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Sex and outcomes of patients with microsatellite instability-high and *BRAF* V600E mutated metastatic colorectal cancer receiving immune checkpoint inhibitors

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ABSTRACT

Background Immune checkpoint inhibitors (ICIs) are the gold standard therapy in patients with deficient mismatch repair (dMMR)/microsatellite instability-high (MSI-H) metastatic colorectal cancer (mCRC). A significant proportion of patients show resistance, making the identification of determinants of response crucial. Growing evidence supports the role of sex in determining susceptibility to anticancer therapies, but data is lacking for patients with MSI-H CRC.

Methods In this real-world cohort comprising 624 patients with MSI-H mCRC receiving ICIs, we investigated the impact of sex on patients' outcomes, overall and according to *RAS-BRAF* mutational status or type of treatment (anti-PD-(L)1 with or without anti-CTLA-4 agents). We then investigated these associations also in two independent cohorts of patients with early-stage or advanced MSI-H CRC unexposed to ICIs. Finally, we explored two public microarray and RNA-seq datasets from patients with non-metastatic or metastatic MSI-H CRC to gain translational insights on the association between sex, *BRAF* status and immune contextures/ICI efficacy.

Results Although no differences were observed between females and males either overall or in the *BRAF* wild-type cohort, male sex was associated with inferior progression-free survival (PFS) and overall survival (OS) in the *BRAF* mutated cohort (in multivariable models, HR for PFS: 1.79, 95% CI: 1.13 to 2.83, $p=0.014$, and for OS: 2.33, 95% CI: 1.36 to 3.98, $p=0.002$). Males receiving anti-PD-(L)1 monotherapy had the worst outcomes, with a 3-year PFS and 3-year OS of 23.9% and 41.8%, respectively, while the addition of anti-CTLA-4 agents rescued such a worse outcome. We also observed that females experienced a higher frequency of any-grade immune-related adverse

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Deficient mismatch repair (dMMR)/microsatellite instability-high (MSI-H) metastatic colorectal cancer (mCRC) is sensitive to immune checkpoint inhibitors (ICIs), but a portion of patients display primary or acquired resistance. Sex has been found to determine susceptibility to ICIs in other disease settings, but data is scarce for dMMR/MSI-H CRC.

WHAT THIS STUDY ADDS

⇒ Male sex was associated with worse outcomes in patients with *BRAF* mutated dMMR/MSI-H mCRC treated with ICIs, especially when receiving anti-PD1 monotherapy. Contrarily, it was not found to be prognostic in independent cohorts of early-stage and advanced dMMR/MSI-H CRC unexposed to ICIs. An enrichment of androgen receptor signature and an immune-depleted microenvironment observed in males with *BRAF* mutated MSI-H mCRC may be responsible for the poorer sensitivity to ICIs.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ A complex interplay between sex and *BRAF* status may modulate ICIs activity MSI-H mCRC, potentially paving the way to sex-tailored strategies.

events. Conversely, sex was not prognostic in the independent cohorts of patients with MSI-H CRCs not treated with ICIs. Exploratory transcriptomic analyses suggest that tumors of males with *BRAF* mutated MSI-H metastatic CRC are characterized by an enrichment of androgen receptor signature and an immune-depleted

microenvironment, with a reduction in memory B cells, activated natural killer cells, and activated myeloid dendritic cells.

Conclusions Overall, our findings suggest a complex interplay between sex and *BRAF* mutational status that may modulate the activity of ICIs in patients with MSI-H mCRC and pave the way to novel tailored strategies.

BACKGROUND

Incorporating sex and gender medicine and adopting sex-specific prevention or treatment strategies in oncology is imperative, given the growing evidence indicating distinct genetic, metabolic, and immunological differences between female and male patients with cancer.^{1,2} In colorectal cancer (CRC), female sex is strongly associated both with *BRAF*V600E mutations and deficient mismatch repair (dMMR)/microsatellite instability-high (MSI-H) status. *BRAF* mutations and microsatellite instability are also associated with each other but given their relatively uncommon frequency in patients with metastatic colorectal cancer (mCRC), they co-occur in only 2% of the overall population. This unique scenario represents a promising opportunity for the application of precision medicine.^{3,4} Testing for dMMR/MSI-H is recommended to guide the upfront choice of immune checkpoint inhibitors (ICIs) in patients with mCRC regardless of *RAS* and *BRAF* mutational status.⁵ In patients with dMMR/MSI-H tumors, even in the presence of distant metastases, ICIs achieve long-term disease control or even cure in a significant proportion of patients.⁶ Yet, a significant proportion of patients exhibit primary or secondary resistance to single agent anti-PD-(L)1 therapy. To overcome this, combination strategies are being investigated, and CTLA-4/PD-1 dual blockade has shown better efficacy compared with PD-1 blockade alone, at price of higher costs and adverse events (AEs) burden.⁷ Recent studies have investigated predictive factors of ICI response, including clinical features such as ascites, liver metastases, worse Eastern Cooperative Oncology Group performance status (ECOG PS), as well as molecular biomarkers (eg, tumor mutational burden, DNA mutational signatures, or gene expression signatures).^{8–13} With compelling data in other cancers,¹⁴ sex is another putative factor modulating immunotherapy efficacy. This may stem from sex-specific genetic and hormonal differences influencing innate and acquired immunity. The effects of oestrogens are dose- and context-dependent: physiological levels are associated to a greater production of type 1 interferon (IFN) and efficient antigen presentation, while high levels of oestrogens and progesterone (eg, in pregnancy) induce an immune-tolerant and anti-inflammatory state.^{15,16} Contrarily, androgens may lead to immunosuppression by interfering with the IFN- γ signaling pathway and by promoting T cell exhaustion.^{1,17} However, the relationship of sex and ICI response in MSI-H CRC has not been thoroughly investigated. Further, the clinical application of determinants of response or resistance is challenging, particularly in rare patient subgroups such as those with co-occurring dMMR/MSI-H mCRC and *BRAF* V600E mutations, where both ICIs and dual *BRAF*/EGFR

targeting are viable treatment options. Clinical trials often fail to report sex-associated outcomes, as this objective is rarely pre-specified and the limited statistical power of subgroup analyses. In such instances, real-world data emerges as a valuable resource, providing the opportunity to investigate patient features, such as sex, in rare tumors or small subgroups. These findings may then inform the clinical practice or the design of dedicated clinical trials.

