Scientific Reports

https://doi.org/10.1038/s41598-026-56380-1

Article in Press

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Received: 12 March 2026

Accepted: 31 May 2026

Published online: 05 June 2026

Cite this article as: Gandini A., Tosi F., Carmisciano L. *et al.* Absence of co-occurrence between *HER2* amplification and dMMR/MSI in a large multicentric colorectal cancer cohort. *Sci Rep* (2026). https://doi.org/10.1038/s41598-026-56380-1

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Absence of co-occurrence between *HER2* amplification and dMMR/MSI in a large multicentric colorectal cancer cohort.

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Abstract

Mismatch repair deficiency (dMMR)/Microsatellite instability (MSI) plays a central role in colorectal cancer (CRC) as a predictive and prognostic biomarker and as a "red flag" for diagnosis of Lynch Syndrome (LS). *HER2* is emerging as a therapeutic target and clinical trials are ongoing. Several reports suggested that *HER2* amplification could be mutually exclusive with dMMR/MSI, which is mostly associated with *HER2* mutations or other alterations. Clinical and molecular aspects in this field mostly derive from subgroup analyses of trials and real-life data are lacking. We retrospectively collected data on 350 dMMR/MSI CRC patients who underwent HER2 testing in two Italian referral centers from 2016 to 2024. *HER2* amplification was evaluated with immunohistochemistry (IHC) and FISH for IHC 2+ patients. Median age was 75 years, 84% were stage II and stage III. *BRAF* and *RAS* mutation occurred in 60% and 14% of the patients with available molecular data, respectively. No patients harbored *HER2* amplification, suggesting that the co-occurrence of HER2 amplification and dMMR/MSI is essentially anecdotal, making therefore HER2 testing in this subgroup less compelling.

Keywords: colorectal cancer, dMMR/MSI, HER2, precision medicine.

Highlights:

• No *HER2* amplification detected in 350 dMMR/MSI CRCs from two different centers.

• Co-occurrence of *HER2* amplification and dMMR/MSI appears anecdotal in CRC.

• Routine HER2 testing in dMMR/MSI CRC may be unnecessary.

Background

The panorama of colorectal cancer (CRC) treatment has evolved in the last few decades thanks to the identification of molecular biomarkers able to predict response to specific therapies. The major determinant in CRC molecular classification is the identification of mismatch repair deficiency (dMMR)/microsatellite instability (MSI), which has both prognostic and therapeutic implications. dMMR/MSI is due to a deficit in the DNA mismatch repair system (MMR), which accounts for around 15% of all CRCs. Immunohistochemistry (IHC) is considered the most cost-effective methodology for testing as it allows the identification of the loss of expression of MMR proteins (MLH1, PMS2, MSH2, or MSH6), leading to the dMMR phenotype. Molecular biology defines the MSI phenotype, and it is usually recommended in equivocal IHC results in CRCs [1]. The identification of dMMR/MSI CRCs has now become mandatory at the time of diagnosis, regardless of the stage of the disease, due to two main reasons. First, it allows the identification of patients at risk of being affected by Lynch Syndrome (LS), an autosomal dominant inherited disorder caused by a germline pathogenic variant in the MMR genes [2]. Moreover, it predicts sensitivity to immunotherapy, both in early and advanced disease, which has revolutionized the therapeutic algorithm and the prognosis of these patients [3]. dMMR/MSI CRCs show a different clinical behavior when compared to proficient MMR (pMMR)/microsatellite stable (MSS) CRCs, and the impact on prognosis seems to vary between different stages of disease. While in advanced disease (5% of all CRCs), before the advent of immunotherapy, it identified a subpopulation with poorer prognosis [1], in localized stages, it is associated with better prognosis when compared to pMMR/MSS, even if this better prognostic impact seems to be lost in stage III high-risk patients [4]. Due to the deficit in the DNA repair mechanism, and the subsequent accumulation of mutations, dMMR/MSI patients seem to be enriched in targetable genomic alterations, such as *HER2* mutations, *NTRK* fusions [5], as well as *BRAF V600E* mutation, which excludes the possibility of LS [6]. In this regard, although *BRAF V600E* represents an actionable alteration, microsatellite instability remains the dominant therapeutic driver, making immunotherapy the preferred upfront therapeutic choice. Accordingly, ongoing first-line trials such as SEAMARK are evaluating the combination of immunotherapy with anti-BRAF and anti-EGFR inhibitors [7]. However, the known clinical and molecular aspects of dMMR/MSI CRCs mostly derive from subgroup analyses of selected clinical trials, and data on large real-life cohorts are lacking. With the increasing identification of targetable alterations in CRC, such as *KRAS G12C* mutations and *HER2* amplification, optimizing patient selection for molecular testing has become a relevant clinical issue. Current ESMO guidelines recommend extended next-generation sequencing (NGS) in advanced disease only when it does not generate additional costs, and not in localized CRC [8]. While all*RAS* and *BRAF* testing are routinely performed in the metastatic setting because of their predictive value for

