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The efficacy of adjuvant chemotherapy with 5-fluorouracil in colorectal cancer depends on the mismatch repair status

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ABSTRACT

Aims: The aim of this study is to evaluate if mismatch repair (MMR) defective colorectal cancer has a different response to adjuvant 5-fluorouracil (5-FU) chemotherapy in a cohort of patients prospectively followed during 5 years.

Methods: The cohort included 754 surgically treated patients with colorectal cancer. MMR status was diagnosed by MLH1 and MSH2 immunohistochemistry and microsatellite instability analysis. Median follow-up was 49.2 months (range 1–73). At inclusion, 505 patients were diagnosed as TNM II or III stage, analysis of the efficacy of adjuvant chemotherapy was made on this population. Adjuvant chemotherapy was applied to 248 patients (98.2% 5-FU based).

Results: MMR deficiency was found in 76 patients (10.1%). No differences were found in overall survival (log-rank $p = 0.3$) or disease-free survival (log-rank $p = 0.3$) regarding MMR status. Adjuvant chemotherapy improves survival in patients in the II or III stage, but this improvement is only evident in patients with MMR-competent tumours (log-rank $p = 0.00001$). Survival of patients with MMR-defective tumours does not improve with adjuvant chemotherapy (log-rank $p = 0.7$). A multivariate analysis showed an independent

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effect of the interaction between MMR status and adjuvant chemotherapy (Hazard ratio 2.04; 95% confidence interval: 1.42–2.93).

Conclusion: In a cohort of colorectal cancer patients, those with MMR-deficient tumours seem not to benefit from 5-FU-based chemotherapy.

1. Background

In the last 15–20 years, adjuvant chemotherapy for colorectal cancer has demonstrated efficacy and it is commonly used.[1] To date, the appropriateness of chemotherapy is decided according to the TNM stage. Since the early 1990s, 5-fluorouracil (5-FU) is the mainstay drug in adjuvant chemotherapy in colorectal cancer, but in the last years, new drugs, such as oxaliplatin and irinotecan, as well as target drugs acting in key points in the tumourigenic cascade, such as cetuximab or bevacizumab have demonstrated their efficacy in the treatment of colorectal cancer.

With the recent availability of these newer chemotherapeutic agents, the potential use of molecular markers to identify which patient may respond better to particular drugs could be specially important. One of the most studied markers is microsatellite instability (MSI). Almost all hereditary non-polyposis colorectal cancers and around a 10–15% of sporadic colorectal cancers develop through a pathway that results from a failure of the DNA mismatch repair (MMR) system, an enzymatic machinery that recognises and repairs damaged DNA.[2,3] These tumours display insertions and deletions of nucleotide repeats at microsatellite sequences, and they are said to have MSI or MMR deficiency. Several studies have demonstrated that patients with MMR-deficient tumours have a better prognosis, and a systematic review has confirmed these results.[4] Early reports showed that MMR-deficient tumours have also a better response to adjuvant chemotherapy but these studies were probably biased by the intrinsic good prognosis of these tumours.[5,6] More recently, retrospective studies have suggested that patients with MMR-deficient tumours do not benefit from 5-FU-based chemotherapy.[7–10] A prospective work from our group also showed lack of efficacy of adjuvant 5-FU chemotherapy in patients with MMR-deficient colorectal cancer after a short-term follow-up.[11] However, until now, there is not any long-term prospective study that confirms this possible lack of efficacy of 5-FU in patients with MMR-defective tumours. The aim this study is to evaluate if MMR-defective colorectal cancers have a different response to adjuvant 5-FU chemotherapy in the same cohort of patients prospectively followed during 5 years.

2. Patients and methods

Between November 2000 and October 2001, all newly diagnosed colorectal cancer patients in 25 hospitals were included in the EPICOLON study, a clinical epidemiology survey aimed at establishing the incidence of hereditary non-polyposis colorectal cancer in Spain.[12,13] Ten of these 25 centres agreed to participate in a nested prospective follow-up investigation. Exclusion criteria were familial adenomatous polyposis, personal history of inflammatory bowel disease and patient's refusal to participate in the study. The study was approved by the institutional Ethics Committee of each participating hospital, and written informed consent was obtained from all patients.

The integrity of the mismatch repair system was evaluated by MSI testing and immunostaining for MSH2 and MLH1 proteins. Both tests were performed in all patients and centralised in two separate centers. Tumour MMR deficiency was defined by either MSI-high (see below) or immunohistochemistry showing a loss of MLH1 or MSH2 protein expression. Analysis of germline mutations in both *MLH1* and *MSH2* genes was done following the method previously described.[13]

Adjuvant chemotherapy was administered according to standard clinical criteria.[14–17] regardless of the MMR status of the tumours. Adjuvant chemotherapy with 5-fluorouracil (5-FU) was given to patients with stages II and III tumours under standard schedules and doses. Oncologists that decided adjuvant treatment were blinded to MMR tumour status.

