Introduction

Colorectal cancer is a heterogeneous disease arising through different pathways (1, 2). Three molecular pathways are well known to be involved in the multistep process of colorectal carcinogenesis, including the chromosomal instability (CIN) pathway, the mutator pathway [microsatellite instability (MSI)], and the epigenetic instability pathway or CpG island methylator phenotype (CIMP), the latter of which has substantial overlap with the other two.

MSI is the result of a deficient DNA mismatch repair (dMMR) system. A germline mutation in one of the MMR genes, most often MLH1 or MSH2, is the cause of dMMR in patients with Lynch syndrome, which comprises 0.8% to 5% of all colorectal cancers (3). dMMR is also observed in 10% to 20% of patients with sporadic colorectal cancer, of which the majority of dMMR tumors are due to inactivation of MLH1 (~95%), caused by hypermethylation of the gene promoter, with MSH2 and MSH6 accounting for a smaller percentage (3–5). These dMMR tumors have distinct features, such as origin in the proximal colon, prominent lymphocytic infiltrate, poorly differentiated morphology, mucinous or signet ring differentiation (6), and association with a favorable prognosis in early-stage colorectal cancer (7). In metastatic colorectal cancer (mCRC), the prevalence of dMMR is low (3.5%; refs. 8, 9). This supports the hypothesis that dMMR tumors have a reduced metastatic
the overall prevalence of diagnosis of Lynch syndrome (12, 13). In colorectal cancer, tumor indicates a sporadic origin, and essentially excludes a BRAF (14).

COIN (19, 20), and FOCUS (21). Large prospective phase III studies: CAIRO (17), CAIRO2 (22), COIN, and FOCUS studies. We show that the prevalence of deficient MMR (dMMR) and BRAF<sup>MT</sup> is low in patients with mCRC. Both biomarkers confer an inferior prognosis. We observed a higher incidence of BRAF<sup>MT</sup> in dMMR tumors than reported for patients with early-stage dMMR colorectal cancer, and our data suggest that the poor prognosis of dMMR is driven by BRAF<sup>MT</sup> status.

Translational Relevance
This is the first pooled analysis on individual patient data to assess the role of the mismatch repair (MMR) status in relation to the BRAF mutation (BRAF<sup>MT</sup>) status in respect to prevalence and outcome in patients with metastatic colorectal cancer (mCRC). These patients participated in four large randomized prospective phase III studies, namely the CAIRO, CAIRO2, COIN, and FOCUS studies. We show that the prevalence of deficient MMR (dMMR) and BRAF<sup>MT</sup> is low in patients with mCRC. Both biomarkers confer an inferior prognosis. We observed a higher incidence of BRAF<sup>MT</sup> in dMMR tumors than reported for patients with early-stage dMMR colorectal cancer, and our data suggest that the poor prognosis of dMMR is driven by BRAF<sup>MT</sup> status.

Materials and Methods

Patients and treatment
Data were derived from patients with mCRC included in four large phase III studies in first-line treatment: CAIRO (ClinicalTrials.gov; NCT00312000), CAIRO2 (ClinicalTrials.gov; NCT0208546), COIN (ISRCTN; 27286448), and FOCUS (ISRCTN; 79877428), of which the results have been published previously (17–21). Collection of formalin-fixed paraffin-embedded material (FFPE) of the primary tumors from small subsets of selected patients. The current study was initiated to assess the role of MMR status in relation to the BRAF<sup>MT</sup> status in respect to prevalence and outcome in patients with mCRC who participated in four large prospective phase III studies: CAIRO (17), CAIRO2 (18), COIN (19, 20), and FOCUS (21).

MMR status
For samples of both CAIRO studies, immunohistochemistry (IHC) was performed on FFPE tissue with antibodies against MMR proteins hMLH1, hMSH2, hMSH6, and hPMS2. In addition, MSI analysis was performed where there was an absence of MMR protein expression or equivocal IHC results. dMMR status was determined using two microsatellite markers (BAT25 and BAT26). If only one of these markers showed instability, the analysis was extended with four additional markers (BAT40, D2S123, DSS346, and D17S250). A tumor was defined as dMMR if at least two of the six markers showed instability or pMMR if none of the markers showed instability. Tumors with only one of the markers showing instability were defined as dMMR-low and included in the pMMR category. For samples from the COIN study, dMMR status was assessed using two microsatellite markers (BAT25 and BAT26). If only one of these markers showed instability, the tumor was defined as dMMR, and as pMMR if no instability was observed. For samples from the FOCUS study, dMMR status was based on loss of MLH1 and MSH2 protein expression, assessed by IHC. If either protein showed loss of expression, the tumor was defined as dMMR, and pMMR if no loss of expression was observed.