To investigate the role of sex as a determinant of the efficacy of ICIs in patients with dMMR/MSI-H mCRC, we assembled a multinational cohort of 624 patients receiving ICIs as any line of treatment and with available *RAS* and *BRAF* mutational status.

METHODS

Study population

Patients with dMMR/MSI-H metastatic CRC treated with anti-PD-(L)1 monotherapy or anti-CTLA-4-based combination therapy in any line were retrospectively retrieved from 11 academic hospitals in European Union and USA. MMR and/or MSI status were locally assessed using immunohistochemistry, multiplex PCR and/or next-generation sequencing as per standard institutional practices. Clinical and pathological baseline characteristics before ICI therapy were age, sex, ECOG PS, primary tumor sidedness (right vs left), primary tumor resection, mucinous histotype, time-to-metastases (synchronous vs metachronous; synchronous metastatic disease was defined by diagnosis of metastases within 6 months from surgery or de novo diagnosis of metastatic or locally advanced unresectable disease), number of metastatic sites (≥ 2 vs 1), metastatic sites, ICI treatment line and ICI treatment type (anti-PD-(L)1 monotherapy vs anti-CTLA-4-based combination). Immune-related adverse events (irAEs) were defined as the AE that occurred after ICI start, considered to result from immunological dysfunction by treating physicians. irAEs were graded based on National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) V.5.0. We grouped pruritus (without skin rash), anemia, infusion reactions, xerpthalmia, etc under the category 'Other'.

Statistical analysis

Continuous variables were expressed as the median and IQR, and categorical variables were expressed as absolute values and percentages. Progression-free survival (PFS) was defined as the time from the start of ICI treatment to the evidence of disease progression or death from any cause. Overall survival (OS) was defined as the time from the start of ICI treatment to death from any cause or last follow-up. PFS and OS analyses were estimated according to the Kaplan-Meier method, and treatment groups were compared using the log-rank test. Cox proportional hazard model was used to estimate hazard ratios and 95% CI. Follow-up time was estimated using the reverse Kaplan-Meier method. In Cox proportional hazards regression models, all the covariates showing a

statistically significant association with PFS and OS in the univariable model were included in the multivariable model. P values <0.05 were considered statistically significant. The Kaplan-Meier estimator and Cox proportional hazards regression were used for survival analysis using the survival, survminer, and survMisc packages. All statistical analyses were performed using R statistical software (R V4.1.1).

Analyses of independent datasets of non-metastatic BRAF mutated, dMMR/MSI-H CRC

The Molecular and Cellular Oncology Study (MCO, Australia, n=1395)^{18,19} was a prospective study of participants undergoing curative resection for CRC from 1994 to 2010. Clinical and pathological data were collected for all cases, including clinical follow-up collected annually up to 5 years. The Cancer Genome Atlas (TCGA) project plans to profile genomic changes in 20 different cancer types, we here used the colorectal cancer cohort (TCGA-COADREAD, online supplemental material section 1).²⁰ DACHS ('Darmkrebs: Chancen der Verhütung durch Screening' (colorectal cancer: chances for prevention through screening))²¹ is an epidemiological case-control study conducted by the German Cancer Research Center (DKFZ) in Heidelberg.

Analyses of independent datasets of metastatic dMMR/MSI-H CRC

Patients with dMMR/MSI-H mCRC treated with chemotherapy were selected from a French multicenter real-world data cohort that included dMMR/MSI-H mCRC patients in 2007–2017 in 18 French hospitals, a Dutch nationwide population-based real-world data cohort that included dMMR/MSI-H mCRC patients in 2014–2019 from all Dutch hospitals, and all the dMMR/MSI-H mCRC patients from three randomized phase III studies (CAIRO, CAIRO2 and CAIRO3) in the period 2003–2012. Details are available elsewhere.^{22–25}

Transcriptomic analyses

Two publicly available datasets were used for the analyses: a cohort of patients with ICI-treated metastatic MSI-H/dMMR CRC and available formalin-fixed, paraffin-embedded (FFPE) blocks (Gallois *et al.*)¹⁰, and a cohort of patients with non-metastatic MSI-H/dMMR CRC who underwent upfront surgery and available FFPE blocks (GSE39582, Marisa *et al.*)²⁶. In both instances, patients with co-occurring BRAF V600E mutation were included for the analyses. Methods of gene expression normalization were previously documented in the original publications providing these datasets and are summarized in online supplemental table 1. To ensure that pathway alterations and immune cell differences between genders were unique to BRAF profiles rather than from gender alone, we excluded pathways or immune cell differences exhibiting concordant changes across both the BRAF mutated and BRAF wild-type subgroups.

Dimension reduction was performed with Uniform Manifold Approximation and Projection (UMAP). Ellipses stratified by gender were included and computed using the ggbiplot package. Differential gene expression across gender and BRAF status subgroups were retrieved using the limma²⁷ package, with omission of the voom transformation as the gene expression data were already normalized. limma fits linear models to the data and applies empirical Bayes methods to moderate the standard errors of the estimated log-fold changes. Pathway analyses were undertaken using Gene Set Enrichment Analysis (GSEA). Gene set signatures queried the Molecular Signatures Database (MSigDB) HALLMARK database and the Gene Ontology (GO) Biological Process (BP) database. We also included the androgen receptor (AR) signature score as described by Mendiratta *et al.*²⁸, and an immune filtration gene signature described by Yoshihara *et al.*²⁹. For GSEA, statistical significance was determined by a lenient false discovery rate (FDR) q-value threshold of <0.25 to ensure potentially biologically relevant pathways are captured in this exploratory analysis. P value adjustment was conducted with the Benjamini & Hochberg method.³⁰ For immune cell type deconvolution, the LM22 signature matrix implemented through CIBERSORT was used. Cytokine signaling activity was retrieved with CytoSig as described by Jiang *et al.*³¹. Comparisons of CIBERSORT cell abundance scores and cytokine signaling activity were retrieved with an unpaired Student's t-test. A two-sided p value of 0.05 was considered to be statistically significant.