2.1. *Tumour MSI analysis*

Tissue samples from tumour and normal colonic mucosa were obtained from each patient, and DNA was extracted from them. In those cases in which fresh tissue was not available, archival formalin-fixed, paraffin-embedded samples were used. Genomic DNA was isolated using the QiaAmp Tissue Kit® (Qiagen, Courtaboeuf, France).

Microsatellite instability testing was performed using either the 5-marker panel proposed by the National Cancer Institute or a pentaplex of mononucleotide repeats.[18,19] Tumours were classified as stable if none of the markers showed instability. Tumours with >30% unstable markers were classified as MSI-high and tumours with <30% unstable markers were classified as low-level MSI. Primers were fluorescently labelled and analysed on ABI 310 Genetic Analyser using GeneScan Analysis software (Applied Biosystems, Foster City, CA).

2.2. *Tumour MSH2 and MLH1 protein expressions*

One block of formalin-fixed, paraffin-embedded tumour tissue was selected per case. Before immunostaining, antigen retrieval was performed by immersing sections in a 10-mmol/l concentration of citrate buffer, pH 6.0 and boiling in a pressure cooker for 5 min. Sections were then incubated for 20 min at room temperature with mouse monoclonal antibodies against MLH1 protein (clone G168-15, dilution 1:40;

PharMingen, San Diego, CA) and MSH2 protein (clone FE11, dilution 1:35; Oncogene Research Products, Boston, MA). Ultra-Vision streptavidin-biotin peroxidase detection kit (DAKO, Carpinteria, CA) was used as secondary detection system. The peroxidase reaction was developed using diaminobenzidine tetrachloride as chromogen. Tumour cells were judged to be negative for protein expression only if they lacked staining in a sample in which normal colonocytes and stroma cells were stained. If no immunostaining of normal tissue could be demonstrated, the results were considered ambiguous.

2.3. Statistical analysis

Continuous variables are reported as mean ± standard deviation and categorical variables as frequency or percentages. Statistical differences of basal characteristics between groups were analysed using the χ^2-test for categorical data applying the Yate's correction when required and the Mann–Whitney *U* test for quantitative data.

The primary outcomes were overall survival and disease-free survival. Both analyses were performed in the whole series as well as in the subset of patients with stages II and III tumours to specifically evaluate the effect of MMR status on the benefit from adjuvant chemotherapy. Patients that received adjuvant chemotherapy without 5-FU were excluded from the benefit of 5-FU chemotherapy analysis. Overall survival was defined as the time from study entry to death, and disease-free survival was defined as the time from study entry to death from any cause or the first relapse. Data on overall and disease-free survival were censored at 73 months from the date of diagnosis. Differences in the probability of overall survival or disease-free survival were analysed using the χ^2-test. Survival curves were generated according to the Kaplan and Meier method, and univariate survival distributions were compared with the use of the log-rank test. Intervals of confidence were calculated using the standard error of survival according to the Greenwood method.

A multivariant analysis of hazard risk of death or tumour recurrence, adjusted for TNM stage of disease, age and gender, was performed using Cox proportional hazards regression in a stepwise manner to test the effect of microsatellite instability, adjuvant chemotherapy and interaction term between MMR status and chemotherapy. Hazard ratios and 95% confidence intervals (95% CI) for death were computed using the Cox survival modelling.

All reported *p*-values are two-sided, and *p*-values of less than 0.05 were considered to indicate significance. All calculations were performed using the SPSS 10.0 software (SPSS inc, Chicago, Il, USA).

3. Results

3.1. Relation between mismatch repair status and colorectal cancer prognosis

The cohort included 754 patients. Median follow-up was 49.2 months (range 1–73). Sixteen patients (2.1%) were not operated on due to advanced disease. The rest were surgically treated. Seventy-six (10.1%) tumours were MMR-deficient and 678 (89.9%) were MMR-proficient. Seven patients had germline mutations in *MLH1* (*n* = 5) or *MSH2* (*n* = 2) gene. Characteristics of patients at diagnosis, according to mismatch repair status can be seen in Table 1. We found no significant differences between patients regarding MMR status for age, use of chemotherapy and rates of mortality or recurrence of neoplastic disease. MMR-deficient patients were more frequently females. Forty-two patients (5.6%) were lost to follow-up and their data were included in the survival analysis until the date of loss.