target therapies, *HER2* amplification testing is not yet standard in Europe, despite emerging targeted options [9]. *HER2* amplification occurs in approximately 2–3% of metastatic CRCs and has been associated with poor prognosis and resistance to anti-EGFR therapies [10–12], while its prevalence in localized disease appears to be even lower [13,14]. Importantly, preliminary evidence suggests that *HER2* amplification may be exceptionally rare in dMMR/MSI tumors, raising the hypothesis of a potential mutual exclusivity between these two molecular alterations [15]. If confirmed, this would have practical implications by identifying patient subsets in whom *HER2* testing is unlikely to be informative, thereby avoiding low-yield analyses and improving the efficiency of precision medicine strategies.
The aim of this study is to evaluate clinical and molecular characteristics of a large multicentric real-world cohort of unselected dMMR/MSI CRC patients, focusing on the co-occurrence of dMMR/MSI status and *HER2* amplification as proof-of-concept.

Results

Study population

Overall, 350 dMMR/MSI CRCs were identified, 293 from Genoa and 57 from Milan. Patients were mostly female (66%), with a median age of 75 years. Most patients had a localized CRC at the time of the diagnosis (stage I to III = 93%), and proximal tumors (86%), defined as right-sided or transverse colon cancer. More than half of CRCs were high grade - G3 and G4 (193/350 cases – 56%). Focusing on the IHC results in this cohort of dMMR/MSI CRC patients, loss of MLH-1/PMS2 was the most frequently detected (87%). Complete patients' characteristics are reported in Table 1.

Characteristics	N = 350 (%)

Age – mean (SD)	74.93 (11.41)

Sex	
Male	119 (34)
Female	231 (66)

Primary tumor location	
Right/transverse colon	297 (86.1)
Left colon	40 (11.6)
Rectum	6 (1.7)
Multiple	2 (0.6)
Unknown	5

Disease stage at diagnosis	
I	32 (9.1)
II	189 (54)
III	105 (30)
IV	24 (6.9)

Histotype	
Adenocarcinoma	310 (88.6)
Other histology	40 (11.4)

Grade	
Low	153 (44)
High	193 (56)
Unknown	4

***BRAF* status**	
Mutated	171 (48.9)
Wild type	110 (31.4)
Unknown	69 (19.7)

***RAS* status**	
Mutated	10 (2.9)
Wild type	59 (16.9)
Unknown	281 (80.3)

Loss of MMR Proteins by IHC	
MLH-1 PMS-2	304 (86.9)
MSH-2 MSH-6	24 (6.9)
Other	22 (6.2)

HER2	
Positive	0
Negative	349
Unknown	1

Table 1: *Patient characteristics. Acronyms: dMMR= deficit of the Mismatch repair system. MSI= Microsatellite instability. IHC= Immunohistochemistry. SD= standard deviation.*

Pathological and molecular variables

Among the 350 patients, *RAS* status was available for 69 patients (20%) and, among these, 14% were mutated. Around 80% of the patients were tested for *BRAF* status, and 60% of these were mutated. None of the 350 patients tested for *HER2* showed protein overexpression/gene amplification. Only one patient with a HER2 IHC 2+ was not tested through FISH and thus should be considered as "unknown". Details on HER2 testing results are available in Table 2.

Assuming the prevalence of HER2 in the general CRC population is 2% [10], and the prevalence of dMMR/MSI in non-metastatic CRC is around 15% [16], then, under the assumption of independence, their joint prevalence would be approximately 0.3%. Under this same independence assumption, approximately 7 HER2 positive cases would be expected among 350 dMMR/MSI patients. In our cohort of 350 CRC dMMR/MSI patients, we identified no HER2 positive cases. We estimate a prevalence of HER2 in MSI cases between 0% and 0.9% with 95% confidence. The probability of observing a number of HER2 cases of 0 under the assumption of independence between HER2 and MSI is ~ 0.085%. Although we cannot formally establish mutual exclusivity, the absence of any HER2-positive cases in our MSI cases series indicates that their co-occurrence is essentially anecdotal.