All bioinformatic analyses were conducted in R-4.4.2 with packages umap, ggplot2, limma, immunedeconv, ggbiplot, and gsea.

RESULTS

Patients and disease baseline characteristics

Baseline features in the overall population, BRAF V600E mutated or wild-type subgroups, are summarized in table 1. Briefly, no significant differences in patient and disease baseline characteristics including sex were observed among patients with BRAF V600E mutation; some imbalances were instead evident in the BRAF wild-type subgroup.

Impact of sex on ICI efficacy and toxicity according to RAS/BRAF mutational status

In the whole cohort, after a median follow-up of 49.9 months (IQR: 33.1–69.2), no significant differences in PFS and OS were found between males compared with females, with HR 1.01 (95% CI 0.79 to 1.26, p=0.99) and 1.03 (95% CI 0.79 to 1.36, p=0.83), respectively (online supplemental figure 1a,b).

Conversely, in BRAF V600E mutated cohort (n=161), after a median follow-up of 46.8 months (IQR: 31.6–71.1), male patients had inferior PFS (3-year PFS: 42.2% vs 56.7%; HR=1.42, 95% CI: 0.92 to 2.19, p=0.11) and OS (3-year OS: 53.2% vs 70.1%; HR=1.82, 95% CI: 1.10 to 3.00, p=0.019) compared with females (figure 1a,b). To

Table 1 Baseline patients' and disease characteristics of the overall cohort (n=624) and of patients with *BRAF* wild-type (n=463) and *BRAF* mutated (n=161) status grouped by sex

Characteristics		Overall n=624 n (%)	<i>BRAF</i> wt n=443		P value*	<i>BRAF</i> mut n=161		P value*
			Females n=199 n (%)	Males n=264 n (%)		Females n=96 n (%)	Males n=65 n (%)	
Sex	Male	329 (53)						
	Female	295 (47)						
Age at ICI start	Median (IQR)	60 (18–91)	60 (21–86)	51 (18–91)	<0.001	70.5 (64–76.3)	70 (64–77)	1
ECOG PS at ICI 0 start		305 (49)	96 (47)	141 (53)	0.184	47 (49)	24 (34)	0.178
	1–2	319 (51)	106 (53)	123 (47)		49 (51)	41 (64)	
Primary tumor location	Right colon	421 (67)	130 (65)	147 (56)	0.046	87 (91)	57 (88)	0.739
	Left colon/rectum	203 (33)	69 (35)	117 (44)		9 (9)	8 (12)	
Primary tumor resection	Yes	515 (83)	160 (80)	218 (83)	0.634	80 (83)	57 (88)	0.592
	No	109 (17)	39 (20)	46 (17)		16 (17)	8 (12)	
Mucinous histotype	Yes	238 (38)	66 (33)	101 (38)	0.308	44 (46)	27 (42)	0.837
	No	375 (60)	128 (64)	157 (60)		52 (54)	38 (58)	
	NA	11 (2)	5 (3)	6 (2)		/	/	
<i>RAS</i> status†	Wild-type	315 (50)	141 (71)	174 (66)	0.3			
	Mutated	148 (24)	58 (29)	90 (34)				
Time to metastases	Synchronous	327 (52)	116 (58)	120 (46)	0.007	52 (54)	39 (60)	0.568
	Metachronous	297 (48)	83 (42)	143 (54)		44 (46)	26 (40)	
Number of metastatic sites	1	281 (45)	83 (42)	128 (48)	0.175	44 (46)	26 (40)	0.568
	≥2	343 (55)	116 (58)	136 (52)		52 (54)	39 (60)	
Liver metastases	Yes	236 (38)	84 (42)	103 (39)	0.55	28 (29)	21 (32)	0.836
	No	388 (62)	115 (68)	161 (61)		68 (71)	44 (68)	
Lung metastases	Yes	114 (18)	32 (18)	48 (18)	0.64	23 (25)	11 (17)	0.363
	No	510 (82)	167 (82)	216 (82)		73 (75)	55 (83)	
Nodal metastases	Yes	358 (57)	110 (55)	150 (57)	0.813	58 (60)	41 (63)	0.82
	No	266 (43)	89 (45)	114 (43)		38 (40)	24 (27)	
Peritoneal involvement	Yes	255 (41)	89 (45)	93 (35)	0.048	42 (44)	31 (48)	0.74
	No	369 (59)	110 (55)	171 (65)		54 (56)	34 (52.3)	
ICI treatment line	1st	174 (28)	60 (30)	64 (24)	0.188	30 (31)	20 (31)	1
	≥2nd	450 (72)	139 (70)	200 (76)		66 (69)	45 (69)	
ICI regimen	aPD-(L)1 mono	423 (68)	150 (75)	165 (62)	0.005	66 (69)	42 (65)	0.706
	aCTLA-4 combo	201 (32)	49 (25)	99 (38)		30 (31)	23 (35)	

Continued

Table 1 Continued

Characteristics	Overall n=624 n (%)	<i>BRAF</i> wt n=443		P value*	<i>BRAF</i> mut n=161		P value*
		Females n=199 n (%)	Males n=264 n (%)		Females n=96 n (%)	Males n=65 n (%)	

Significant (<0.05) p values are displayed in bold.

*Fisher test or Mann-Whitney test as appropriate.

†Typical *RAS* driver mutations (codons 12, 13, 59, 61, 117, and 146 in *KRAS* and *NRAS*).