There were 315 (41.8%) deaths over the mean follow-up time of 39.7 months. The median survival for dead patients was 16.3 months (range 1–68). Twenty patients died in the post-operative period (the first 30 d post-surgery), and 244 patients died because of tumour progression. The causes of death of the remaining patients were late post-surgical complications (12 patients), complications of chemotherapy (10 patients) and other causes (29 patients). Tumour recurrence was seen in 170 (22.5%) patients, with a median of 32.5 months after surgery (range 1–68). Chemotherapy was given to 353 patients after surgical treatment and it was 5-FU based in 320 of these patients (90.6%).

To investigate the relationship between MMR status and prognosis, we included the whole cohort of 754 patients. We found no differences in overall survival or disease-free survival on the basis of MMR status (Table 1, Fig. 1A and B). This lack of difference in survival and disease-free survival remained unchanged after the exclusion of patients who died in the post-operative period (data not shown). We did not find any difference in survival or disease-free survival regarding MMR status for any of the TNM stages (Table 2). No differences were found in survival or disease-free survival in patients with stages II or III disease regarding MMR status (Table 3, Fig. 1C and D).

Table 1 – Characteristics of patients

	MMR-proficient (*n* = 678)	MMR-deficient (*n* = 76)	*p*-Value
Age, mean (SD)	70.0 (11.5)	68.4 (13.8)	0.3
Sex, n (%)			
Males	427 (63)	29 (46)	0.0001
Females	251 (37)	47 (62)	
TNM, n (%)			
Stage I	98 (14)	5 (7)	0.08
Stage II	258 (38)	38 (50)	
Stage III	187 (28)	22 (29)	
Stage IV	135 (20)	11 (14)	
Follow-up(mean, months)	39.9	38.5	0.6
Chemotherapy, *n* (%)	322 (47)	31 (41)	0.3
Mortality, *n* (%)	289 (43)	26 (34)	0.2
Recurrence, *n* (%)	157 (23)	13 (17)	0.3

p: chi-square; MMR: mismatch repair; SD: standard deviation.

