 3). Of these 23 patients, 9 were tested for germline mutations, and 5 harbored a pathogenic alteration suggestive of Lynch syndrome.

Table 2. Molecular profile according to *MLH1* methylation status.

dMMR/MSI-H Samples	N° (Frequency)
<i>MLH1</i> MET	77 (77.0%)
<i>MLH1</i> MET & <i>BRAF</i> WT	30 (39.0%)
<i>MLH1</i> MET & <i>BRAF</i> MUT	47 (61%)
<i>MLH1</i> UMET	23 (23.0%)
<i>MLH1</i> UMET & <i>BRAF</i> WT	23 (100%)
<i>MLH1</i> UMET & <i>BRAF</i> MUT	0 (/)
TOTAL	100

MUT: Mutated; WT: Wild-type; MET: Methylated; UMET: Unmethylated; *BRAF* MUT: *BRAF* mutated—all these cases were *BRAF* p.V600E.

Following the ACG/ASCO algorithm, currently used to screen for Lynch syndrome dMMR/MSI-H CRC patients, 53 of 100 (53.0%) specimens (i.e., samples without the *BRAF* mutation) would have needed a “second-level” analysis for *MLH1* promoter methylation, for a total of 153 molecular tests (100 samples analyzed for *BRAF* and 53 analyzed also for *MLH1* methylation status) (Figure 4). On the contrary, starting the dMMR/MSI-H CRC analysis with an evaluation of the *MLH1* methylation status would lead to performing a “second-level” analysis for the *BRAF* alteration in only 23 of 100 (23.0%) samples (i.e., those samples without *MLH1* promoter methylation), for a total of 123 molecular tests (100 samples analyzed for *MLH1* methylation status and 23 specimens analyzed also for *BRAF* mutational status) (Figure 4).