CT, chemotherapy; ECOG PS, Eastern Cooperative Group Performance Status; ICI, immune checkpoint inhibitor.

better understand the interaction of ICI intensity and sex in the *BRAF* mutated cohort, we investigated the outcomes according to both sex and the type of ICI treatment (anti-PD-(L)1 monotherapy vs anti-CTLA-4-based combination). Males with *BRAF* mutations receiving anti-PD-(L)1 monotherapy had the worst outcomes, with a 3-year PFS and 3-year OS of 23.9% and 41.8%, respectively (figure 1c,d). Interestingly, when patients received a combination of anti-CTLA-4 and anti-PD-1 therapies, overall outcomes were better and no significant survival differences were observed between male and female patients (3-year PFS: 76.3% vs 68.8%, and 3-year OS: 75.0% vs 82.6%, respectively) (figure 1c,d). In multivariable models including both treatment type and sex, as well as the other candidate prognostic features (online supplemental table 2), male sex was significantly associated with worse PFS (HR=1.79, 95% CI 1.13 to 2.83, p=0.014) and OS (HR=2.33, 95% CI 1.36 to 3.98, p=0.002), along with anti-PD-(L)1 monotherapy, presence of peritoneal and lung metastases, and mucinous histotype (figure 1e,f). In contrast, in the *BRAF* wild-type subgroup, PFS (p=0.56) and OS (p=0.40) were not significantly different in male vs female patients (online supplemental figure 2a,d), irrespective of the type of ICI treatment (online supplemental figure 2b,e) and *RAS* mutational status (*RAS* mutated vs *RAS/BRAF* wild-type) (online supplemental figure 2c,f). Therefore, the interaction test p values between sex and *BRAF* mutational status were 0.106 and 0.019 for PFS and OS, respectively.

Finally, we compared the frequency of irAEs, overall and by organ involved, according to sex (online supplemental figure 3). In terms of any-grade overall toxicity, there were no differences between females vs males in the entire cohort (47% vs 45%; p=0.6), nor in the *BRAF* wild-type subgroup (43% vs 48%, p=0.4), but females with *BRAF* mutation did experience significantly more irAEs than males (57% vs 35%, OR=2.49, 95% CI 1.11 to 5.88, p=0.02). In the *BRAF* mutated population, we observed better outcomes in terms of PFS and OS for both females and males experiencing irAEs compared with those who did not experience irAEs, with the worse OS showed by male patients without toxicities (online supplemental figure 4).

Association of sex and survival outcomes in patients with MSI-H CRC unexposed to ICIs

To investigate whether sex may be prognostic in patients with dMMR/MSI-H and *BRAF* V600E mutated CRC not receiving ICIs, we took advantage of three independent cohorts of patients with non-metastatic disease (Methods): Molecular and Cellular Oncology study^{18 19} (MCO, n=108), the DACHS²¹ (n=194), and The Cancer Genome Atlas²⁰ (TCGA, n=59). With consistency across the three cohorts, sex was not associated with OS either in *BRAF* mutated or in *BRAF* wild-type subgroups (online supplemental figure 5). Then, we focused on advanced disease and pooled different cohorts (Methods): patients enrolled in the CAIRO, CAIRO-2, and CAIRO-3 trials evaluating chemotherapy-based regimes; and a real-world data cohort of Dutch and French patients (figure 2a), altogether reaching a total of 364 patients with available *BRAF* status (online supplemental table 3). PFS and OS were not significantly different both in the *BRAF* mutated and wild-type subgroup (figure 2b-e).

Sex differences in transcriptomic analyses of MSI-H CRC

To gain translational insights on the influence of sex in *BRAF* V600E mutated and dMMR/MSI-H CRC, we explored two publicly available datasets (Methods), GSE39582²⁶ (microarray, non-metastatic setting) and Gallois *et al.*¹⁰ (RNA-seq, metastatic setting). In the cohort of patients with metastatic disease, 22 females and 16 males from a total of 103 patients were identified as carrying *BRAF* mutation and dMMR/MSI-H status. From the cohort of patients with non-metastatic CRC, 22 females and 9 males out of 566 patients had both *BRAF* mutation and dMMR/MSI-H status (online supplemental table 4).

Broad differences in dimensionally reduced gene expression profiles were appreciated across sex in *BRAF* mutated dMMR/MSI-H CRC regardless of stage; however, the converse was not appreciated in *BRAF* wild-type subgroup (figure 3a). Gene expressions of putative targets such as PD-1 (*PDCD1*), PD-L1 (*CD274*), and CTLA4 were inspected and compared across gender and *BRAF* status. A non-significant trend towards reduced gene expression of PD-L1 (p=0.37), PD-1 (p=0.39), and CTLA4 (p=0.22) was found in males in the *BRAF* mutated