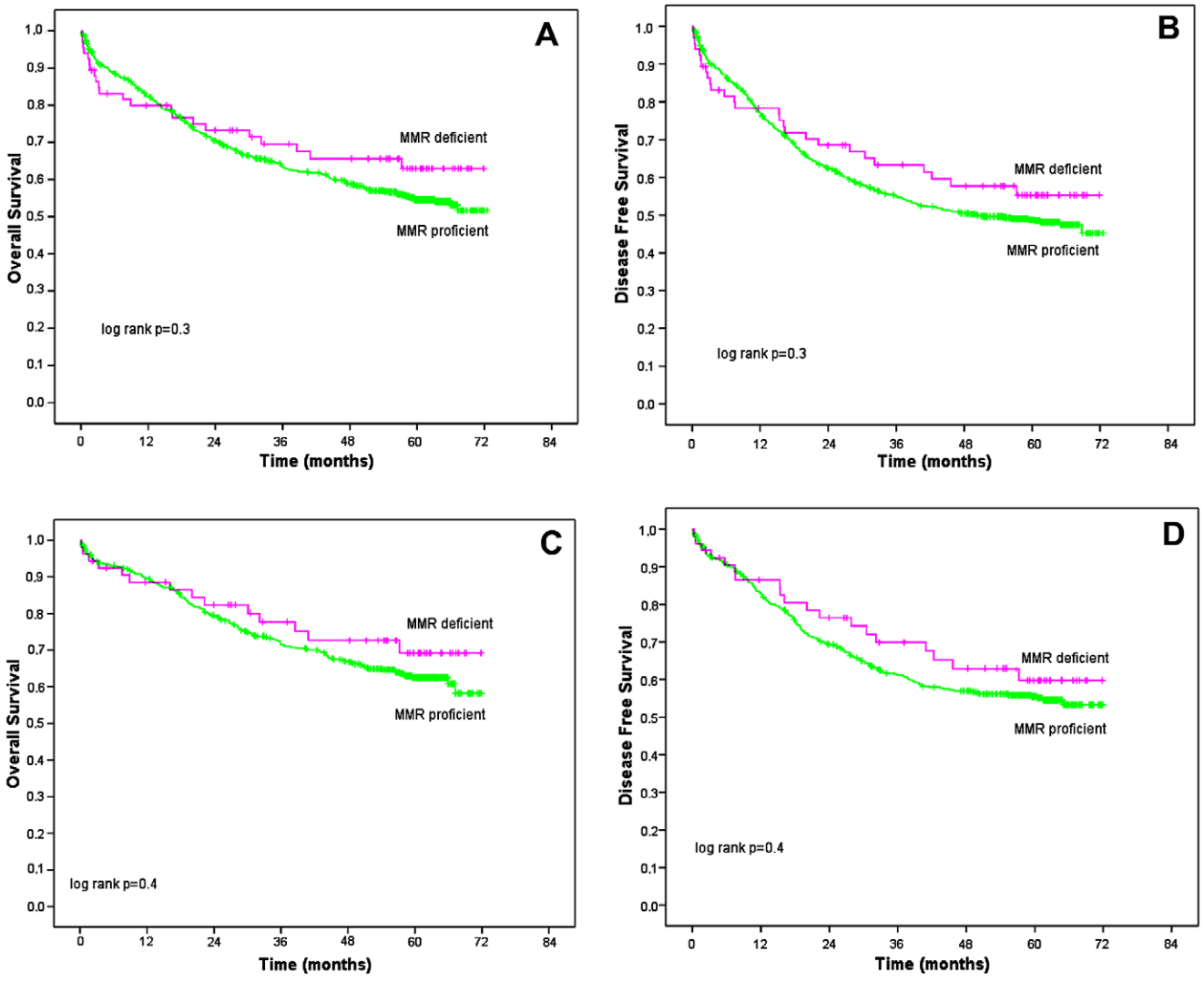

Fig. 1 – (A and B) Overall survival (A) and Disease-free survival (B) in the whole series of mismatch repair (MMR) deficient and proficient tumours. (C and D) Overall survival (C) and disease-free survival (D) in stages II and III disease patients, according to mismatch repair (MMR) status.

Table 2 – Overall survival and disease-free survival according to TNM stage

	MMR-proficient (%)	MMR-deficient (%)	*p*-Value
Stage I			
Overall survival	79/98 (81)	5/5 (100)	0.3
Disease-free survival	73/98 (74)	5/5 (100)	0.2
Stage II			
Overall survival	182/258 (71)	29/38 (76)	0.5
Disease-free survival	163/258 (63)	27/38 (71)	0.3
Stage III			
Overall survival	108/187 (58)	14/22 (64)	0.6
Disease-free survival	91/187 (49)	11/22 (50)	0.9
Stage IV			
Overall survival	20/135 (15)	2/11 (18)	0.7

p: log-rank value; MMR: mismatch repair.

Table 3 – Characteristics of stage II and III patients with MMR-competent versus MMR-deficient

	MMR-proficient (n = 445)	MMR-deficient (n = 60)	*p*-Value
Age	70.9 (10.7)	68.6 (13.6)	0.1
Sex, n (%)			
Males	262 (59)	23 (39)	0.004
Females	183 (41)	37 (61)	
Adjuvant 5-FU Chemotherapy (%)	225 (51)	26 (43)	0.2
Mortality, *n* (%)	155 (35)	17 (28)	0.3
Recurrence, *n* (%)	129 (30)	13 (22)	0.4

p: chi-square; MMR: mismatch repair.

3.2. *Relation between mismatch repair status and benefit of 5-FU adjuvant chemotherapy in stages II and III tumours*

Out of the whole cohort of 754 patients, 505 had stages II and III diseases. Analysis of the benefit of adjuvant chemotherapy was made on this population. After a median follow-up of 44.0 months (range 1–73), 172 (34.1%) patients had died and 142 (28.1%) showed disease recurrence. Two-hundred and sixty stages II and III patients received adjuvant chemotherapy, 125 with stage II (42.2%) and 135 with stage III (64.6%). Adjuvant chemotherapy was based on 5-FU in all but 9 patients (98.2%), all 9 were with MMR-competent tumours and they were excluded from the benefit of 5-FU chemotherapy analysis. Patients who received chemotherapy were younger (65.5 ± 10.2 *versus* 76.1 ± 9.3 years-old; $p = 0.0001$). There were significant differences in the rate of patients treated with adjuvant chemotherapy between the different hospitals participating in the study (χ^2, $p = 0.04$). There were no differences regarding sex, days of follow-up or percentage of lost in follow-up between patients who received adjuvant chemotherapy and patients who did not.

Adjuvant chemotherapy provided an improvement in overall survival and disease-free survival in patients with TNM stages II and III (Table 4). Patients with MMR-proficient tumours showed a better survival when treated with 5-FU adjuvant chemotherapy, with significant increases in the duration (Fig. 2A–C) and the rates of overall survival and disease-free survival (Table 4). In contrast, among patients with MMR-deficient tumours, adjuvant 5-FU chemotherapy did not improve the outcome compared with no adjuvant treatment, neither in terms of overall survival nor in disease-free survival (Fig. 2B–D; Table 4). These differences in the survival benefit were also seen in a multivariate analysis controlled for age, gender and TNM stage (Table 5) and there was a significant and independent effect of the interaction between MMR status and adjuvant chemotherapy. Interaction between both variables was only seen in patient with MMR-proficient tumours. Thus, 5-FU treatment was not associated with better overall survival and disease-free survival in stage II or III patients with MMR-deficient tumours.