Genoa						Milan			
GC scoring		Heracles scoring				Heracles scoring			
IHC results		IHC results		FISH performed	FISH ampl.	IHC results		FISH performed	FISH ampl.
Score 0	197	Score 0	197	1	0	Score 0	41	0	0
Score 1+	45	Score 1+ Score 2+ <50%	45 35	0 35	0 0	Score 1+ Score 2+ <50%	13 0	0 0	0 0
Score 2+	49	Score 2+ >50%	14	13*	0	Score 2+ >50%	3	3	0
Score 3+	2	Score 3+ <50% Score 3+ >50%	2 0	2 0	0 0	Score 3+ <50% Score 3+ >50%	0 0	0 0	0 0

Table 2: *Results of HER2 testing in Genoa and Milan, according to both scoring systems (GC and Heracles). *One patient with an IHC score of 2+ did not undergo FISH analysis due to technical issues.*

Survival and genetic data

After a median follow-up of 31.2 months (95%CI from 28.0 to 35.0), median overall survival (OS) was 76.9 months (95%CI: from 70.5, upper confidence limit not reached) across all stages. OS was strictly related to the stage at diagnosis, as expected, with 3-year OS rate ranging from 92.3% in stage I to 24.6% in stage IV (figure 1).

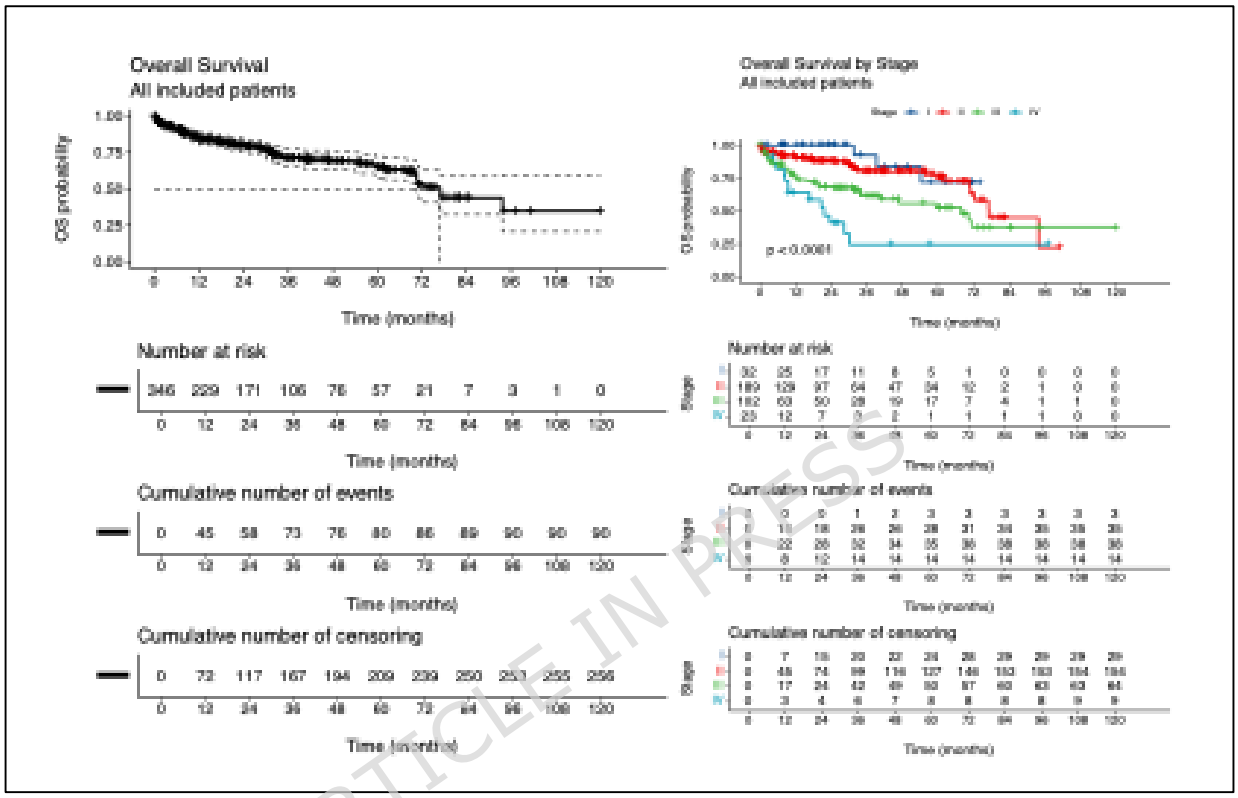

Figure 1: Survival data. *Overall survival in all population and divided by stage. 3-year OS rate was 92.3%, 80.1%, 61.5%, and 24.6% in stage I, II, III, and IV, respectively.*

Among all patients, 31% (n=109) were referred for genetic counseling, and 17 patients were found to be Lynch Syndrome carriers (16% of those tested and 5% of the study cohort). Most patients presented with CRCs with loss of MLH1/PMS2 (304 patients representing 87% of the entire cohort); data on *BRAF* mutation with subsequent *MLH1* promoter methylation status, if performed, according to the Lynch Syndrome diagnostic algorithm, are available for 259 patients. Two-thirds of these patients (211/259 – 81%) had CRCs that showed either *BRAF* mutation or were *BRAF* wild-type with *MLH1* promoter methylation, in line with the literature [17]. On the other hand, among the 46 patients with loss of MMR proteins other than MLH1 (loss of MSH2/MSH6 or isolated loss of either PMS2 or MSH6), 30 underwent genetic counseling (65%), and testing, and 12 were LS carriers (about 40%).

Discussion

The therapeutic landscape of mCRC is rapidly evolving with increasing availability of targeted treatments thus eliciting a growing demand for molecular testing. European guidelines [8] indicate that *HER2* amplifications occur in approximately 2% of mCRC and carry an ESCAT IIB actionability level. Several HER2-targeting regimens (i.e., trastuzumab-lapatinib [18], trastuzumab-tucatinib [9], trastuzumab deruxtecan [19,20]) have reported objective response rates (ORR) of 30–40% in non-randomized studies, and are currently being evaluated in earlier lines of therapy [21]. However, a careful balance between the benefits of identifying actionable alterations and the associated costs is necessary — especially in centers where financial and logistical constraints limit access to comprehensive NGS testing, and where the effort required to detect very rare alterations may not be justified by their low probability of occurrence.