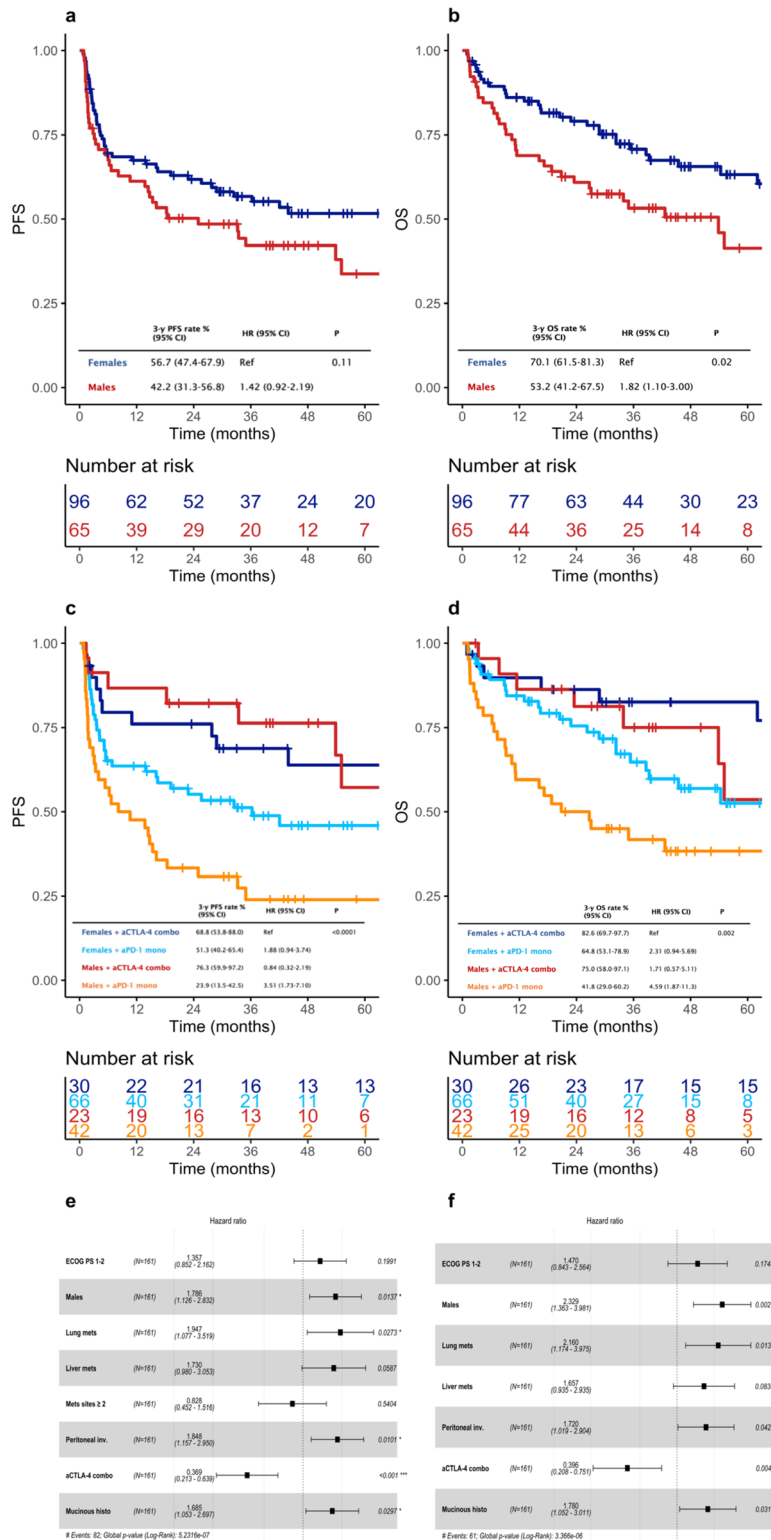


Figure 1 Male sex is associated with worse outcomes in *BRAF* mutated deficient mismatch repair (dMMR)/microsatellite instability-high (MSI-H) metastatic colorectal cancer (mCRC) treated with ICIs. (a–d) Exploratory Kaplan-Meier analysis of the PFS and OS probability (with 95% CIs) and 3-year PFS and OS rates (as percentages) according to sex (a, b) and type of immune checkpoint inhibitors (ICIs) treatment (c, d) in patients with *BRAF* V600E mutated dMMR/MSI-H mCRC (n=161). (e, f) Multivariable Cox proportional hazards regression models of the PFS (e) and OS (f) in patients with *BRAF* V600E mutant dMMR/MSI-H mCRC (n=161). Covariates showing a statistically significant (p<0.20) association with PFS and OS at univariable models were further included in the multivariable models. At multivariable models, p values <0.05 were considered statistically significant. Abbreviations: aCTLA-4 combo, combination; aPD-1 mono, monotherapy; ECOG, Eastern Cooperative Oncology Group; histo, histology; inv, invasion; mets, metastasis; OS, overall survival; PFS, progression-free survival; PS, performance status; Ref, reference.

