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CpG island methylator phenotype identifies high risk patients among microsatellite stable *BRAF* mutated colorectal cancers

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The prognostic value of CpG island methylator phenotype (CIMP) in colorectal cancer remains unsettled. We aimed to assess the prognostic value of this phenotype analyzing a total of 1126 tumor samples obtained from two Norwegian consecutive colorectal cancer series. CIMP status was determined by analyzing the 5-markers *CAGNA1G*, *IGF2*, *NEUROG1*, *RUNX3* and SOCS1 by quantitative methylation specific PCR (qMSP). The effect of CIMP on time to recurrence (TTR) and overall survival (OS) were determined by uni- and multivariate analyses. Subgroup analyses were conducted according to MSI and *BRAF* mutation status, disease stage, and also age at time of diagnosis (<60, 60-74, ≥75 years). Patients with CIMP positive tumors demonstrated significantly shorter TTR and worse OS compared to those with CIMP negative tumors (multivariate hazard ratio [95% CI] 1.86 [1.31-2.63] and 1.89 [1.34-2.65], respectively). In stratified analyses, CIMP tumors showed significantly worse outcome among patients with microsatellite stable (MSS, $P < 0.001$), and MSS *BRAF* mutated tumors ($P < 0.001$), a finding that persisted in patients with stage II, III or IV disease, and that remained significant in multivariate analysis ($P < 0.01$). Consistent results were found for all three age groups. To conclude, CIMP is significantly associated with inferior outcome for colorectal cancer patients, and can stratify the poor prognostic patients with MSS *BRAF* mutated tumors.

Introduction

Colorectal cancer is one of the most common malignancies throughout the world, with almost 1.4 million new cases and close to 700 000 deaths each year.[1] Clinicopathological staging according to the Tumor–Node–Metastases (TNM) system[2] remains the most important prognostic factor. However, the TNM system fails to accurately predict the outcome of patients within stages,[3] and robust markers to stratify these

Key words: Age of Onset, CIMP, Colon Cancer, DNA Methylation, Prognostic Factor

Abbreviations: CIMP: CpG island methylator phenotype; FFPE: formalin fixed paraffin embedded; HR: hazard ratio; MSI: microsatellite instability; MSS: microsatellite stable; mut: mutation; OS: overall survival; PMR: percent methylated reference; TTR: time to recurrence; qMSP: quantitative methylation specific polymerase chain reaction; wt: wild type

Additional Supporting Information may be found in the online version of this article.

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What's new?

As many as one-fifth of colorectal cancers have a CpG island methylator phenotype (CIMP) involving widespread promoter DNA methylation. CIMP is associated with key factors related to disease outcome, including microsatellite instability and *BRAF* mutations. In this study, CIMP was found to be significantly associated with worse prognosis in colorectal cancer patients, particularly those with microsatellite stable (MSS) *BRAF*-mutated tumors. In stratified analyses, trends toward worse survival were identified for CIMP-positive stage III and stage IV patients in the MSS *BRAF*-mutated group. The findings suggest that CIMP status should be included in prognostic analyses at time of diagnosis.

patients to improve prognostication and choice of therapy are clearly needed.

CpG islands are found in 50–70% of human gene promoters and are normally unmethylated. In cancer however, these islands frequently become hypermethylated, which is associated with aberrant silencing of several genes, including tumor suppressors.[4] Approximately 15–20% of colorectal cancers are characterized by widespread promoter DNA methylation, referred to as the CpG island methylator phenotype (CIMP). CIMP positive tumors are associated with microsatellite instability (MSI), *BRAF* mutations, wild type *TP53*, poor differentiation, proximal location, and also female gender and older age.[5–7]

In addition to CIMP, there are two other well known molecular phenotypes of colorectal cancer, characterized by either MSI or chromosomal instability (CIN).[8–10] Patients diagnosed with MSI generally have a good prognosis,[11–14] whereas patients with CIN have a much poorer prognosis,[15] although with great stage dependent variation. In contrast, the prognostic effect of CIMP remains unsettled.[16–20] Several studies fail to reach statistical significance due to insufficient sample size, and the effect of CIMP is often diminished after adjusting for other factors.[21] We have analyzed the prognostic value of CIMP in two Norwegian population representative patient series ($n > 1100$), using a well-established and validated CIMP panel,[5,22] adjusting for other molecular- and clinical variables, including stage, *BRAF* and MSI status.