Hypermethylation status of the MLH1 gene promoter
Hypermethylation of the MLH1 gene promoter in patients with a dMMR tumor was analyzed in samples from the CAIRO and CAIRO2 studies only and therefore not included in the pooled analysis. The DNA methylation status of the MLH1 promoter region was determined after bisulfite treatment of the DNA using the EZ DNA Methylation Kit (Zymo Research), as described previously (8).

BRAF<sup>MT</sup> status
The BRAF V600E mutation status was assessed in duplicate by high-resolution melting (HRM) sequencing analysis for tumor material in the CAIRO study (22) and by direct sequencing analysis in the CAIRO2 study (23). For samples of the COIN and FOCUS studies, the BRAF V600E mutation status was determined by Pyrosequencing (and Sequenom in COIN), and verified by Sanger sequencing as described previously (19, 24). Non-V600E BRAF<sup>MT</sup> detected by these assays (n = 19) were not included in the current analyses on outcome.

Statistical methods
Individual patient data were included in the pooled analysis. Progression-free survival (PFS) was defined as the time from the date of randomization to first progression or death, whichever came first. Overall survival (OS) was defined as the time from randomization to the date of death. The primary outcome measure was the hazard ratio (HR) for PFS and OS in relation to MMR and BRAF<sup>MT</sup> status. For PFS and OS, all studies were included in a Cox regression model (proportional hazard model) by using the study as a factor in the model. In this way, dependence of the hazard on study could be modeled. The HR was corrected for study effect. Survival curves were plotted and log-rank tests were performed to compare survival for the different groups defined. A statistical interaction analysis for survival data of dMMR and BRAF<sup>MT</sup> status was performed. All analyses were conducted using the SAS system version 9.2; P < 0.05 was considered statistically significant.

Results

Study population and MMR/BRAF<sup>MT</sup> status
Tumor and normal samples from 3,063 out of 6,155 randomized mCRC patients were available and suitable for analysis.
for analysis of both MMR and \(BRAF^{MT}\) status. Of these 3,063 patients, 322 patients participated in the CAIRO study, 516 patients in the CAIRO2 study, 1,461 patients in the COIN study, and 764 patients in the FOCUS study.

The prevalence of MMR status and \(BRAF^{MT}\) status and their correlation are presented in Tables 1 and 2, respectively. dMMR was found in tumors of 153 (5.0%) patients and 250 (8.2%) patients had a \(BRAF^{MT}\) (Table 1). There was no evidence of heterogeneity for the prevalence of dMMR and \(BRAF^{MT}\) in the four studies; \(P = 0.614\) and \(P = 0.943\), respectively (Table 1). A \(BRAF^{MT}\) was observed in 53 (34.6%) of patients with dMMR tumors compared with 197 (6.8%) of patients with pMMR tumors (\(P < 0.001\); Table 2). There was heterogeneity for the prevalence of combined MMR and \(BRAF^{MT}\) status between the four studies. In the CAIRO study, there were significantly more patients with a combined dMMR and \(BRAF^{MT}\) (dMMR/\(BRAF^{MT}\)) tumor compared with the other three studies (\(P = 0.002\); Table 2).

Patient and tumor characteristics (sex, age, location of the primary tumor, performance status, and number of metastatic sites involved) for the different subgroups defined by the combined MMR and \(BRAF^{MT}\) status are summarized in Supplementary Table S1. Hypermethylation of \(MLH1\) was the main cause of dMMR in both CAIRO and CAIRO2 studies (30 out of 45 patients), this was associated with a high frequency of \(BRAF^{MT}\) (73%) compared with tumors without \(MLH1\) hypermethylation (7%).