In patients who received adjuvant 5-FU chemotherapy, there were no differences in the rates of overall survival and disease-free survival regarding MMR status (Table 4). However, when we analysed only patients that did not receive adjuvant chemotherapy, patients with MMR-deficient tumours showed better rates of both overall survival and disease-free survival, compared with patients with MMR-competent tumours (Table 4). Fig. 3 shows the Kaplan–Meier charts for overall survival and disease-free survival of patients regarding treatment received.

4. Discussion

The main finding of this study is that the efficacy of adjuvant 5-FU chemotherapy, in terms of mortality or tumour recurrence, is significantly different depending on the mismatch repair status of colorectal cancer. Patients in stage II or III disease, which tumours show a competent mismatch repair status, obtained an important benefit from 5-FU chemotherapy, improving their overall survival and disease-free survival to almost a 20%. However, patients having MMR-deficient tumours seem not to benefit from 5-FU adjuvant chemotherapy. These results remain unchanged when we apply a multivariate model adjusted for age, gender and the disease stage.

Table 4 – Univariate survival analyses according to treatment received or mismatch repair status in stage II and III patients

	N	Probability of survival (95% CI)	*p*-Value	Probability of disease-free survival (95% CI)	*p*-Value
According to adjuvant chemotherapy status					
All patients[a]	496				
Adjuvant 5-FU	251	74.2 (68.8–79.6)		64.2 (58.3–70.1)	
No adjuvant 5-FU	245	57.1 (50.8–63.4)	0.0001	51.0 (44.6–57.4)	0.003
MMR-proficient	436				
Adjuvant 5-FU	225	74.8 (69.1–80.5)		65.0 (58.8–71.2)	
No adjuvant 5-FU	211	54.5 (47.6–61.4)	0.0001	48.3 (41.4–55.2)	0.001
MMR-deficient	60				
Adjuvant 5-FU	26	69.2 (51.1–87.3)		57.7 (38.3–77.1)	
No adjuvant 5-FU	34	73.5 (58.4–88.6)	0.8	67.6 (51.5–83.7)	0.6
According to mismatch repair status					
All patients[a]	496				
MMR-proficient	436	65.2 (60.7–69.7)		57.1 (52.4–61.8)	
MMR-deficient	60	71.7 (60.1–83.3)	0.3	63.3 (50.9–75.7)	0.4
Adjuvant 5-FU chemotherapy	251				
MMR-proficient	225	74.8 (69.1–80.5)		65.0 (58.8–71.2)	
MMR-deficient	26	69.2 (51.1–87.3)	0.5	57.7 (38.3–77.1)	0.5
No adjuvant 5-FU chemotherapy	245				
MMR-proficient	211	54.5 (47.6–61.4)		48.3 (41.4–55.2)	
MMR-deficient	34	73.5 (58.4–88.6)	0.03	67.6 (51.5–83.7)	0.03

p: chi-square; MMR: Mismatch repair; 5-FU: 5-fluorouracil.

a Nine patients with MMR proficient tumours that did not receive 5-FU adjuvant chemotherapy were taken out of the analysis.