As combination strategies with immunotherapy and targeted therapies are currently being evaluated in the dMMR/MSI population as well [7], we were interested in understanding whether *HER2* amplification should be looked for in this population, by collecting data from two large tertiary referral centers for mCRC management. Only a few studies have evaluated this co-alteration [15,22,23], and most included dMMR/MSI patients within much larger pMMR/MSS mCRC cohorts. In contrast, our series is the first and the largest one to focus exclusively on dMMR/MSI CRC across all disease stages.

In the present cohort, no HER2-positive tumors were identified among 350 dMMR/MSI CRCs. Although a formal demonstration of mutual exclusivity would require explicit verification that no individuals with HER2 also exhibit dMMR/MSI, as well as larger sample size, the observed absence of co-occurrence is highly unlikely under the assumption of independence and suggests that HER2 positivity in dMMR/MSI tumors is exceedingly rare. Importantly, our conclusions apply specifically to *HER2* amplification, while *HER2* mutations, which were not assessed in this study, may represent a distinct and potentially more frequent molecular alteration in dMMR/MSI tumors, as a consequence of their hypermutated phenotype. From a clinical perspective, our results indicate that routine HER2 testing in dMMR/MSI CRC could be omitted or can be limited to research setting. However, it should be acknowledged that the great majority of patients included in our cohort had localized disease (93%), whereas *HER2* amplification seem to occur more frequently in metastatic CRC than in earlier stages [24]. Larger datasets would be required to definitively assess mutual exclusivity.

The inclusion of two high expertise CRC centers using two different HER2 scoring systems enabled a direct comparison between the two approaches. In Genoa, among the 49 patients classified as IHC 2+ according to the GC score, none showed gene amplification by FISH. If patients would be tested

according to the HERACLES criteria, only 14 of these 49 would have undergone FISH testing, resulting in a reduction of costs and turnaround time. Based on our findings, the HERACLES criteria may help reduce the number of FISH analyses required in clinical practice, while no *HER2*-amplified cases were identified among tumors that would not have met HERACLES criteria for reflex FISH testing [25].

