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ORIGINAL REPORT

Evaluation of *PIK3CA* Mutation As a Predictor of Benefit From Nonsteroidal Anti-Inflammatory Drug Therapy in Colorectal Cancer

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ABSTRACT

Purpose
Aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) protect against colorectal cancer (CRC) and are associated with reduced disease recurrence and improved outcome after primary treatment. However, toxicities of NSAIDs have limited their use as antineoplastic therapy. Recent data have suggested that the benefit of aspirin after CRC diagnosis is limited to patients with *PIK3CA*-mutant cancers. We sought to determine the predictive utility of *PIK3CA* mutation for benefit from both cyclooxygenase-2 inhibition and aspirin.

Methods
We performed molecular analysis of tumors from 896 participants in the Vioxx in Colorectal Cancer Therapy: Definition of Optimal Regime (VICTOR) trial, a large randomized trial comparing rofecoxib with placebo after primary CRC resection. We compared relapse-free survival and overall survival between rofecoxib therapy and placebo and between the use and nonuse of low-dose aspirin, according to tumor *PIK3CA* mutation status.

Results
We found no evidence of a greater benefit from rofecoxib treatment compared with placebo in patients whose tumors had *PIK3CA* mutations (multivariate adjusted hazard ratio [HR], 1.2; 95% CI, 0.53 to 2.72; $P = .66$; $P_{INTERACTION} = .47$) compared with patients with *PIK3CA* wild-type cancers (HR, 0.87; 95% CI, 0.64 to 1.16; $P = .34$). In contrast, regular aspirin use after CRC diagnosis was associated with a reduced rate of CRC recurrence in patients with *PIK3CA*-mutant cancers (HR, 0.11; 95% CI, 0.001 to 0.832; $P = .027$; $P_{INTERACTION} = .024$) but not in patients lacking tumor *PIK3CA* mutation (HR, 0.92; 95% CI, 0.60 to 1.42; $P = .71$).

Conclusion
Although tumor *PIK3CA* mutation does not predict benefit from rofecoxib treatment, it merits further evaluation as a predictive biomarker for aspirin therapy. Our findings are concordant with recent data and support the prospective investigation of adjuvant aspirin in *PIK3CA*-mutant CRC.

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INTRODUCTION

Despite modest improvements in the systemic therapy of colorectal cancer (CRC) over the last two decades, almost half of all patients who undergo surgical resection with curative intent experience relapse, and there remains a pressing unmet need for more effective adjuvant therapies informed by our increasing knowledge of CRC biology. One attractive therapeutic target is cyclooxygenase-2 (COX-2; PTGS2), a member of the cyclooxygenase family of enzymes, which act to mediate inflammation by conversion of arachidonic acid to prostaglandins.[1] COX-2 is frequently overexpressed in CRC,[2,3] resulting in promotion of tumor progression, invasion, and metastasis.[4] Preclinical models have shown that COX-2 inhibition attenuates tumor growth[5,6] and that pharmacologic targeting of COX-2 by aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs) is thus a rational strategy for the prevention and treatment of CRC. Studies have demonstrated that regular, low-dose aspirin or NSAID use reduces the risk of CRC development,[7-13] decreases the incidence of adenomas after adenoma or CRC resection,[14-17] and is associated with a decreased frequency of metastases when taken after CRC diagnosis.[13,18]

Although regular aspirin and NSAID use is generally well tolerated, a proportion of patients suffer toxicities, particularly GI bleeding, with associated morbidity and mortality. Consequently, the identification of biomarkers that predict benefit from COX-2 inhibition would be of substantial clinical utility. Although tumor COX-2 expression[19] and germline *UGT1A6* polymorphisms[20] have been suggested as candidates, these await validation. However, a recent study has suggested that the benefit of regular aspirin therapy after CRC diagnosis is confined to the 12% to 15% of patients whose tumors harbor *PIK3CA* mutations.[21] *PIK3CA* encodes the p110α catalytic subunit of PI3 kinase, and *PIK3CA* mutations result in constitutive activation of the kinase and the downstream AKT pathway. One of the consequences of AKT pathway activation is COX-2 upregulation,[22] thus providing a potential mechanistic explanation for these findings, although interestingly no correlation between *PIK3CA* mutation and COX-2 expression was evident in the recent study.[21] Although noteworthy, the exploratory nature of the analyses in that study means that external validation is essential before conclusions on the predictive utility of tumor *PIK3CA* status can be reached. Therefore, we have investigated the correlation between COX-2 inhibition with either rofecoxib or aspirin, *PIK3CA* mutation, and clinical outcome in patients from the Vioxx in Colorectal Cancer Therapy: Definition of Optimal Regime (VICTOR) study.[23]