Materials and Methods

Patient samples

Samples from a total of 1126 patients with stage I-IV colorectal cancer were analyzed, obtained from two Norwegian series (Oslo 1,[23,24] and Oslo 2[25,26]). The Oslo 1 series comprised 762 formalin fixed paraffin embedded (FFPE) colorectal cancer tissue samples, collected from patients undergoing surgical resection at the Oslo University Hospital- Aker (OUH) in the period 1993–2003. The median age of the patients was 73 years (range 30–94 years). The Oslo 2 series included 364 fresh frozen samples from patients undergoing surgery at OUH in the period 2005–2011. The median age of the patients was 71 years (range 27–97 years).

The study was carried out according to the Helsinki declaration, and the research biobanks have been registered according to national legislation (numbers 2781 and 236–2005–16141). The study has been approved by the Regional Committee for Medical and Health Research Ethics (numbers 1.2005.1629 and S-09282c 2009/4958), which requires that informed consent is obtained from patients enrolled in the study.

DNA extraction, bisulfite treatment and determination of CIMP status

DNA from the fresh frozen colorectal tissue samples (Oslo 2) was extracted using either a standard phenol/chloroform protocol[27] or magnetic beads (Maxwell 16, Promega). For the FFPE tissue samples (Oslo 1) the QIAmp DNA Mini kit (Qiagen, Hilden, Germany) was applied for DNA extraction as previously described.[23] DNA from both series was bisulfite treated using the EpiTect Bisulfite kit (Qiagen) with 1.3 μg DNA as input, and purified using the QIAcube (Qiagen). For the FFPE tissue samples, an extra ethanol step was included during clean-up. Quantitative methylation specific PCR (qMSP) was performed as previously described.[28] In brief, the PCRs (95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 60 sec) were carried out in triplicates in 384 well plates in the 7900HT Real-Time PCR System (Life Technologies, Carlsbad, CA), and included 1xTaqMan Universal PCR Mastermix No AmpErase UNG (Life Technologies), 0.9 μM of each primer, 0.2 μM probe, and approximately 32.5 ng bisulfite treated template DNA. Water was included as template negative control, bisulfite treated DNA isolated from the whole blood of healthy individuals was included as methylation negative control, and bisulfite-converted *in vitro* methylated DNA (IVD, Chemicon; Millipore) was used as a methylation positive control. A standard curve was generated from IVD consisting of 1:5 serial dilutions (32.5 ng- 0.052 ng). To distribute template and master mix to the 384-well plates, the EpMotion 5075 pipetting robot (Eppendorf, Hamburg, Germany) was used.

The threshold for censoring was cycle 35 and cycle 40 for the fresh frozen (Oslo 2) and FFPE (Oslo 1) samples, respectively. The median quantity value of the triplicates was used for data analysis. To normalize for DNA input the ALU-C4 element was used as a control.[29] The percent of methylated reference (PMR) values were calculated by dividing the median GENE:ALU ratio of the sample by the median GENE:ALU ratio of the positive control (IVD), and multiply by 100.

In accordance with the CIMP panel described by Weisenberger *et al.*[5] including *CACNA1G, IGF2, NEUROG1, RUNX3* and *SOCS1*, samples with a PMR value $\geq$10 for $\geq$3/5 markers were considered CIMP positive. PMR $\geq$ 10 was also used for scoring *MLH1* methylation positive samples.[30] CIMP and *MLH1* status was successfully determined for 1122 samples, including all 364 fresh frozen samples and 758/762 = 99.5% of the FFPE samples.

Determination of MSI status

Of the 1122 samples successfully analyzed for CIMP, MSI status was available for 1113 samples, including 933 MSS and 180 MSI tumors.[23–25] As previously described,[23] MSI status was determined by fragment analyses of the five microsatellites BAT25, BAT26, D2S123, D5S346, and D17S250 (the Bethesda markers).

Mutation analyses of BRAF

BRAF exon 15 was amplified using the primers: sense 5'-TCATAATGCTTGCTGTGATAGGA-3'and antisense 5'-GGCCAAAAATTTAATCAGTGGA-3'. The PCR products were purified enzymatically by illustra ExoStar 1-step (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, US) prior to sequencing using the BigDye Terminator v.1.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, US). The sequencing products were purified with Big Dye Xterminator (Applied Biosystems) or Sephadex (GE healthcare) and subjected to sequencing on an ABI 3730 DNA Sequencer (Applied Biosystems).