Regarding genetic testing, the identification of only 5% of patients with Lynch syndrome in our series is markedly lower than the expected prevalence of this hereditary condition among dMMR/MSI CRCs (approximately 10–20%) [26,27]. The high proportion of CRCs with MLH1 loss and positive reflex testing (i.e., *BRAF* mutation or MLH1 promoter hypermethylation), together with the relatively high median age of our population, might be responsible for the large proportion of non-hereditary cases observed in our cohort. However, the underdiagnosis of hereditary syndromes remains a well-known issue in oncology, with important consequences in terms of delayed cancer detection and avoidable mortality. Indeed, identifying patients at increased genetic risk enables targeted surveillance protocols for second primary tumors, and cascade testing of relatives has been associated with a substantial reduction in cancer mortality—estimated at around 60% in some series [28]. Reflex testing, through the analysis of *BRAF* mutation analysis and MLH1 promoter hypermethylation in CRC patients with loss of MLH1 at IHC, effectively excludes LS. This approach may markedly reduce inappropriate referrals for genetic counseling and improve the detection of truly at-risk individuals. Its feasibility and clinical impact are currently being evaluated in the Italian ItaLynch study, coordinated by our group [29]. Preliminary results were presented at the ASCO Annual Meeting 2025 [30], although final results will be available in 2026.

The main strength of our study lies in the large number of dMMR/MSI CRC patients with available HER2 analysis, all assessed in two major referral centers for pathology and compared using two different scoring systems, ensuring the reliability of the results. Among the limitations, we acknowledge its retrospective design and lack of data on systemic treatments, which prevented a proper evaluation of survival, as well as the fact that FISH confirmation was not performed systematically in all cases, but only in selected equivocal IHC results according to diagnostic algorithms.

In conclusion, this study represents the largest series focusing exclusively on dMMR/MSI and *HER2* amplification in CRC. Our findings, together with available evidence, suggest that the co-occurrence of *HER2* amplification in dMMR/MSI CRC is extremely rare and likely anecdotal. Larger

collaborative datasets are needed to confirm these results and refine clinical recommendations for HER2 testing strategies in this subgroup.

Materials and methods

We have included patients with dMMR/MSI CRC consecutively referred to the University Pathology Unit of IRCCS Azienda Ospedaliera Metropolitana Genoa, University of Genoa, and ASST Grande Ospedale Metropolitano Niguarda, Milan. MMR test was performed by immunohistochemistry (IHC); indeterminate/inadequate cases were sent to molecular MSI testing by PCR. *BRAF* and *allRAS* analyses were conducted in PCR. *RAS* status was analyzed only in selected patients (metastatic, recurrent, or at high risk of recurrence), while *BRAF* was systematically tested after February 2019, after the introduction of the reflex testing for the Lynch Syndrome [30]. Starting in 2016 in Genoa and 2019 in Milan, HER2 IHC was systematically performed in all consecutive CRC cases evaluated at the two centers during the inclusion period. The cases from Genoa were categorized following two different scoring systems for HER2 evaluation: i) Gastric Cancer (GC) scoring system [31], where a cut-off of 10% was used for complete or basolateral membranous reactivity with absent expression in score 0, faint/barely perceptible in score 1, weak to moderate in score 2 and moderate to strong expression in score 3; ii) the HERACLES study [25] scoring system designed specifically for HER-2 evaluation in colorectal cancer where both intensity (as seen above) and percentage of expression are considered (<10%, 10-50%, ≥50%). The cases from Milan were all evaluated with the Heracles criteria. Table 3 shows the differences between the two scoring systems.

GC HER-2 Scoring System			**HERACLES Scoring system**		
			Intensity	**Percentage (%) of cells**	
Score 0	No reactivity or membranous reactivity in <10% of cells	Negative	No staining (0)		Negative
Score 1+	Faint/barely perceptible membranous reactivity in >10% of cells; cells are reactive only in part of their membrane	Negative	Faint staining (1+)	Any	Negative
Score 2+	Weak to moderate complete or basolateral membranous	Equivocal	Moderate (2+)	<50%	Negative

	reactivity in >10% of tumour cells		Moderate (2+)	≥50%	Equivocal
Score 3+	Moderate to strong complete or basolateral membranous reactivity in >10% of tumour cells	Positive	Intense (3+)	≤ 10%	Negative
			Intense (3+)	11 - 49%	Equivocal
			Intense (3+)	≥ 50%	Positive