Survival data

The survival data of the individual studies, the pooled dataset, and the pooled analysis for patients with dMMR, pMMR, \(BRAF^{MT}\), and \(BRAF^{WT}\) tumors are presented in Table 3. The median PFS and OS were significantly worse for patients with dMMR compared with pMMR tumors [PFS: 6.2 vs. 7.6 months, respectively; HR, 1.33; 95% confidence interval (CI) 1.12–1.57; \(P = 0.001\); OS: 13.6 vs. 16.8 months, respectively; HR, 1.35; 95% CI, 1.13–1.61; \(P = 0.001\)]. Median PFS and OS were also significantly worse for patients with \(BRAF^{MT}\) compared with \(BRAF^{WT}\) tumors (PFS: 6.2 vs. 7.7 months, respectively; HR, 1.34; 95% CI, 1.17–1.54; \(P < 0.001\); OS: 11.4 vs. 17.2 months, respectively; HR, 1.91; 95% CI, 1.66–2.19; \(P < 0.001\)).

To determine a possible interaction between MMR and \(BRAF\) status, with respect to the survival, a Cox regression was performed by using the study as a factor in the model. For PFS and OS, all studies were included in a Cox regression model.
(proportional hazard model) by using the study as a factor in the model. Results are presented for MMR status in a BRAF<sup>MT</sup> and BRAF<sup>WT</sup> background, and vice versa for BRAF status in ad M M Ra n dp M M Rb a c k g r o u n di nT a b l e4 . S u r v i v a l c u r v e s , as estimated by the Cox regression, are presented in Fig. 1. In BRAF<sup>MT</sup> tumors stratified by MMR status, there was no significant survival difference for patients with dMMR compared with pMMR tumors (PFS: 6.1 vs. 6.2 months, respectively; HR, 0.95; 95% CI, 0.62–1.46; P = 1.000). Also in BRAF<sup>WT</sup> tumors stratified by MMR status, there was no significant survival difference for patients with dMMR compared with pMMR tumors (PFS: 6.3 vs. 7.8 months, respectively; HR, 1.32; 95% CI, 1.00–1.75; P = 0.051; OS: 15.0 vs. 17.3 months, respectively; HR, 1.22; 95% CI, 0.91–1.65; P = 0.463). In dMMR tumors stratified by BRAF status, there was no significant survival difference for patients with BRAF<sup>MT</sup> compared with BRAF<sup>WT</sup> tumors (PFS: 6.1 vs. 6.3 months, respectively; HR, 1.07; 95% CI, 0.67–1.70; P = 1.000; OS: 11.7 vs. 15.0 months, respectively; HR, 1.51;
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NOTE: Statistically significant results are shown in bold.
Abbreviations: mo., median PFS or OS time in months; mt, mutant tumor; wt, wild-type tumor.
95% CI, 0.93–2.46; \( P = 0.155 \). In pMMR tumors stratified by \( \text{BRAF} \) status, there was a significantly decreased median PFS and OS for patients with \( \text{dMMR/BRAF}^{\text{MT}} \) tumors, \( \text{dMMR/BRAF}^{\text{WT}} \) tumors, \( \text{pMMR/BRAF}^{\text{MT}} \) tumors, and \( \text{pMMR/BRAF}^{\text{WT}} \) tumors.

**Figure 1.** PFS (A) and OS (B) curves of all patients included in the pooled dataset comparing patients with \( \text{dMMR/BRAF}^{\text{MT}} \) tumors, \( \text{dMMR/BRAF}^{\text{WT}} \) tumors, \( \text{pMMR/BRAF}^{\text{MT}} \) tumors, and \( \text{pMMR/BRAF}^{\text{WT}} \) tumors.

**Discussion**

This study presents the largest dataset on the role of tumor MMR status and \( \text{BRAF}^{\text{MT}} \) status in respect to prevalence and outcome in a population of patients (\( n = 3,063 \)) with mCRC who participated in four prospective phase III studies. We found that dMMR and \( \text{BRAF}^{\text{MT}} \) in mCRC each have a low prevalence (5% and 8.2%, respectively), and that both biomarkers indicate a
poor prognosis. Given the absence of a statistically significant interaction between BRANMT and dMMR, our data suggest that the poor prognostic value of dMMR is driven by the BRANMT status.

Several aspects of our study warrant further discussion. In this pooled analysis, different methods for detecting dMMR were applied, which, however, have all been validated for the detection of dMMR in colorectal cancer. In both CAIRO studies, an approach based on test methods described in the Bethesda criteria, used for standard clinical practice for patients suspected for Lynch syndrome, has been applied (25). The COIN study analyzed the BAT125 and BAT26 mononucleotide markers, which have a high sensitivity (94%) and specificity (98%), and the use of these two markers alone identifies 97% of MSI tumors (26). The FOCUS study evaluated MLH1 and MSH2 protein expression by IHC, which is a sensitive (92.3%) and specific (100%) method for screening for dMMR (27).