METHODS

Patients and Study Details

Details and outcome data from the VICTOR trial have been previously reported.[23] After completion of therapy (surgery ± radiotherapy ± chemotherapy), patients with histologically proven stage II or III CRC were randomly assigned to rofecoxib or placebo. Patients receiving long-term NSAID therapy were ineligible, with the exception of those taking low-dose aspirin (≤ 100 mg per day), the use of which was documented at random assignment. A minimization strategy was taken to ensure balance of prognostic variables (stage, disease site, age, and prior chemotherapy and radiotherapy) between the rofecoxib and placebo groups. The trial recruited 2,434 patients between April 2002 and September 2004, at which point study treatment was discontinued after the demonstration of adverse cardiovascular events associated with rofecoxib and the withdrawal of the drug from the market.[24] The median duration of rofecoxib treatment overall was 7.4 months. Patients underwent clinical assessment, physical examination, and measurement of serum carcinoembryonic antigen at 3, 6, 12, 18, and 24 months after random assignment and annually thereafter. Details of concomitant medications and low-dose aspirin use were recorded at each follow-up visit. Patients who were taking regular low-dose aspirin at random assignment or who started during follow-up were classified as aspirin users. Computed tomography scanning was required 1 year after surgery and was performed according to local practice thereafter.

Clinicopathologic Variables

Clinicopathologic variables at presentation were obtained from the trial database and treated as either binary (ie, location [left *v* right], sex, stage [II *v* III]) or continuous (ie, age, differentiation [well, moderate, poor]), as appropriate.

Tumor Molecular Analysis

Formalin-fixed, paraffin-embedded (FFPE) blocks were available for 965 VICTOR study cancers, 906 of which contained adequate amounts of carcinoma for molecular analysis.[25] With the exception of 69 rectal carcinomas treated with neoadjuvant radiotherapy, all tumor blocks were obtained before nonsurgical therapy. DNA was extracted from areas containing more than 80% tumor cells by microdissection of FFPE tissue using the DNeasy FFPE Kit (Qiagen, Hilden, Germany). Sanger sequencing of *PIK3CA* mutational hotspot exon 9 (codons 513 to 554) and the majority of exon 20 (codons 992 to 1,068) was successful in 896 cancers (see Appendix and Appendix Table A1, online only, for primers and polymerase chain reaction conditions). Sequencing of mutation hotspots in *KRAS*, *BRAF*, *FBXW7*, and *TP53* (exons 5 to 8) was performed as previously detailed (details of polymerase chain reaction primers and reaction conditions available from authors on request).[25] Microsatellite instability was assessed according to published criteria, and chromosomal instability was determined as previously reported.[25] Tumor COX-2 expression was evaluated by immunohistochemistry in 798 cancers on tissue microarrays containing triplicate cores for each specimen, using rabbit polyclonal anti–COX-2 antibody (Abcam, Cambridge, United Kingdom) at 1:1,000 dilution. COX-2 expression was scored independently by two researchers as 0 (absent staining), 1+ (weak staining), 2+ (moderate staining), or 3+ (strong staining) according to published criteria.[19,23] Sections graded as 3+ were regarded by the study pathologists as sufficiently distinct from the other categories to be categorized separately for statistical analysis (defined as COX-2 positive). Concordance between pathologists for classification of 3+ staining was more than 85%, with discrepancies resolved by discussion. Patients included in this biomarker study were similar to the main study population with respect to age, sex, disease stage, tumor site, and prior chemotherapy, although fewer patients in this biomarker study had received prior radiotherapy (9.2% *v* 12.7%, respectively; $P = .0065$).