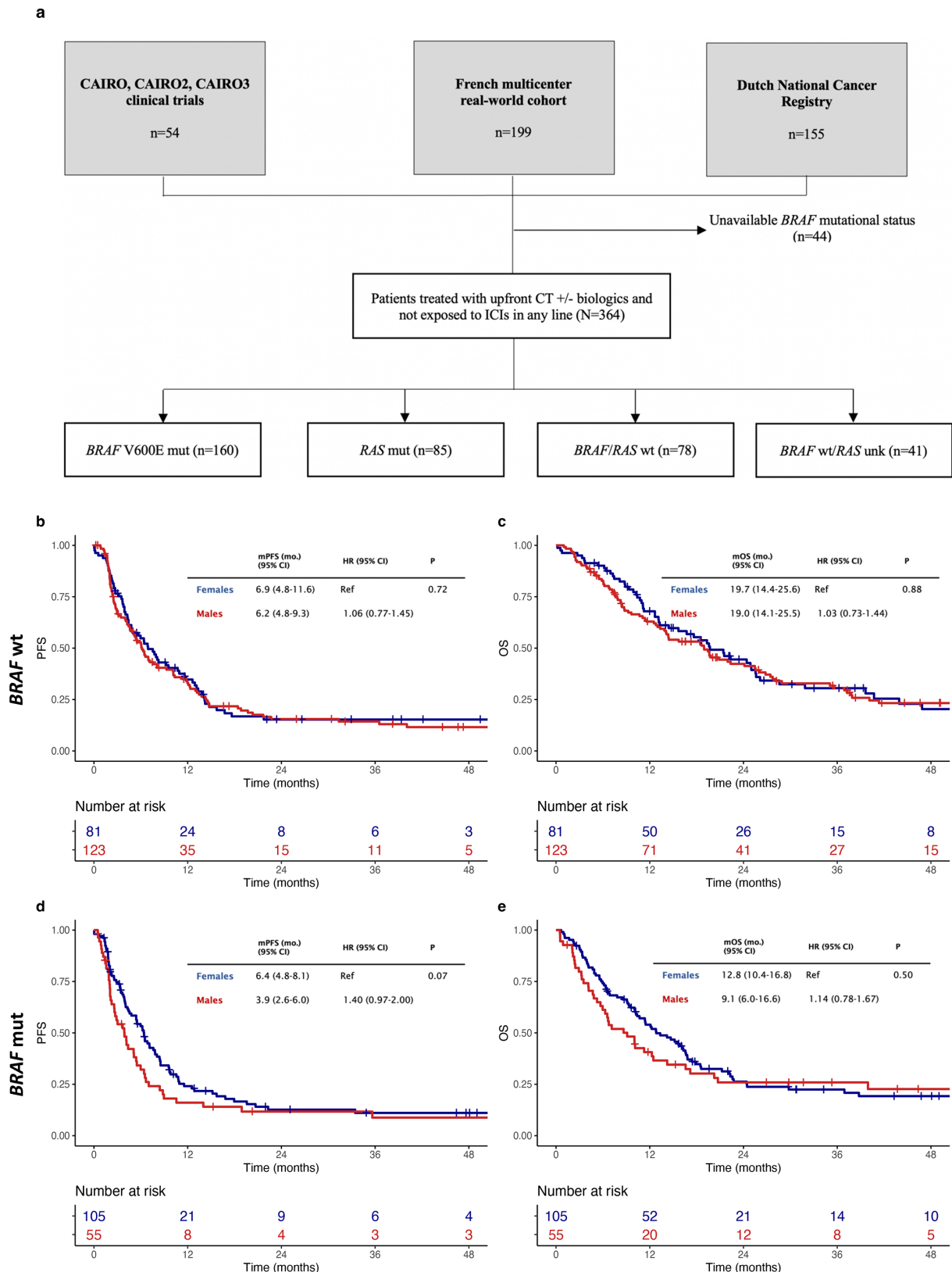


Figure 2 Sex is not associated with different prognosis in patients with metastatic deficient mismatch repair (dMMR)/microsatellite instability-high (MSI-H) and *BRAF* V600E mutated CRC treated in the pre-immunotherapy era. (a) Flow chart of collection of a large independent cohort of patients with metastatic dMMR/MSI-H CRC who were treated with chemotherapy with or without biologics in the pre-immunotherapy era (n=408), further selected for availability of *BRAF* mutational status (n=364). (b–e) Exploratory Kaplan-Meier analysis of PFS and OS according to sex in patients with dMMR/MSI-H CRC with (d, e) or without (b, c) *BRAF* V600E mutation. Abbreviations: mOS, median OS; mPFS, median PFS; mut, mutated; OS, overall survival; PFS, progression-free survival; Ref, reference; wt, wild-type.