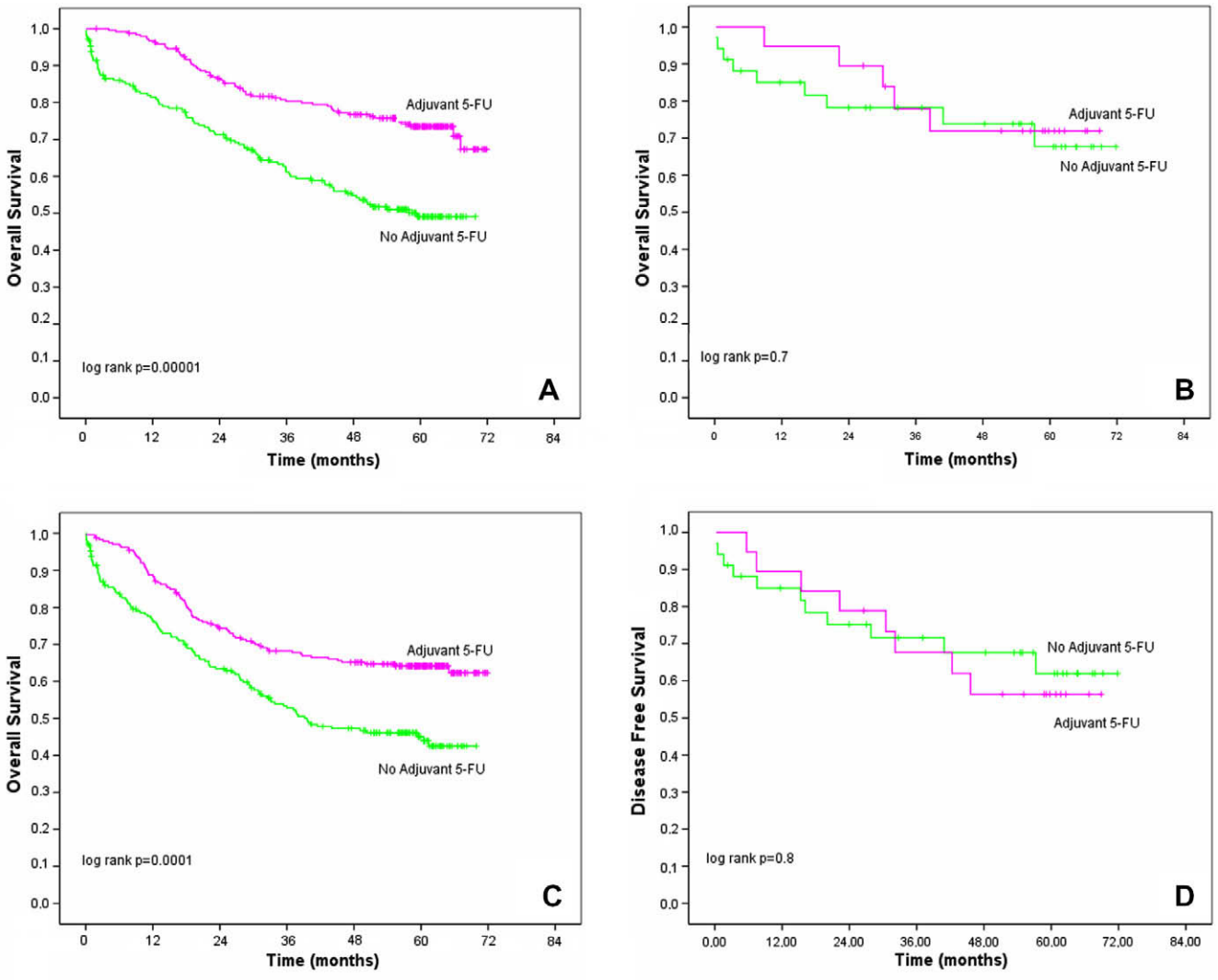

Fig. 2 – (A and B) Overall survival of patients with mismatch repair (MMR) proficient tumours (A) or MMR-deficient tumours (B) regarding treatment received. (C and D) Disease-free survival of patients with mismatch repair (MMR) competent tumours (C) or MMR-deficient tumours (D) according to the treatment received.

Table 5 – Multivariate analysis of overall survival and disease-free survival in stages II and III patients

	Overall survival (Hazard ratio[a](95% CI))	*p*-Value	Disease-free survival (Hazard ratio[a](95% CI))	*p*-Value
MMR status (deficient/proficient)	1.16 (0.68–1.96)	0.588	1.15 (0.73–1.83)	0.545
Adjuvant 5-FU chemotherapy (yes/no)	2.01 (1.37–2.94)	0.0001	1.92 (1.37–2.69)	0.0001
Interaction MMR status × 5-FU chemotherapy	2.04 (1.42–2.93)	0.0001	1.96 (1.42–2.70)	0.0001
Adjuvant 5-FU chemotherapy in deficient MMR status (yes/no)	0.93 (0.25–3.53)	0.915	0.98 (0.30–3.17)	0.971
Adjuvant 5-FU chemotherapy in proficient MMR status (yes/no)	2.17 (1.46–3.24)	0.0001	2.06 (1.45–2.93)	0.0001

p: chi-square; MMR: mismatch repair.
a Adjusted for age, sex and TNM stage Cox proportional hazards regression.

Previous studies had compared the efficacy of 5-FU according to MMR status. Initial reports showed a presumably better response to adjuvant chemotherapy for patients with MMR-defective colorectal cancer;[5,6,20] however, these studies did not include a control group of non-treated patients for comparative purposes and were probably affected by a confounding effect. Without a control group it was not clear if the survival benefit was derived from the chemotherapy or from