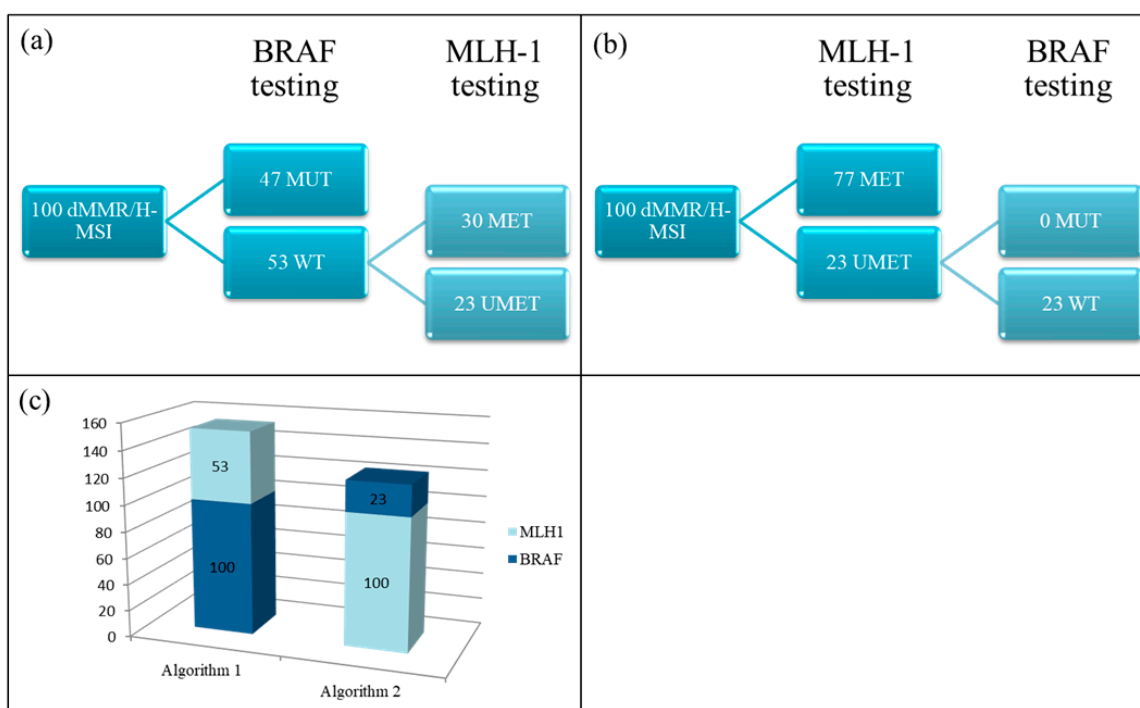


Figure 4. Comparison of the total number of molecular tests for dMMR/MSI-H CRC: (a) first *BRAF* and then *MLH1* methylation analysis (Algorithm 1) vs. (b) performing first *MLH1* methylation and then *BRAF* analysis (Algorithm 2); (c) total number of tests following Algorithm 1 and Algorithm 2. MET: Methylated; MUT: Mutated; WT: Wild-type; UMET: Unmethylated; *MLH1*: *MLH1* analyses; *BRAF*: *BRAF* analyses.

4. Discussion

Considering the clinical relevance of Lynch syndrome diagnosis, routine CRC screening for the disease is of absolute importance [21].

To date, recommendations for the evaluation of Lynch syndrome’s risk mandate that the loss of *MLH1* IHC in patients with colorectal cancer should be followed by *BRAF*-mutation analysis. If no *BRAF* alteration is found, promoter methylation analysis of the *MLH1* promoter region is then performed to discriminate sporadic cases from those potentially associated with Lynch syndrome [21,27].

In patients with *BRAF* wild-type and *MLH1* unmethylated CRC, genetic counseling is recommended to identify Lynch syndrome patients [28]. In the screening algorithm proposed by Chen et al. [24], the first step for dMMR/MSI-H CRC evaluation is *BRAF* p.V600E mutational analysis, and when no *BRAF* mutation is identified *MLH1* promoter methylation is tested.

In our cohort of CRC specimens following the classical scheme—*BRAF* followed by *MLH1* analysis—we performed a total of 153 tests (100 *BRAF* and 53 *MLH1*).

Starting the evaluation with *MLH1* methylation analysis would have allowed us to perform a total of only 123 tests (100 *MLH1* and 23 *BRAF*), saving the laboratory 30 unnecessary tests for *MLH1* out of a total of 53 dMMR/MSI-H *BRAF*-WT CRCs that needed *MLH1* evaluation according to current guidelines. The frequency of *BRAF* mutation found in our cohort is in accordance with previously published data [24,29].

In a previous paper, Xiao and colleagues [30] compared four types of different screening approaches: (i) only *BRAF* testing; (ii) only *MLH1* promoter region methylation testing; (iii) *MLH1* testing and *BRAF*; and (iv) *MLH1* methylation testing and revised Bethesda criteria. From their data, the best approach for Lynch syndrome pre-screening is the combination of *MLH1* methylation and *BRAF* testing, reducing the rate of unnecessary referral to genetic counseling.

Newton and colleagues demonstrated that performing only *BRAF* testing has a lower specificity (66%) in detecting possible Lynch syndrome patients than performing only *MLH1* analyses (88%) [31]. These data are also in accordance with those by Adar and colleagues: in CRC with loss of *MLH1* staining, using *BRAF* as the only test has a 31% rate of sending to genetic counseling, compared with *MLH1* methylation testing alone, which has a rate of 13.5% (2.3-fold lower) [32]. Moreover, Adar and colleagues stated that a hybrid approach further reduces the referral rate for genetic tests [32].