Statistical Analysis

The primary aim of this biomarker study was to validate the predictive utility of tumor *PIK3CA* mutation for benefit from COX-2 inhibition measured by recurrence-free survival (RFS). Secondary objectives were to evaluate the effect of COX-2 inhibition on overall survival (OS) according to *PIK3CA* mutation and to explore the relationship between COX-2 expression and outcome in aspirin users (Appendix Table A2, online only). RFS was defined as the time from random assignment to recurrence and/or CRC death; patients who were alive and recurrence free were censored at their last known recurrence-free date. OS was measured as the time from random assignment to death from any cause; for analysis, patients who were not reported dead were censored at the last date they were known to be alive. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. Cox proportional hazards models were used to determine the effect of rofecoxib treatment and aspirin use according to tumor *PIK3CA* mutation status by univariate analysis and, after adjustment for baseline characteristics and known prognostic factors (age, sex, disease stage, tumor location, tumor grade, microsatellite instability, and prior chemotherapy or radiotherapy), by multivariate analysis. Firth's correction was applied for analysis of aspirin subgroups because of the absence of events in the *PIK3CA* mutant/aspirin use group. The significance of interaction between *PIK3CA* mutation and COX-2 inhibition was assessed using the Wald test on the cross product term of the rofecoxib/aspirin and *PIK3CA* variables. Statistical analyses were performed using Stata (StataCorp, College Station, TX) and R (http://www.r-project.org/). All *P* values were two-sided. Further details are provided in the Appendix.

Ethical Approval

The VICTOR trial protocol was peer reviewed by the United Kingdom Cancer Research Campaign, the multicenter Research Ethics Committee, and research ethics committees at participating centers, and the study was conducted according to the tenets of the Declaration of Helsinki. Approval for the molecular analysis was given by the Oxfordshire Research Ethics Committee B (Approval No. 05\Q1605\66).

RESULTS

Patient Characteristics and Tumor PIK3CA Mutation Frequency

Characteristics of the 896 study patients for whom tumor *PIK3CA* mutations were successfully screened are listed by *PIK3CA* mutation status, aspirin use, and rofecoxib treatment in Table 1 and Appendix Table A3 (online only). Clinicopathologic variables, duration of treatment, and outcomes in this group were similar to the

Table 1. Demographics and Clinicopathologic Characteristics of Patients According to *PIK3CA* Mutation Status and Aspirin Use