Statistical analysis

The statistical analyses were performed using IMB SPSS Statistics 21 and R version 3.2.2 together with the "survival"[31] and "bootStepAIC"[32] packages. χ^2 or Fisher's exact tests (when expected frequencies were less than five) were applied to evaluate associations between CIMP and clinicopathological features (categorical variables). In survival analyses, the end points were five-year time to recurrence (TTR) and five-year overall survival (OS). TTR, defined according to Punt *et al.*[33] was calculated from the date of surgery until the first of death from the same cancer, local recurrence or distal metastasis. Patients were censored at last follow-up, death caused by other events than colorectal cancer and death due to postoperative complications (<3 months). OS was calculated from time of surgery until death of any cause, and cases were censored at last follow-up. The effect of CIMP and *BRAF* were first independently evaluated in an univariate approach. Kaplan-Meier method was used to estimate the survival curves and univariate Cox's proportional hazard models were fitted to the data. Hazard Ratios (HRs) and 95% confidence intervals (CIs) were derived from the model, and significance of the parameters was assessed using a Wald's test. The multivariate approaches were also based on Cox's proportional hazard model and preceded by a stepwise selection procedure by Akaike information criterion (AIC), in order to identify a subset of relevant predictor variables from the set of available clinicopathological data, both for the whole series and for the MSS tumors alone. To ensure robustness in the selection procedure, a Bootstrap approach with 1,000 iterations was implemented. A total of 1035 patients, including 872 with MSS tumors, had information on all predictor variables, and were included in the multivariate analyses. A χ^2 test was first performed to evaluate whether the proportional hazards assumption for a Cox regression model fit was met. The significance of the variables included in the final model was then assessed by a Wald's test and p values <0.05 was considered statistically significant.

Finally, three alternative ways of stratifying patients according to age at diagnosis were tested, 1) <70 and $\geq$70, 2) <55, 55–74, $\geq$75, and 3) <60 years, 60–74 years, and $\geq$75 years. The third alternative achieved the lowest AIC value and was used for further analyses.

Results

CIMP and clinical- and molecular features

Among the 1122 samples successfully analyzed for CIMP status, 207 (18%) were CIMP positive, including sixty-two of 933 (7%) MSS tumors and 143 of 180 (79%) MSI tumors. Associations between CIMP and clinical- and molecular features are shown separately for the MSS and MSI groups (Table 1), and across the whole series (Supporting Information Table 1). For all three groups, CIMP was significantly associated with $BRAF^{V600E}$ mutation, promoter methylation status of *MLH1*, right sided tumor localization, and female gender. In the MSS group, CIMP positive tumors were more frequently of advanced stages (III and IV) compared with CIMP negative tumors. An association between CIMP and age was only observed in the MSI group (Table 1). Associations between CIMP and clinical and molecular features stratified by age are summarized in Supporting Information Tables 2–4.

CIMP and survival analyses

Data from a total of 1118 patients (758 from Oslo 1 and 360 from Oslo 2) were available for survival analyses. Across all samples, CIMP alone was not significantly associated with OS or TTR (Table 2, and Supporting Information Table 5, respectively). In stage stratified analyses, there was a tendency towards worse survival for those with stage III CIMP positive tumors and significantly worse survival among patients with stage IV CIMP positive tumors, compared to those with CIMP negative tumors (Supporting Information Figure 1).

The multivariate models with OS and TTR as end points are shown in Table 2 and Supporting Information Table 5, respectively. Following the selection procedure and the coefficient testing CIMP was, in contrast to the univariate analysis, included and significant in both models after adjusting for stage, R status, age, MSI or *MLH1* and *BRAF* mutation status.

Table 1. Associations between CIMP and clinical- and molecular features stratified by MSI status.

	MSS				MSI			
	Total n	CIMP− n (%)	CIMP+ n (%)	*P* value	Total n	CIMP− n (%)	CIMP+ n (%)	*P* value
No. of patients	933	871 (93)	62 (7)		180	37 (21)	143 (79)	
Gender				0.004				0.024
Male	474	454 (96)	20 (4)		50	16 (32)	34 (68)	
Female	459	417 (91)	42 (9)		130	21 (16)	109 (84)	
Age				0.597				<0.001
<60	170	159 (94)	11 (6)		23	13 (57)	10 (43)	
60–74	367	339 (92)	28 (8)		63	11 (17)	52 (83)	
≥75	396	373 (94)	23 (6)		94	13 (14)	81 (86)	
Stage				<0.001				0.475
I	166	165 (99)	1 (1)		22	6 (27)	16 (73)	
II	333	316 (95)	17 (5)		105	20 (19)	85 (81)	
III	258	236 (91)	22 (9)		40	10 (25)	30 (75)	
IV	173	151 (87)	22 (13)		13	1 (8)	12 (92)	
Localization				<0.001				<0.001
Right colon	301	260 (86)	41 (14)		151	22 (15)	129 (85)	
Left colon	329	314 (95)	15 (5)		18	7 (39)	11 (61)	
Rectum	289	284 (98)	5 (2)		8	6 (75)	2 (25)	
BRAF				<0.001				<0.001
BRAF wt	825	792 (96)	33 (4)		53	30 (57)	23 (43)	
BRAF mut	55	28 (51)	27 (49)		111	3 (3)	108 (97)	
*MLH1*methylation				0.024				<0.001
MLH1 unmeth	929	869 (94)	60 (6)		35	25 (71)	10 (29)	
*MLH1*meth	4	2 (50)	2 (50)		145	12 (8)	133 (92)	

Meth, methylated; mut, mutation; No., number; unmeth, unmethylated; wt, wild type.