Our data confirm that performing *MLH1* methylation analysis as the first step, followed by *BRAF* mutation analysis would decrease by 56.6% ($[(23 \text{ } MLH1 \text{ UMET} - 53 \text{ } BRAF \text{ WT}) / 53 \text{ } BRAF \text{ WT}] \times 100$) the number of “second-level” tests performed by the laboratory (Figure 4). As we have not observed *MLH1*-UMET/*BRAF*-MUT specimens in a series of 100 consecutive dMMR/H-MSI CRCs consideration should be given to the possibility of using *MLH1* methylation analysis as a stand-alone test for Lynch syndrome risk screening, as is the case for endometrial carcinoma [33]. However, it should be considered that some very rare cases of CRC with the *BRAF* mutation but without *MLH1* methylation have been reported in the literature [31,32]. From the point of view of molecular epidemiology, the relationship between *BRAF* status (*BRAF* WT vs. *BRAF* p.V600E) and *MLH1* promoter methylation status (*MLH1* MET vs. *MLH1* UMET) in the dMMR/MSI-H CRC population of a given geographic region is currently unclear. A reasonable approach would be to first define the proportion of dMMR/MSI-H CRC cases that are: (i) *BRAF* MUT and *MLH1* MET, (ii) *BRAF* MUT and *MLH1* UMET, (iii) *BRAF* WT and *MLH1* MET, and (iv) *BRAF* WT and *MLH1* UMET, in a given medical center. On the basis of these data, the molecular pathology laboratory can then adopt the most cost-effective protocol: either to test first for *BRAF* and then perform *MLH1* methylation analysis (Algorithm 1 of Figures 2 and 4) or to perform first *MLH1* methylation evaluation and then *BRAF* analysis (Algorithm 2 of Figures 4 and 5).

It should be further considered that even larger studies underline the superiority of *MLH1* promoter hypermethylation over *BRAF* testing; somatic analysis of mismatch repair genes helps to identify patients with *MLH1* IHC loss but without germline alterations [34]. Even if the *MLH1* methylation test by pyrosequencing is more technically labor-intensive than *BRAF* analysis, in our medical center, it is clearly more convenient to use the second approach (Algorithm 2 of Figures 4 and 5). In a medical center where *BRAF* is the “first-level” analysis for Lynch syndrome risk screening of CRC patients it is crucial to perform *MLH1* analysis of all *BRAF*-WT specimens, without directly addressing patients with *BRAF*-WT tumors to genetic counseling, as stated by current guidelines (https://www.nccn.org/professionals/physician_gls/pdf/genetics_colon.pdf, accessed date 21 January 2022).