Demographic or Clinicopathologic Characteristic	All Patients (N = 896)		No *PIK3CA* Mutation				*PIK3CA* Mutation				*P*
			No Aspirin Use (n = 681)		Aspirin Use (n = 111)		No Aspirin Use (n = 90)		Aspirin Use (n = 14)		
	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%	
Age, years											< .001
Median	64.6		63.7		69.7		64.3		65.6		
Range	24.6-89.1		24.6-89.1		46.5-82.9		37.1-77.7		55.8-86.3		
Sex											.033
Male	577	64.4	424	62.3	78	70.3	62	68.9	13	92.9	
Female	325	35.6	257	37.7	33	29.7	28	31.1	1	7.1	
Stage											.867
II	443	49.4	332	48.7	57	51.4	46	51.1	8	57.1	
III	453	50.6	349	51.2	54	48.6	44	48.9	6	42.9	
Location											.551
Right	305	34.0	224	32.9	43	38.7	33	36.7	5	35.7	
Left	568	63.4	441	64.8	64	57.7	54	60.0	9	64.3	
Not known	23	2.6	16	2.3	4	3.4	3	3.3	0	0	
Grade											.660
1	72	8.0	54	7.9	11	9.9	7	7.8	0	0	
2	717	80.0	542	79.6	86	77.5	76	84.4	13	92.9	
3	87	9.7	70	10.3	11	9.9	5	5.6	1	7.1	
Not known	20	2.2	15	2.2	3	2.7	1	1.1	0	0	
Chemotherapy											.318
No	337	37.6	250	36.7	49	44.1	31	34.4	7	50	
Yes	558	62.3	430	63.1	62	55.9	59	65.6	7	50	
Not known	11	1.2	1	0.1	0	0	0	0	0	0	
Radiotherapy											.049
No	814	90.8	609	89.4	107	96.4	84	93.3	14	100	
Yes	82	9.2	72	10.6	4	3.6	6	6.7	0	0	
Rofecoxib therapy											.816
Placebo	445	49.7	344	50.5	51	45.9	43	47.8	7	50.0	
Rofecoxib	451	50.3	337	49.5	60	54.1	47	52.2	7	50.0	
COX-2 expression											.636
No (0-2+)	630/798*	78.9	476/606	78.5	76/99	77.1	67/80	83.8	11/13	84.6	
Yes (3+)	168/798	11.1	130/606	21.5	23/99	23.2	13/80	16.3	2/13	15.4	
CIN											.279
No CIN	275/856	32.1	203/644	31.5	33/109	30.3	36/89	40.4	3/14	21.4	
CIN	581	68.0	441/644	68.5	76/109	69.7	53/89	59.6	11/14	78.6	
MSI											.323
MSS	767/882	87.0	585/669	87.4	98/110	89.1	72/89	80.9	12/14	85.7	
MSI	115/882	13.0	84/669	12.6	12/110	10.9	17/89	19.1	2/14	14.3	
KRAS											< .001
Wild type	588/890	66.1	456/676	67.5	82/110	74.5	43/90	47.8	7/14	50	
Mutant	302/890	33.9	220/676	32.7	28/110	25.5	47/90	52.2	7/14	50	
BRAF											.847
Wild type	805/894	90.0	609/679	89.7	100/111	90.1	83/90	92.2	13/14	92.9	
Mutant	89/894	10.0	70/679	10.3	11/111	9.9	7/90	7.8	1/14	7.1	
TP53											.200
Wild type	424/752	56.4	314/571	55.5	49/94	52.1	51/76	67.1	7/11	63.6	
Mutant	328/752	43.6	251/571	44.5	45/94	47.9	25/76	32.9	4/11	36.4	
FBXW7											.892
Wild type	713/748	95.3	540/567	95.2	90/94	95.7	73/76	96.1	10/11	90.9	
Mutant	35/748	5.7	27/567	4.8	4/94	4.3	3/76	3.9	1/11	9.1	

Abbreviations: CIN, chromosomal instability; COX-2, cyclooxygenase-2; MSI, microsatellite instability; MSS, microsatellite stable.
*No. of patients/total No. of patients.

overall trial population.[23] Of the 896 patients, 445 patients were randomly assigned to placebo, and 451 were randomly assigned to rofecoxib. Sixty-nine patients (7.7%) were regular users of low-dose aspirin (≤ 100 mg daily) at time of random assignment, and an additional 56 patients commenced aspirin therapy during follow-up, for a cumulative total of 125 aspirin users (14.0% of the study population). Aspirin at doses ≥ 100 mg daily or use of concomitant NSAIDs other than study medication was not permitted. The overall