Poor prognosis for patients with MSS CIMP positive tumors. Patients with CIMP positive MSS tumors displayed a significantly worse OS and shorter TTR compared to those with CIMP negative MSS tumors (Table 3 and Supporting Information Table 6, respectively. Visualized in Figure 1A). Further stratification by stage showed that CIMP was significantly associated with worse outcome among patients with MSS stage III and stage IV tumors (Figure 1C-D).

CIMP status stratifies the poor prognosis group of patients with MSS and BRAF mutated tumors. $BRAF^{V600}$ mutation was significantly associated with worse survival among patients with MSS tumors (univariate analysis; Table 3 and Supporting Information Table 6. Visualized in Figure 1E). The same trend was observed among patients with stage II, III or IV tumors (Figure 1F-H).

To evaluate the effect of CIMP on *BRAF*, patients with MSS tumors were divided into four groups; 1: CIMP negative/*BRAF* wild type, 2: CIMP negative/*BRAF* mutated, 3: CIMP positive/*BRAF* wild type, and 4: CIMP positive/*BRAF* mutated. Results are presented in Figure 2 and Table 4, and show that patients with type 1, 2, or 3 tumors all had a significantly improved OS compared to patients with type 4 tumors (both CIMP positive and *BRAF* mutated). Although there were few patients within each stage, this finding was in general maintained in stage II-III. For stage IV, no difference in survival was observed between patients with CIMP positive tumors with or without *BRAF* mutation (type 4 and type 3, respectively; Figure 2D).

The worse survival for patients with CIMP positive *BRAF* mutated tumors remained significant after adjusting for stage, R status, and age (Table 4). Comparable findings were observed for TTR (not shown).

When looking specifically at the effect of CIMP in patients with MSS, *BRAF* mutated tumors, we observed that also within this poor prognostic group, CIMP was significantly associated with worse OS (HR = 4.39, 95% CI (2.21–8.70), $P < 0.001$; Figure 2) and shorter TTR (HR = 4.02, 95% CI (4.84–8.77), $P < 0.001$). The same trend was maintained among patients with stage II, III or IV tumors (Figure 2B-D), showing that the worse survival observed was not only due to later stages.

Table 2. Univariate and multivariate Cox proportional hazard analyses with overall survival as endpoint.

	Patients, n	Events, n	Univariate HR (95% CI)	*P* value	Patients, n	Multivariate HR (95% CI)	*P* value
Gender							
Male	525	227	1.00 (ref)			Not included	
Female	593	262	1.05 (0.88–1.26)	0.575		Not included	
Age							
<60	194	50	1.00 (ref)		181	1.00 (ref)	
60–74	434	178	1.78 (1.30–2.44)	<0.001	403	1.97 (1.42–2.73)	<0.001
≥75	490	261	2.51 (1.85–3.40)	<.0001	451	3.51 (2.56–4.83)	<0.001
Stage							
I	188	37	1.00 (ref)		175	1.00 (ref)	
II	439	138	1.73 (1.20–2.49)	0.003	408	1.43 (0.98–2.09)	0.061
III	301	144	3.08 (2.14–4.42)	<0.001	275	2.75 (1.90–4.00)	<0.001
IV	188	169	11.96 (8.34–17.14)	<.0001	177	3.29 (1.90–5.68)	<0.001
Localization							
Right	454	202	1.00 (ref)			Not included	
Left	350	164	1.06 (0.86–1.30)	0.602		Not included	
Rectum	298	116	0.81 (0.65–1.02)	0.073		Not included	
CIMP							
CIMP−	912	392	1.00 (ref)		846	1.00 (ref)	
CIMP+	206	97	1.18 (0.94–1.47)	0.144	189	1.89 (1.34–2.65)	<0.001
*MLH1*methylation							
MLH1 unmeth	970	439	1.00 (ref)		899	1.00 (ref)	
MLH1 meth	148	50	0.67 (0.50–0.90)	0.008	136	0.35 (0.23–0.56)	<0.001
MSI status							
MSS	930	423	1.00 (ref)			Not included	
MSI	179	61	0.69 (0.53–0.90)	0.007		Not included	
BRAF							
BRAF wt	882	379	1.00 (ref)		871	1.00 (ref)	
BRAF mut	167	78	1.17 (0.91–1.49)	0.216	164	1.32 (0.92–1.9)	0.128
R status							
R0	905	306	1.00 (ref)		836	1.00 (ref)	
R1	24	10	1.39 (0.74–2.61)	0.304	24	1.28 (0.68–2.41)	0.451
R2	186	170	6.75 (5.55–8.21)	<0.001	175	4.30 (2.77–6.66)	<0.001

Meth, methylated; mut, mutation; unmeth, unmethylated; wt, wild type.