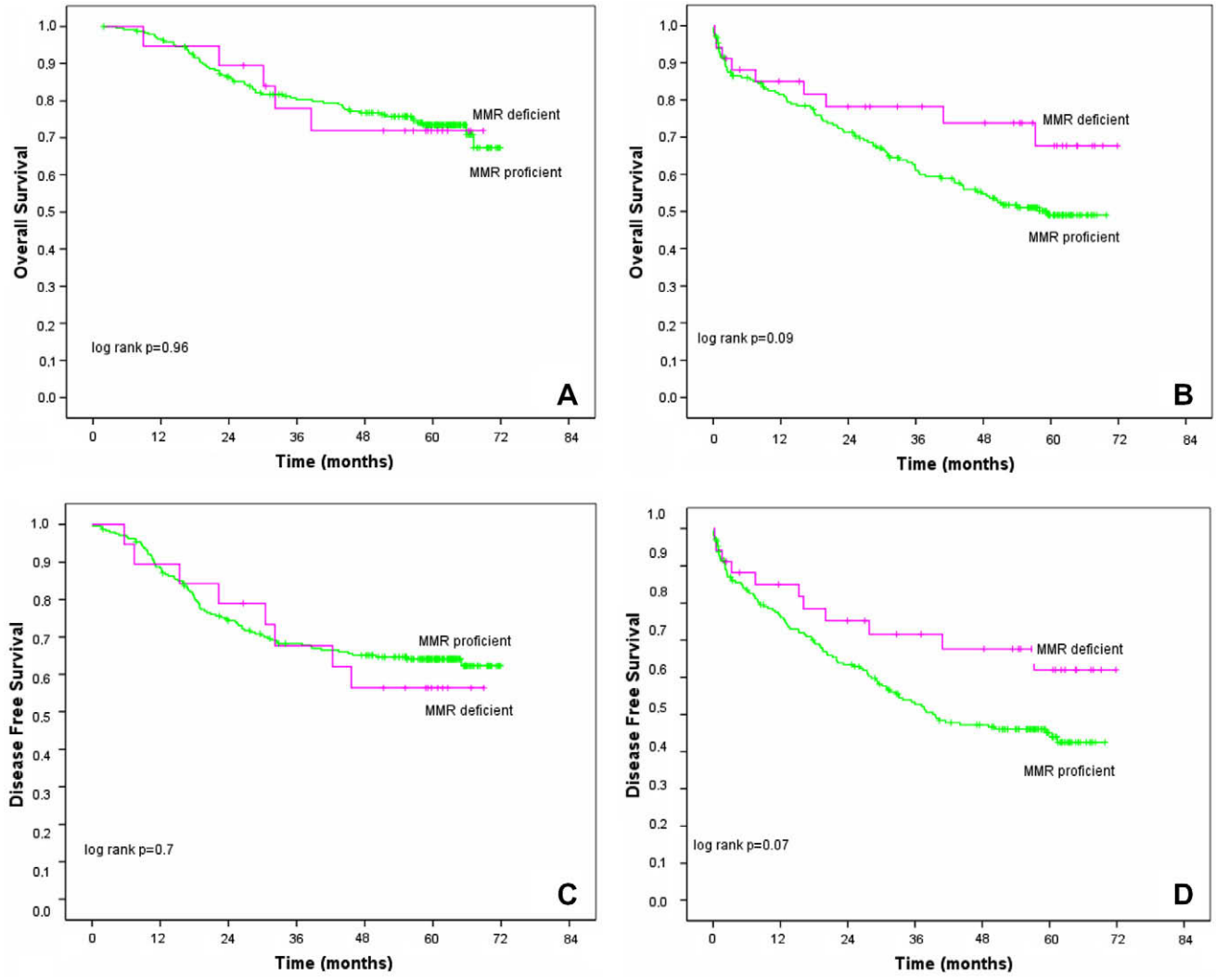

Fig. 3 – (A and B) Overall Survival of patients that received (A) or did not receive (B) adjuvant 5-FU chemotherapy, according to their mismatch repair (MMR) status. (C and D) Disease-free survival of patients that received (C) or did not receive (D) adjuvant 5-FU chemotherapy, according to their mismatch repair (MMR) status.

the intrinsic better survival of MMR-deficient tumours itself. The majority of studies that included a non-treated control group showed that 5-FU adjuvant chemotherapy benefited patients with stages II or III colon cancer with MMR-proficient tumours but not those with MMR-deficient tumours.[7,8,10,21] All these studies were retrospective, a fact that can limit their applicability in clinical practice. One of the strengths of our study is that we present the first long-term prospective data on the use of 5-FU-based chemotherapy analysed according to patient's tumour MMR status. Our results agree with the previous retrospective results, as well as with the findings obtained in the same cohort using a shorter follow-up,[11] confirming the probable lack of efficacy of 5-FU adjuvant chemotherapy in patients with MMR-defective colorectal cancer. A possible limitation of our study is that treatment assignment was not random, because adjuvant chemotherapy was decided using clinical criteria, and a selection bias cannot be excluded. Moreover, these clinical criteria were heterogeneous between the different hospitals participating in the study. However, we did not find significant clinical differences between MMR-competent and deficient patients that could indicate an association between MMR and another good prognosis marker able to explain the interaction between MMR status and chemotherapy effect. Another limitation is the small number of MMR-deficient patients included in our sample, decreasing the statistical power of our study.