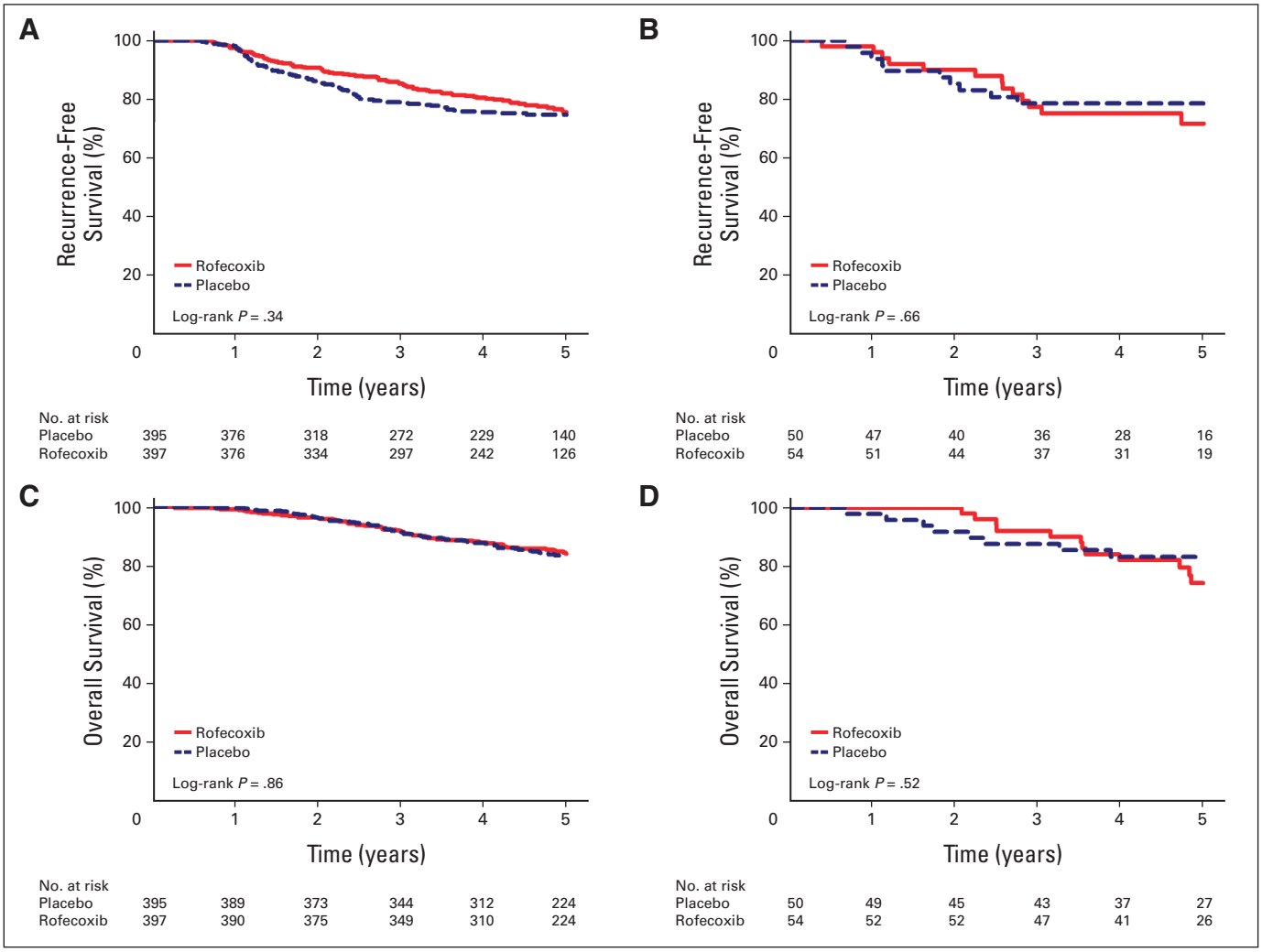

Fig 1. Recurrence-free survival of patients treated with rofecoxib or placebo according to the (A) absence or (B) presence of tumor *PIK3CA* mutation. Overall survival of patients treated with rofecoxib or placebo according to the (C) absence or (D) presence of tumor *PIK3CA* mutation. The number of patients at risk is indicated below each panel. Comparison between groups was made using the log-rank test.

frequency of tumor *PIK3CA* mutation was 11.6% (104 patients; Appendix Table A4, online only), with no significant difference in mutation frequency between rofecoxib and placebo groups (12.0% *v* 11.2%, respectively; $P = .76$) or between aspirin users and nonusers (12.6% *v* 11.7%, respectively; $P = 1.0$). *PIK3CA*-mutant tumors demonstrated an increased frequency of *KRAS* mutation but not COX-2 overexpression, in keeping with previous data (Tables 1 and A3).[21]