From multivariate analyses, both CIMP and *BRAF* were included in the final model with OS as end point, suggesting that both variables have independent prognostic value. However, only CIMP was included in the model with TTR as endpoint (Supporting Information Table 6).

No effect of CIMP or BRAF among patients with MSI tumors. Among patients with MSI tumors, neither CIMP nor *BRAF* status was alone associated with survival (CIMP: OS, HR = 1.34, 95% CI (0.68–2.65); TTR, HR = 1.13, 95% CI (0.54–2.35); *BRAF*: OS, HR = 1.00, 95% CI (0.56–1.77); TTR, HR = 1.13, 95% CI (0.54–2.35)). Also when stratified by stage, no significant associations were seen, neither for CIMP nor for *BRAF*.

The effect of CIMP on survival is consistent across different age groups. The univariate effect of CIMP and *BRAF* on OS in the three analyzed age groups (<60, 60–74, ≥75) are summarized in Supporting Information Table 7 and Supporting Information Figure 2. The associations with survival were in general consistent across all three age groups.

Discussion

In this population representative series of colorectal cancer patients, we showed that patients with CIMP positive tumors had a significantly worse prognosis compared to patients with CIMP negative tumors after adjustment for age, stage, R

Table 3. Univariate and multivariate Cox proportional hazard analyses with OS survival as end point in patients with MSS tumors.

	Total, n	Events, n	Univariate HR (95% CI)	*P* value	Total, n	Multivariate HR (95% CI)	*P* value
Gender							
Male	472	210	1.00 (ref)			Not included	
Female	458	213	1.10 (0.91–1.33)	0.346		Not included	
Age							
<60	170	45	1.00 (ref)		163	1.00 (ref)	
60–74	365	161	1.92 (1.38–2.67)	<0.001	342	2.10 (1.49–2.95)	<0.001
≥75	395	217	2.57 (1.86–3.54)	<0.001	367	3.55 (2.54–4.96)	<0.001
Stage							
I	165	34	1.00 (ref)		156	1.00 (ref)	
II	333	111	1.78 (1.21–2.62)	0.003	311	1.45 (0.98–2.16)	0.064
III	257	123	2.94 (2.01–4.29)	<0.001	240	2.71 (1.83–4.00)	<0.001
IV	173	154	10.94 (7.51–15.92)	<0.001	165	2.72 (1.52–4.86)	<0.001
Localization							
Right	301	152	1.00 (ref)			Not included	
Left	328	153	0.87 (0.69–1.09)	0.217		Not included	
Rectum	288	111	0.66 (0.52–0.85)	0.001		Not included	
BRAF							
BRAF wt	822	359	1.00 (ref)		818	1.00 (ref)	
BRAF mut	55	39	2.48 (1.78–3.45)	<0.001	54	1.57 (1.07–2.29)	0.021
CIMP							
CIMP−	868	379	1.00 (ref)		813	1.00 (ref)	
CIMP+	62	44	2.47 (1.80–3.37)	<0.001	59	1.84 (1.28–2.63)	<0.001
*MLH1*methylation							
MLH1 unmeth	926	422	1.00 (ref)			Not included	
MLH1 meth	4	1	0.51 (0.07–6.60)	0.497		Not included	
R status							
R0	738	255	1.00 (ref)		691	1.00 (ref)	
R1	19	10	1.93 (1.02–3.62)	0.042	19	1.55 (0.82–2.94)	0.179
R2	170	155	6.51 (5.28–8.01)	<0.001	162	4.92 (3.07–7.88)	<0.001

Meth, methylated; mut, mutation; unmeth, unmethylated; wt, wild type.

status, and MSI or *MLH1* methylation and *BRAF* mutation. The effect was strongest among patients with MSS tumors. Interestingly, CIMP status could further identify high risk patients among the poor prognosis group of patients with MSS and *BRAF* mutated tumors, a finding that remained significant after adjusting for stage, R status and age.