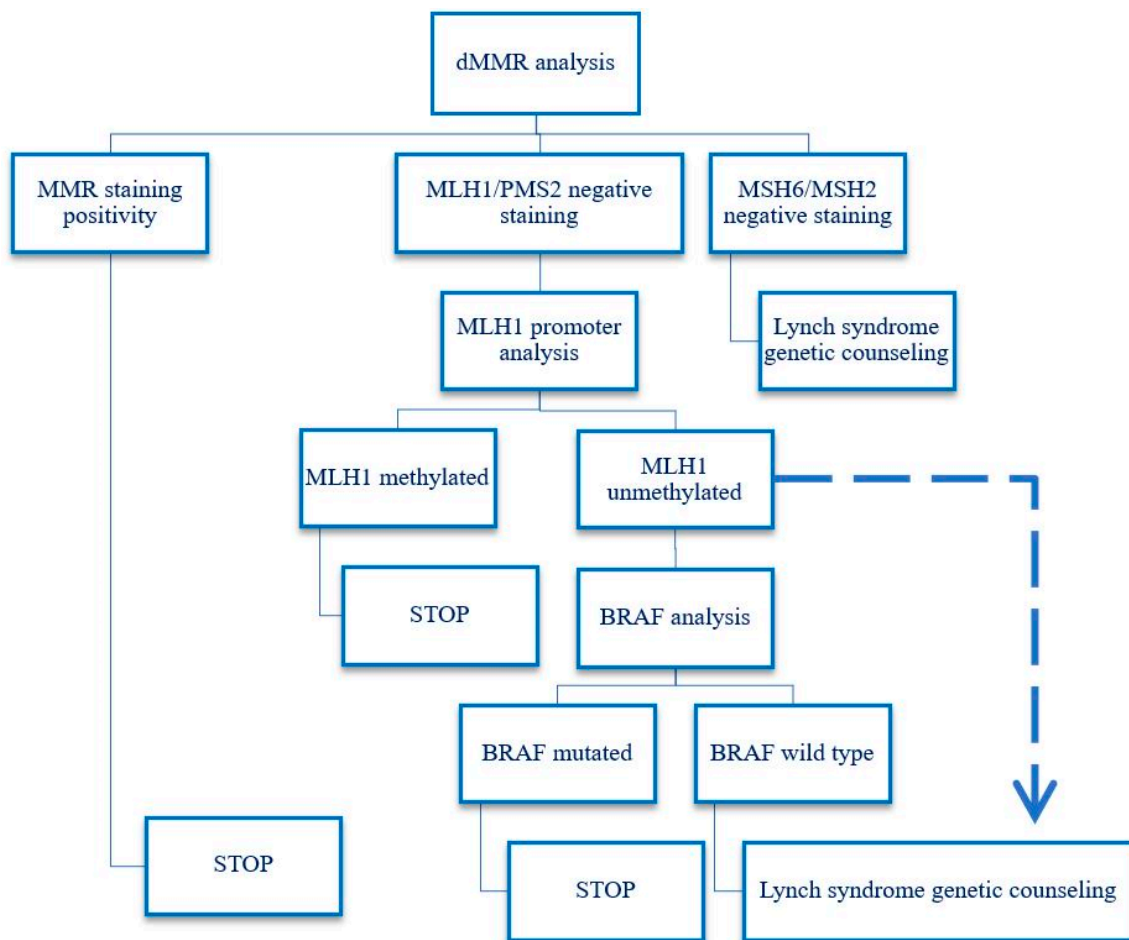


Figure 5. Cost-effective algorithm for *MLH1* promoter methylation and *BRAF* analyses in dMMR/MSI-H CRC at the University of Bologna medical center. The dashed arrow indicates the possible algorithm if *MLH1* promoter methylation analysis is used as a stand-alone test for Lynch syndrome risk screening.

Considering that testing for *BRAF* p.V600E mutation using IHC is not sufficiently sensitive [25], one alternative approach could be testing the *BRAF* mutation status by IHC with *BRAF* p.V600E specific antibodies at the time of pathologic evaluation and immunohistochemical definition of the dMMR status, and then performing *MLH1* promoter methylation analysis in all immunohistochemically *BRAF* p.V600E negative specimens.

In patients with metastatic CRCs, *BRAF* testing of CRC metastases can be integrated with the other biomarkers important for the clinical management of the patients, i.e., *KRAS* and *NRAS* mutational status, by analyzing all three genes using a next-generation-sequencing multi-gene panel [35].

5. Conclusions

Our study highlights the importance of not being limited to *BRAF* analysis in the selection of CRC patients to be addressed to genetic counseling for Lynch syndrome. The best approach should be chosen after the definition of the specific *BRAF* (*BRAF* WT vs. *BRAF* p.V600E) and *MLH1* promoter methylation status (*MLH1* MET vs. *MLH1* UMET) in the dMMR/MSI-H CRC population referred to the medical center. *MLH1* analysis as the first-line test followed by *BRAF* analysis may be more cost-effective than the current protocol that recommends *BRAF* analysis as the first-line test.

Author Contributions: Conceptualization, T.M., A.D.L. and D.d.B.; methodology, T.M., V.S., L.M., S.C., M.V., G.A., A.A., E.G. and S.Z.; formal analysis, T.M., A.D.L. and D.d.B.; data curation, T.M., A.D.L., S.M. and D.d.B.; writing—original draft preparation, T.M. and D.d.B.; writing—review and editing, D.T., M.F., A.D. and G.T.; supervision, D.T., D.d.B. and G.T.; project administration, D.d.B. and G.T.; funding acquisition, G.T. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All the experimental procedures were carried out in accordance with the general authorization to process personal data for scientific research purposes from “The Italian Data Protection Authority” (<http://www.garanteprivacy.it/web/guest/home/docweb/-/docwebdisplay/export/2485392/>, accessed on 7 January 2021). All information regarding the human material was managed using anonymous numerical codes and the study was carried out in accordance with the ethical principles of the Declaration of Helsinki (<https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/>, accessed on 27 August 2021). The study did not affect the clinical management of the involved patients' samples. Follow-up information was not used for this study.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article.

Conflicts of Interest: Dario de Biase has received personal fees (as speaker bureau) from Boehringer Ingelheim, and Eli Lilly, unrelated to the current work.

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