Until recently, 5-FU has been the mainstay of adjuvant chemotherapy in stage III and high-risk stage II colorectal cancer. The efficacy demonstrated by other drugs, like oxaliplatin, is changing the standard regimens of chemotherapy in these patients, and recent studies have shown a further advantage in stage III colorectal cancer patients who undergo adjuvant chemotherapy with oxaliplatin.[22] *In vitro* studies using MSI cell lines had also indicated a difference in response to chemotherapeutic agents. Cells need a competent MMR system to recognise 5-FU and to allow this drug to kill malignant cells.[23–25] Other characteristics of the MMR-deficient tumours could also play a role in the 5-FU resistance,

such as *BAX3* or *TGFβRII* mutations.[26,27] Recent experimental studies have reported new information about the role of MMR, base excision repair system and Smug1 DNA glycosilase in 5-FU resistance,[28,29] confirming that the lack of MMR seems to reduce the effect of this drug.[29] 5-FU is removed from DNA by the base excision and MMR systems, and the absence of MMR would reduce the amount of repair DNA synthesis and thus attenuate the effect of FU through limiting all repair events to the base excision repair system.[29] Other *in vitro* studies have also showed an association between defects in MMR and platinum resistance, probably related with the need of the MMR proteins to recognise platinum adducts.[30,31] However, other drugs such as irinotecan have demonstrated a higher effect against MMR-defective tumours in xenografts and humans.[32,33]

A number of studies have investigated the relationship between MMR status and survival in colorectal cancer patients, and a recent pooled systematic review has demonstrated that colorectal cancers with MSI have a significantly improved prognosis.[4] In our study, we did not find an increase in survival in patients with MMR-defective tumours. This is probably related to the different response to chemotherapy between MMR-deficient and competent tumours. Patients with MMR-competent tumours that receive adjuvant 5-FU show a 20% increase in overall survival, as can be seen in Table 4, allowing these patients to reach the level of survival that patients with MMR-defective tumours show without the influence of adjuvant chemotherapy. An explanation for this beneficial effect of adjuvant chemotherapy, higher than that reported in other studies comparing both populations, could be that ours are patients treated following current standards and under normal conditions of clinical practice during the years 2000 and 2001. Previous studies compare population from clinical trials, with a majority of patients treated in the 1980s and early 1990s, when adjuvant chemotherapy for colorectal cancer patients was still in its beginnings. Combination of a bigger effect of chemotherapy in MMR-competent patients and lack of efficacy in the MMR-defective ones explains the absence of a better survival in the latter.

Despite the potential clinical benefits of determining tumour MMR status in colorectal cancer, these tests have not yet been incorporated into routine clinical practice. In part this relates to the difficulty of testing for MMR deficiency. PCR amplification of microsatellite repeats, the gold standard for the diagnosis of MSI, is not available in the majority of hospital laboratories. However, several studies have demonstrated that immunohistochemical staining for the absence of the MMR proteins has a similar accuracy than molecular biology techniques and is considered a good surrogate for determining the MMR status of colorectal tumours.[13,34–36] The concept of using biological information from tumours to determine treatment approaches is not new as this is routinely done in other tumours, such as breast or lung cancers. Our results support the usefulness of a molecular marker in the decision-making about treatment in patients with colorectal cancer, suggesting that a revision in the guidelines for adjuvant chemotherapy should be considered for patients with MMR-defective colorectal cancers. Moreover, randomised clinical trials stratifying according to MMR status comparing 5-FU and other chemotherapeutic drugs should be granted. In this sense, the ongoing clinical trial E5202 has been designed to address the role of MMR status in the prediction of the response to 5-FU, oxaliplatin and bevacizumab in stage II CRC patients.

In summary, in this prospective study we confirm the previous retrospective results as well as our own preliminary conclusions with a shorter follow-up, suggesting that adjuvant 5-FU-based chemotherapy may not be useful in patients with MMR-defective colorectal cancer with stage II or III disease. Molecular markers related to the biology of tumours should be used in the decision-making process for treatment in colorectal cancer and future clinical trials about the use of chemotherapeutic agents should be stratified, not only by TNM stage, but also by the status of specific molecular markers that can be predictive of treatment response.

Conflict of interest statement

All the authors disclose any financial or personal relationships with other people or organisations that could inappropriately influence (bias) our work in this manuscript.

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