Already in 1993, we reported that patients with MSI showed an improved prognosis compared to those with MSS tumors.[14] This finding was later confirmed in a meta-analysis by Popat *et al.*[12] Within the already good prognostic MSI group, CIMP does not seem to contribute with prognostic value,[34–36] which is in agreement with our results. In the MSS group, however, several reports have demonstrated that patients with CIMP positive tumors have a significantly poorer survival compared to those with CIMP negative tumors.[16–18, 35–38] With some exceptions, including the study by Barault *et al*[36] ($n = 582$ samples), the significant effect of CIMP is often lost in multivariate analyses. In this context a small sample size will be an obvious limitation, reducing the statistical power in subgroup analyses.[21] In the present study, including >1000 patients, we confirmed that CIMP conferred a worse survival in the MSS subgroup. The inferior survival among patients with CIMP positive tumors remained significant in multivariate analyses, both across all samples and in the MSS subgroup.

Furthermore, patients with MSS *BRAF* mutated tumors have been shown to have a particularly poor prognosis.[38,39] We observed the same, and further demonstrated that CIMP was significantly associated with inferior survival among patients with MSS *BRAF* mutated tumors. CIMP thus defined

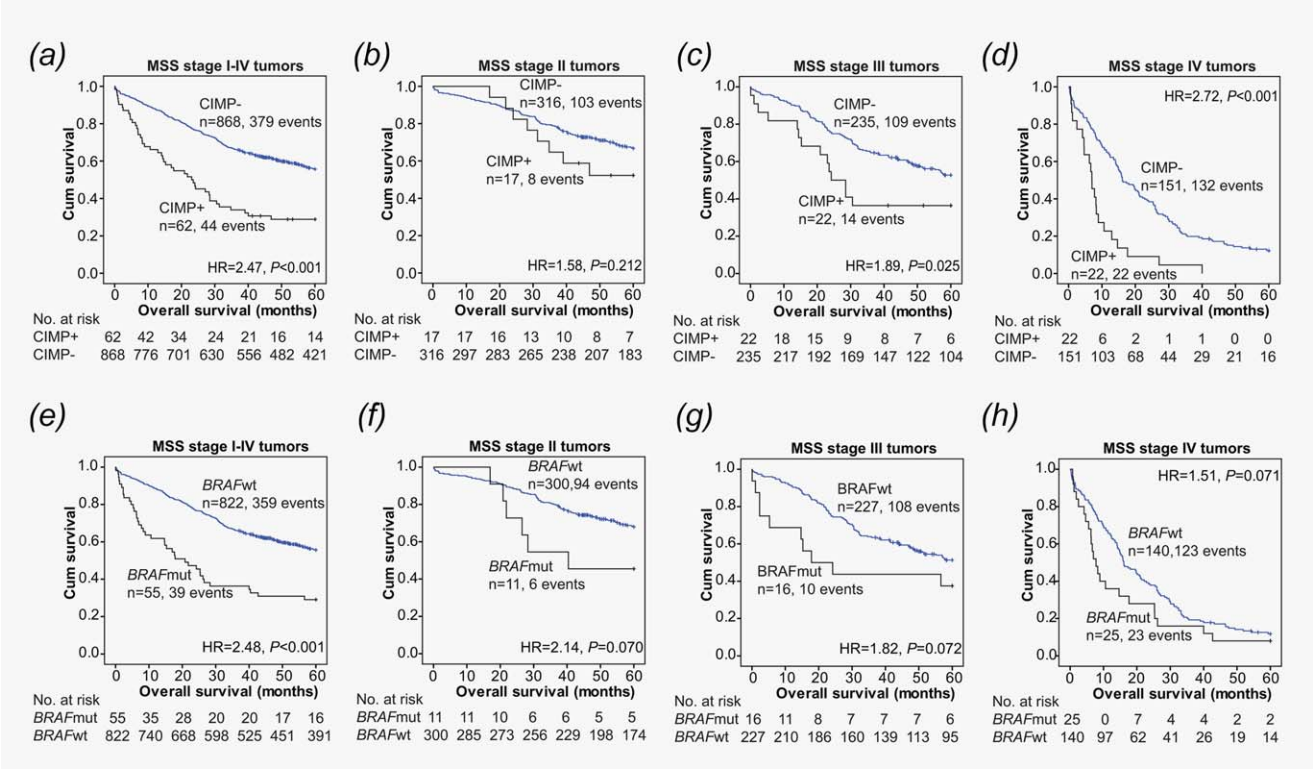

Figure 1. Kaplan Meier curves modeling the effect of CIMP (A-D) and *BRAF* (E-H) on OS among patients with MSS tumors.