Table 3: *Comparison of the two different scoring systems for HER2 analysis (GC and Heracles).Equivocal cases require FISH testing in both scoring systems. Acronyms: GC=Gastric Cancer.*

In brief, IHC results of 0 or 1+ were considered as negative, while 3+ were considered as positive. Results of IHC 2+ (in Genoa, according to GC scoring system, while in Milan, only in score 2+ cases with expression in >50% of the tumor) were investigated by fluorescent in situ hybridization (FISH) for *HER2* amplifications. The few cases scored as IHC 3+ but <50% of expression according to the HERACLES scoring system were further evaluated by FISH.

Data were summarized as frequencies and proportions or as median and range. To assess whether the probability of occurrence of HER2-positivity among patients with dMMR/MSI was consistent with the expected frequency under an assumption of independence, we performed a one-sided exact binomial test under the null hypothesis of a 2% HER2 prevalence in CRC [10]. Assuming independence between HER2 positivity and dMMR/MSI status (15% prevalence in non-metastatic CRC [1]), the expected joint prevalence was 0.3%, corresponding to approximately 7 HER2-positive cases among 350 MSI patients. The probability of observing zero HER2-positive cases under this assumption was estimated using a binomial distribution. Overall survival (OS) was defined as the time from the diagnosis to death from any cause. Survival Curves were estimated by Kaplan-Meier method, and differences among subgroups were evaluated using the log-rank test. Median follow-up was estimated by reverse Kaplan-Meier method. Statistical analyses were conducted in R version 4.4.2 (R Core Team, R Foundation for Statistical Computing, Vienna, Austria; 2024). The study was approved by the Regional Ethics Committee of Liguria (CER Liguria; approval numbers 102/2021 and 513/2020) and complied with the Declaration of Helsinki and good clinical practice guidelines. All methods were carried out in accordance with relevant guidelines and regulations. Informed consent was obtained from all subjects.

Author contribution

AG: conceptualization, data curation, formal analysis, investigation, methodology, project administration, writing – original draft.

FT: data curation, investigation, methodology, writing – review and editing.

LC: formal analysis, investigation, writing – review and editing.

FG: data curation, methodology, writing – original draft.

LM, MCA: data curation, methodology, writing – review and editing.

SP, CP, SN, VM, APa, GM, AA, EB, KB, DP, SSi: data curation, writing – review and editing.

SSc, ASB, APu: conceptualization, investigation, methodology, project administration, supervision, validation, writing – review and editing.

Data availability statement

The datasets generated and/or analysed during the current study are not publicly available due to patient confidentiality constraints but are available from the corresponding author on reasonable request.

Funding

None.

Competing interests statement

Authors declare the following conflict of interests, all not related to this manuscript:

AG: speaker honoraria by Merck

FT: advisory role for AstraZeneca, Daiichi-Sankyo, Bayer

APa: consult and Advisory role honoraria by Merck Sharp & Dohme, Bristo-Myers Squibb, Merck Serono, Takeda, Servier, Bayer, Amgen, BeOne, Nordic Pharma, Pierre-Fabre, Natera, Incyte, Astra Zeneca. Research funding by Astra Zeneca, Bayer. Travel Expenses by Merck Sharp & Dohme, Pierre Fabre, Amgen, BeOne.

GM: speaker honoraria by Amgen.

AA: advisory role honoraria by Amgen and Italfarmaco

SSc: Speaker: MSD, Gentili, Novartis, GSK; Travel/accomodation/expenses for congresses: Novartis, Gentili; Round Table: ALTEMS-GSK

APu: reports consulting or advisory roles for GlaxoSmithKline, Takeda Pharmaceuticals U.S.A, Takeda Italia, Bayer, Daiichi Sankyo Italia, MSD Italia, BeOne, Amgen; Speaker Honoraria for Pierre

Fabre, Servier, Amgen, Bristol Myers Squibb (BMS), Daiichi Sankyo, MSD, Merck Serono, BeOne, Takeda; Institutional funding from GlaxoSmithKline, Amgen; Travel, Accommodations, Expenses from AstraZeneca, Amgen, Merck Serono, BeOne, Takeda.

All other authors declare no conflict of interests.

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