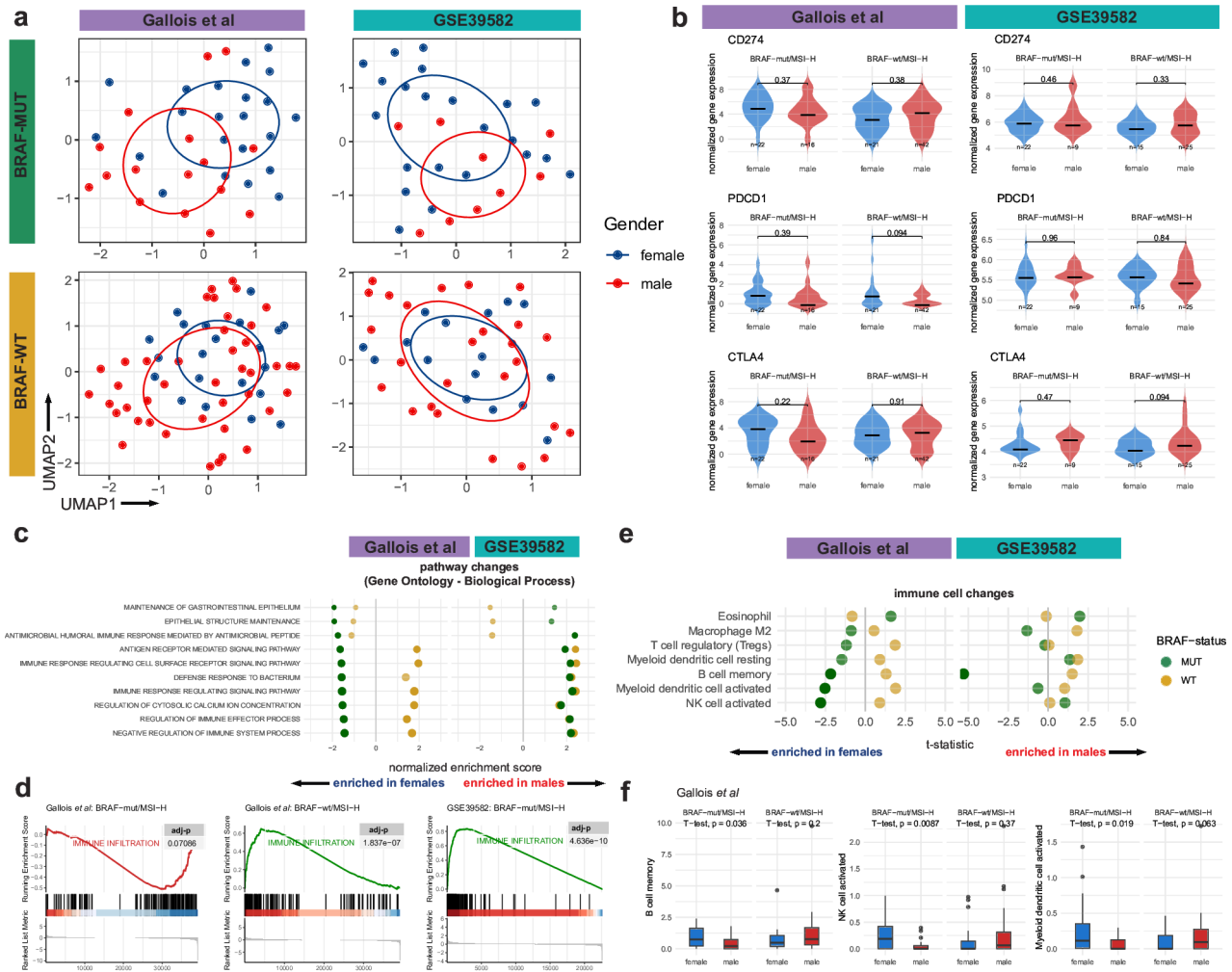


Figure 3 Sex differences between *BRAF* mutated and *BRAF* wild-type deficient mismatch repair (dMMR)/microsatellite instability-high (MSI-H) CRC. (a) UMAP of participants from Gallois *et al* and GSE39582 stratified by *BRAF* mutational status. (b) Gene expression comparisons of PD-1 (*PDCD1*), PD-L1 (*CD274*), and CTLA4. P values were retrieved with an unpaired T-test. (c) GSEA pathway analysis with GO:BP gene signatures. Pathways were included if the comparisons in the *BRAF*-mut subgroup in the metastatic setting had an FDR q-value <0.05 and |NES| greater than 1.00. Raw GSEA results are provided in online supplemental material section 2. NES values were opaque if the FDR q-value was <0.05. (d) GSEA enrichment plots of the immune infiltration signature across gender/*BRAF* subgroups. (e) CIBERSORT deconvoluted immune cell type changes. T-statistic values were opaque if a two sided p value retrieved from an unpaired T-test was <0.05. Raw results are provided in online supplemental material section 2. (f) Boxplot of selected CIBERSORT deconvoluted immune cell type changes in the metastatic cohort. Abbreviations: FDR, false discovery rate; GO:BP, Gene Ontology:Biological Process; GSEA, gene set enrichment analysis; MUT, mutant; NES, normalized enrichments score; UMAP, Uniform Manifold Approximation and Projection; WT, wild-type.

metastatic cohort. Conversely, this phenomenon was not appreciated in the non-metastatic cohort (figure 3b).

Congruent with our clinical findings, pathway analyses suggest an immune-cold tumor microenvironment (TME) in males with *BRAF* mutation in the metastatic setting. Downregulation of gene expression signatures of GO:BP pathways such as antigen receptor-mediated signaling pathway (normalized enrichment score (NES)=-1.64, adjusted p value=0.0078, FDR q-value=0.0078), immune response regulating signaling pathway (NES=-1.55, adjusted p value=0.002, FDR q-value=0.002), and regulation of immune effector process (NES=-1.48, adjusted p value=0.04, FDR q-value=0.04) was appreciated in males with *BRAF*

mutation in the metastatic setting (figure 3c). These changes were not appreciated in males with *BRAF* wild-type status in the metastatic setting or in the non-metastatic setting. In view of these changes, we also inspected an immune infiltration gene signature and found that in the metastatic setting, males carrying *BRAF* mutation had a depleted immune infiltration gene signature compared with females (NES=-1.48, adjusted p value=0.07, FDR q-value=0.07). Conversely, the immune infiltration signature is enriched in male patients with (NES=2.99, adjusted p value=4.64e-10, FDR q-value=1.24e-10) or without (NES=2.15, adjusted p value=1.83e-07, FDR q-value=1.1e-07) *BRAF* mutation in the non-metastatic setting (figure 3d). Other pathway

changes are described in online supplemental material section 2.

AR signaling has been implicated in immunosuppression.³² Analogously, there was a trend towards enrichment in the AR signaling signature among males with *BRAF* mutation, despite this being statistically significant only in the non-metastatic setting (NES=1.26, adj-p=0.097, q-value=0.03) but not the metastatic setting (NES=0.951, adj-p=1.000, q-value=0.949) (online supplemental figure 6); (online supplemental figure 7 and online supplemental material section 2).

To further unravel the tumor immune microenvironment, we inspected immune cell type changes with CIBERSORT deconvolution. Across both cohorts of patients with metastatic and non-metastatic disease, memory B cells were found to be lower in males in *BRAF* mutated subgroup (metastatic: p=0.036; non-metastatic: p=2.25e-05). Unique to the metastatic setting, we found activated myeloid dendritic cells (DCs) (p=0.019) and activated natural killer (NK) cells (p=0.009) were significantly lower in males compared with females in the *BRAF* mutated cohort (figure 3e,f). Next, we inspected cytokine profiles with CytoSig.³¹ We also note an immunosuppressive cytokine profile in males with *BRAF* mutation in the non-metastatic setting, characterized by higher IL10 (p=0.08), CXCL12 (p=0.07), and BMP4 (p=0.01). These cytokine changes were however not appreciated in the metastatic setting (online supplemental figure 8).

DISCUSSION

In this study, we showed that male sex is associated with inferior PFS and OS in patients with dMMR/MSI-H and *BRAF* V600E mutated mCRC. This association was statistically significant in multivariable models with other putative prognostic markers: the type of ICI treatment, specific sites of metastases, ECOG PS, and mucinous histotype, the latter being a well-known factor associated both with MSI-H status and *BRAF* mutations. Along with male sex, the type of ICI strategy (PD-1 blockade alone or combined with CTLA-4 inhibition) had the strongest prognostic effect in multivariable models and the worst outcomes were observed in males receiving anti-PD-(L)1 monotherapy, with almost all patients experiencing disease progression within 3 years. Further, we investigated the association of irAEs with sex, as this aspect is often neglected by randomized clinical trials (RCTs). We showed that females with *BRAF* mutations experience more irAEs than males, particularly thyroid-related AEs; also, despite the limitation of potential immortal time biases, male patients with *BRAF* mutated tumors and no irAEs had the worst OS. This is in line with previous evidence suggesting that irAEs may be associated with higher efficacy of ICIs in several tumor types and endocrine-related toxicity may be associated with better outcomes in patients with dMMR/MSI-H mCRC.³³ Although female sex is associated with development of autoimmune diseases and could be intuitively associated

with higher risk of developing irAEs, conflicting data exist on this topic; the difference in terms of irAEs across sex was not observed in the overall cohort but only in the *BRAF* mutated subgroup.^{34–36} The negative impact of male sex was not replicated in cohorts of patients both with advanced and early stage dMMR/MSI-H CRC irrespective of *BRAF* status, supporting a potential predictive rather than prognostic role of sex and its association with the efficacy of ICIs in the peculiar context of the tumor biology of *BRAF* mutated tumors. Exploratory transcriptomic analyses show that male patients with *BRAF* mutated MSI-H CRC are characterized by an immune-depleted TME, which may explain why these patients experience worse outcomes on PD-1 blockade alone. Indeed, we show that PD-1/CTLA-4 co-inhibition may rescue the worse outcomes observed in male patients with dMMR/MSI-H and *BRAF* mutated tumors, posing the question of whether this patient population should be treated with upfront combinatorial strategies. However, caution must be taken due to the retrospective nature of this work. Further, as dMMR/MSI-H and *BRAF* mutated mCRC are frequently diagnosed in the elderly, several patients may not be fit enough to receive anti-CTLA-4-based combinations, due to the higher risk of adverse events compared with anti-PD-1 monotherapy.⁷