a subgroup with worse prognosis among these patients. By analyzing 236 MSS tumors, Kim *et al*[17] also observed that patients with CIMP positive MSS *BRAF* mutated tumors had a particularly bad prognosis. CIMP was, however, not included in the final multivariate model, and they concluded that the inferior survival among patients with CIMP positive *BRAF* mutated tumors was attributed to the *BRAF* mutation.[17] Samowitz *et al*[38] also looked at CIMP and *BRAF* among patients with MSS tumors. Although they found that patients with *BRAF* mutant CIMP positive tumors had a significantly worse survival compared to those with CIMP negative *BRAF* wild type tumors, they also concluded that the bad prognostic effect on survival was entirely due to the *BRAF* mutation. In their adjusted analysis of MSS tumors, CIMP was not significantly associated with OS or cancer specific survival.[38] This is in contrast to our study, where CIMP is included in the final multivariate model and significant with both TTR and OS as end points. Furthermore, from our results it seems like it is the combination of CIMP and *BRAF* within MSS tumors that provides the worse survival. Patients with either a *BRAF* mutated or a CIMP positive tumor does not seem to have an inferior survival compared to patients with MSS tumors without any of these alterations. Interestingly, Phipps et al[40] recently reported on the survival of 2050 colorectal cancer patients based on classification into five subgroups according to CIMP, MSI and *BRAF* and *KRAS* mutation status.[41] Compared to patients with type four tumors (MSS, CIMP negative, *BRAF*/*KRAS* wild type), patients with type two tumors (MSS, CIMP positive, *BRAF* mutation) had the highest disease-specific mortality.[40] Although this stratification approach did not focus directly at the prognostic effect of CIMP within the MSS, *BRAF* mutated subgroup, it clearly underscores that patients with MSS, *BRAF* mutated CIMP positive tumors have a particularly poor outcome. We further show that this finding persisted in patients with stage II, III or IV disease.

We additionally observed that patients with stage IV CIMP positive tumors had a poorer prognosis, irrespective of *BRAF* and/or MSI status. This has also been observed by others,[42–44] suggesting that CIMP may be used as a poor prognostic marker for advanced disease.

From a subset of the samples included in the Oslo 2 series, data indicated that the prognostic effect of CIMP might depend on the age of the patient. However, these findings were not replicated across a larger sample series (Oslo 1). Instead we observed that CIMP was associated with worse survival independent of age. Nevertheless, this is an interesting observation, and to our knowledge, few studies have looked at the prognostic effect of CIMP in younger (<60) versus old (>75) colorectal cancer patients. Approximately 40% of colorectal cancer patients are older than 75 years. Still, they are underrepresented in clinical trials of adjuvant therapy, mainly due to higher rates of co-morbidities.[45] This results in sparse data on how to best treat this group of patients, including whether they benefit from adjuvant therapy. Forty-four percent of the