We acknowledge that the difference in MMR detection methods represents a weakness of our study; however, the comparable prevalence of the dMMR status among the four studies in this pooled analysis, ranging from 4.4% to 5.6%, argues against this. The results from the individual studies show that the patient population with dMMR tumors is heterogeneous. The observed difference in the prevalence of a BRANMT in dMMR tumors suggests a possible difference in the origin of dMMR, sporadic versus hereditary. Unfortunately, data on the hypermethylation status of the MLH1 gene promoter, which could differentiate between these two groups, are not available of all four studies.

Furthermore, different methods for detecting the BRAF V600E mutation were applied. HRM sequencing, Sanger sequencing, and Pyrosequencing have all shown to be reliable methods (22, 28). Data from systematic studies to assess the test accuracy or reproducibility of the different techniques used for BRANMT testing are not available.

Another issue is the difference in availability of tumor samples among the trials. This is partly caused by nonavailability of an extra paraffin-embedded block for DNA analysis, and partly due to noresected primary tumors in patients with synchronous disease. In these patients, often only a diagnostic biopsy was performed, which does not provide sufficient material for further molecular analysis for research purposes. This is an important, underexposed issue that may introduce a sample/case bias not only in our analysis, but in other translational studies in mCRC as well.

The low prevalence of dMMR in mCRC can be explained by the reduced potential of stage I–III dMMR tumors to metastasize (10, 11). However, the underlying mechanisms of this low metastatic potential are yet to be elucidated. It has been suggested that a greater immunoreactivity of dMMR tumors (29, 30) or decreased tumor cell viability due to excessive DNA damage (31) may play a role. In mCRC, data about the prevalence of BRANMT in dMMR tumors are scarce, but in line with our results (32, 33). The strong inter-relationship between BRANMT and dMMR is well established in early-stage colorectal cancer (14, 34); however, the etiology of both alterations still needs to be elucidated.

We observed a higher prevalence of BRANMT in mCRC dMMR tumors (34.6%) than reported for early-stage dMMR colorectal cancer tumors (24%; 16). Patients with early-stage dMMR in general have a better prognosis compared with patient with early-stage pMMR; however, within the group of dMMR, patients with BRANMT tumors have a worse prognosis (35). Subsequently, this may lead to a shift in the dMMR/BRANMT ratio in patients with mCRC. There is increasing evidence identifying BRANMT as a significant poor prognostic factor in early stage and mCRC (18, 36–38). BRAF is an oncogene and it is known that the mutations constitutively activate the MAPK pathway for cell growth, in the absence of extracellular stimuli. However, by itself BRAF is not sufficient for cancer and must cooperate with other processes to induce the fully cancerous state (39). Another explanation for the inferior prognosis of BRANMT tumors might be their distinct pattern of metastatic spread. Previous studies have demonstrated a significantly increased rate of peritoneal and distant lymph node metastases and a decreased rate of lung metastases compared with BRANMT tumors (9, 40).

It has been speculated that the worse prognostic value of dMMR tumors in mCRC may be related to a difference in metastatic spread. Earlier studies showed a reduced rate of liver metastases for dMMR tumors in mCRC (40), and a higher incidence of peritoneal metastases; these factors are known to be related to prognosis (41, 42). This was confirmed by a previous analysis of the COIN study (9), but these data are not available from the other studies of our analysis.

Finally, due to the different treatment regimens among the four studies of this pooled analysis, the predictive role of dMMR and BRANMT in mCRC could not be addressed.

In conclusion, dMMR and BRANMT each have a low prevalence in mCRC, and both biomarkers confer a poor prognosis. Our data suggest that the poor prognosis of dMMR is driven by the BRANMT status. However, we caution against a firm conclusion on this issue because our study was not sufficiently powered to test this interaction.

Disclosure of Potential Conflicts of Interest
J.P. Cheadle reports receiving a commercial research grant from Merck. No potential conflicts of interest were disclosed by the other authors.

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Mismatch Repair Status and *BRAF* Mutation Status in Metastatic Colorectal Cancer Patients: A Pooled Analysis of the CAIRO, CAIRO2, COIN, and FOCUS Studies


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