Our data are in line with the hypothesis that sexual dimorphism can elicit differential responses to specific treatments. In a previous hallmark study, the efficacy of BRAF/MEK co-targeting was negatively influenced by male sex in patients with both early-stage and advanced melanoma. Neoadjuvant BRAF/MEK co-targeting induced AR overexpression in non-responders, but the use of anti-androgens sensitized both male and female murine models to BRAF/MEK co-inhibition³⁷; however, no data are available yet on *BRAF* V600E mutated CRC. Focusing on the interaction between sex and the efficacy of ICIs, a meta-analysis of RCTs—mostly in melanoma and non-small cell lung cancer (NSCLC)—had inferred that male patients derive greater benefit on ICI treatment compared with females.¹⁴ However, this finding may not be easily transferable to other tumors or molecular subgroups, where sex has a crucial role in cancer risk and development. It is widely accepted that sex hormones possess immunomodulatory properties. Androgens can contribute to immunosuppression by blocking T-cell differentiation into T helper (Th)1 and Th17 cells³⁸ and by promoting expansion of T regulatory lymphocytes.³⁹ They have been further implicated in repression of antigen priming by DCs⁴⁰ and of cytotoxic activity by NK cells.⁴¹ It has also been demonstrated that AR signaling drives T-cell exhaustion through repression of IFN- γ in murine prostate cancer, eventually dampening the efficacy of ICIs, and that this effect can be avoided through AR blockade.⁴² In line with the preclinical evidence that androgen suppression can result in thymic functions' restoration⁴³ and an increase in the number and activity of CD4⁺ and CD8⁺ T lymphocytes,⁴² early clinical data suggest a potentially synergistic action of

ICIs and androgen deprivation therapy in male patients affected by advanced, immune-refractory melanoma.⁴⁴ Similarly, an enrichment of AR signature was observed also in our analyses from tumors of male patients with *BRAF* mutated dMMR/MSI-H CRC. Further, tumors of these patients exhibited a markedly 'cold' TME, characterized by decreased infiltration of DCs, NK, and B cells, an increased level of immunosuppressive chemokines, and a trend for decreased expression of immune checkpoint molecules. This is in line with a previous work in NSCLC showing a more abundant immune infiltration in tumors from female patients.⁴⁵ This may explain why CTLA-4 blockade, primarily acting by enhancing T cells priming and by promoting Treg depletion,⁴⁶ may be able to improve the outcomes of males with *BRAF* mutation refined by an immune-depleted phenotype.

Our study has relevance for clinical practice and research. The CheckMate-8HW trial has compared dual PD-1/CTLA-4 blockade with nivolumab and ipilimumab or nivolumab alone vs chemotherapy as first-line treatment of patients with dMMR/MSI-H mCRC. The initial results on the PFS comparison between ICIs combo vs chemotherapy have been recently published⁴⁷ and ipilimumab/nivolumab combo did not have a differential effect in males vs females. However, further analyses according to both sex and *BRAF* mutational status should be performed to carefully dissect the benefit of dual ICI strategies in specific subpopulations, especially when compared with ICI monotherapy. Indeed, the trial may validate our real-world evidence on the worst outcomes in male patients with *BRAF* mutations receiving PD-1 blockade alone. Accordingly, patients in this subgroup may derive the greatest benefit from the addition of an anti-CTLA-4 agent, although the higher toxicity of a combination regimen may be a critical issue. Additionally, our results may prompt the rational development of new combinatorial strategies concerning the addition of androgenic blockade to ICIs in a sex-oriented personalized algorithm: male patients with *BRAF* mutated MSI-H CRC could benefit from the addition of antiandrogenic drugs, which are usually well-tolerated also in elderly, fragile population, to their PD-1 blockade backbone, to reduce the resistance rate. In this scenario, the role of inhibition of AR signaling and its synergy with ICIs should be further investigated in dMMR/MSI-H and *BRAF* mutated CRC preclinical models, such as tumor-organoid T cell coculture platforms.⁴⁸

Our study has some limitations. First, our study lacks an independent validation cohort of patients with dMMR/MSI-H mCRC receiving ICIs. Second, the predictive impact of sex on the efficacy of immunotherapy should be validated in RCTs, such as the previously mentioned CheckMate-8HW or the KEYNOTE-177 study. In particular, the KEYNOTE-177 compared pembrolizumab to standard chemotherapy as first-line treatment of patients with dMMR/MSI-H mCRC. Data on *RAS/BRAF* mutational status was available in approximately two-thirds of patients, leading to the availability of only 34 and 43

patients with *BRAF* mutated and wild-type status in the experimental and control arms, respectively, who would have been further categorized by sex.⁴⁹ The statistical power of this post-hoc analysis would be limited. Third, while our analyses uncover a significant association of sex and *BRAF* V600E mutation with clinical outcomes, the inherent heterogeneity of real-world evidence represents a constraint for the distinction between the predictive vs prognostic role of sex in *BRAF* mutated and dMMR/MSI-H mCRC. Lastly, although the transcriptomic analyses suggest that an immune-cold TME and increased AR signaling specific of male patients with *BRAF* mutation may contribute to ICI refractoriness, they only offer preliminary insights into the complex interplay between *BRAF* mutation, MSI-H CRC and immune response. Further preclinical investigations may help uncover the mechanistic explanations of our findings.

In conclusion, in the largest global cohort reported to date, we show a significant and functionally reasonable interaction between sex and *BRAF* V600E mutation in patients with dMMR/MSI-H mCRC receiving ICIs. Ultimately, our research may lead to the design of sex-oriented trials focused on the selective use of CTLA-4 co-inhibition in specific patients subgroups or combination of ICIs with hormonal therapy to optimize the treatment strategies of both female and male patients with *BRAF*V600E mutated, dMMR/MSI-H mCRC.

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