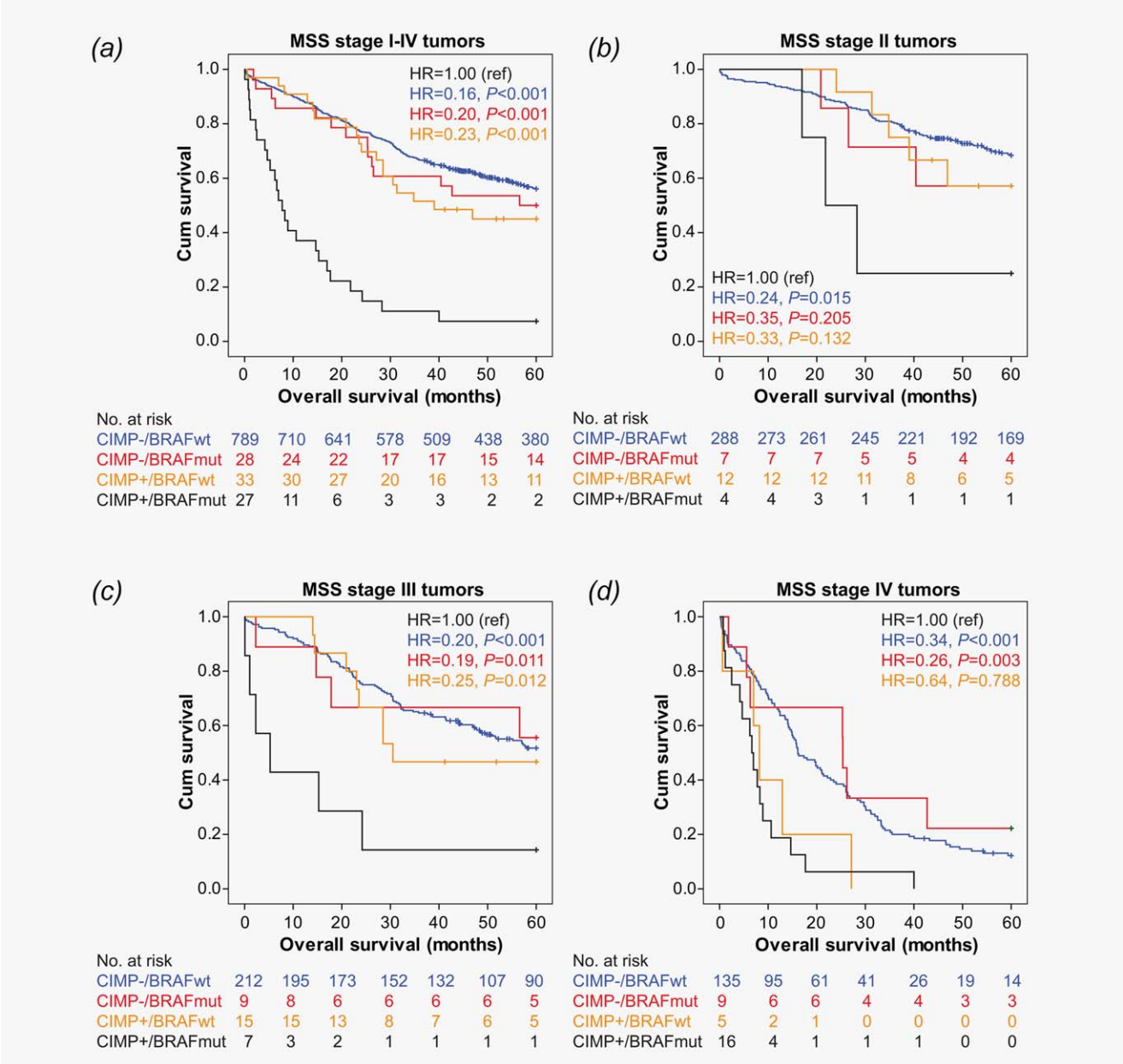

Figure 2. Effect of CIMP and *BRAF* on OS in patients with MSS tumors estimated by Kaplan Meier method.

Table 4. Combined effect of CIMP and *BRAF* in MSS tumors.

		Overall survival					
	Total, n	Events, n	Univariate HR (95% CI)	*P* value	Total, n	Multivariate HR (95% CI)	*P* value[1]
CIMP-*BRAF*							
CIMP−/*BRAF*wt	789	341	0.16 (0.11–0.24)	<0.001	785	0.28 (0.18–0.43)	<0.001
CIMP−/*BRAF*mut	28	14	0.20 (0.10–0.38)	<0.001	28	0.33 (0.17–0.64)	0.001
CIMP+/*BRAF*wt	33	18	0.23 (0.12–0.42)	<0.001	33	0.40 (0.21–0.76)	0.005
CIMP+/*BRAF*mut	27	25	1.00 (ref)		26	1.00 (ref)	

[1]Ajusted for age, stage, and R status. Mut, mutation; wt, wild type.

patients included in the present study were 75 years or older. Worse survival of CIMP within the MSS and MSS *BRAF* mutated subgroup was also seen among these patients, indicating that CIMP status could be applicable to guide the choice of treatment in all colorectal cancer patients, including the elderly.

An apparent challenge within the CIMP literature is the high variation across studies when it comes to choice of gene panel, marker threshold and method to define CIMP, and also the lack of consistent stratification based on molecular and clinical features.[46] These factors may influence the association between CIMP and survival, and also makes meta-analyses challenging.[47] To avoid potential biases introduced by qualitative methods such as methylation specific PCR (MSP),[48] we have used quantitative MSP (qMSP) to analyze an accepted and validated CIMP panel.[5,22] In accordance with other publications,[5,16, 49] we find that CIMP is commonly associated with the *BRAF*^{V600E} mutation, MSI, *MLH1* methylation, female gender, tumor localization, and older age, underscoring that the series used here is compatible to that of others. All patients included have control follow-ups at the same hospital and the clinical data are continuously quality controlled and updated. The high number of samples included in this study combined with the high-quality clinical data provides a unique opportunity for robust analyses, also within patient subgroups.

In the present study, we have implemented a selection procedure by AIC in order to determine the best-fitting model from the initial set of clinicopathological data. This approach enables discarding of spurious variables, which may have added noise to the model and also helps preventing over-fitting. We combined the selection procedure with a bootstrap re-sampling method in order to guarantee the stability of the final model. By running the whole procedure independently for TTR and OS, stage, age, R status and CIMP were all systematically included in both models. In addition to stage, age and R status, which are variables known to be associated with survival, these results strongly suggest that CIMP is also a robust marker for survival among colorectal cancer patients, and should be considered in the prognosis of the disease.

In conclusion, we report that CIMP is associated with worse prognosis in colorectal cancer patients after adjusting for other factors, and specifically among patients with MSS and MSS *BRAF* mutated tumors.

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