Outcome by Tumor PIK3CA *Mutation, Rofecoxib Treatment, and Aspirin Use*

To examine whether tumor *PIK3CA* mutation was associated with differential benefit from COX-2 inhibition, we grouped patients according to *PIK3CA* mutation status and treatment with rofecoxib or low-dose aspirin (Tables 1 and A3). With the exception of *KRAS* mutation, demographic, clinicopathologic, and molecular variables were well matched between rofecoxib and placebo groups irrespective of *PIK3CA* mutation (Table A3). Aspirin users were significantly older than nonusers (69.1 *v* 63.2 years, respectively; $P < .001$), and fewer patients in the aspirin subgroups had received prior radiotherapy (Table 1). The median follow-up time overall was 61.5 months (interquartile range, 49.9 to 69.8 months). There was no significant difference in duration of follow-up between subgroups according to rofecoxib or aspirin use ($P = .79$ and $P = .11$, respectively).

In patients with tumors lacking *PIK3CA* mutations, 91 RFS events occurred in the placebo group and 82 RFS events occurred in the rofecoxib group during the study period. The univariate hazard ratio (HR) for RFS with rofecoxib treatment was 0.87 (95% CI, 0.64 to 1.16; $P = .34$; Fig 1A; Table 2), with minimal change after adjustment for known prognostic variables by Cox regression analysis (HR, 0.85; 95% CI, 0.62 to 1.15). The numbers of RFS events in the placebo and rofecoxib groups in the *PIK3CA*-mutant cohort were 10 and 13, respectively. The univariate HR for disease recurrence with rofecoxib compared with placebo in patients with *PIK3CA*-mutant tumors was 1.20 (95% CI, 0.53 to 2.72; $P = .66$), with almost no alteration after multivariate regression (HR, 1.22; 95% CI, 0.50 to 2.98; $P = .658$; Fig 1B; Table 2). There was no

Table 2. RFS and OS by Tumor *PIK3CA* Mutation and Rofecoxib Therapy

Group	No. of Patients	RFS							OS				
		3-Year RFS (%)	95% CI (%)	Univariate HR	95% CI	Multivariate Adjusted HR	95% CI	*P**	3-Year OS (%)	95% CI (%)	Univariate HR	95% CI	Multivariate Adjusted HR	95% CI	*P**
Wild-type *PIK3CA*															
Rofecoxib	397	86.4	82.7 to 89.4	0.87	0.64 to 1.16	0.85	0.62 to 1.15	.473	91.9	88.8 to 94.3	0.96	0.67 to 1.38	0.91	0.63 to 1.32	.298
Placebo	395	80.3	76.0 to 83.9						91.9	88.8 to 94.2					
Mutant *PIK3CA*															
Rofecoxib	54	79.6	66.9 to 89.0	1.20	0.53 to 2.72	1.22	0.50 to 2.98		92.6	82.0 to 97.6	1.33	0.55 to 1.38	2.39	0.77 to 7.45	
Placebo	50	80.0	66.8 to 89.0						88.0	75.8 to 94.8					

Abbreviations: HR, hazard ratio; OS, overall survival; RFS, recurrence-free survival.
**P* value for interaction of tumor *PIK3CA* mutation and rofecoxib therapy after multivariate analysis.

evidence of an interaction between *PIK3CA* mutation and rofecoxib on RFS ($P = .473$). Thus, we found no indication that the effect of rofecoxib therapy on RFS compared with placebo is altered by tumor *PIK3CA* mutation. Similarly, there was no suggestion that tumor *PIK3CA* mutation modifies OS in rofecoxib-treated patients (Figs 1C and 1D, Table 2).

We next analyzed whether *PIK3CA* mutation predicted benefit from aspirin therapy. In patients with tumors lacking *PIK3CA* mutations, the number of RFS events during the study period was 151 in 681 patients who did not use aspirin and 22 in 111 regular aspirin users. In the *PIK3CA*-mutated tumor cohort, 23 of 90 patients who did not use aspirin suffered CRC recurrence, whereas there were no relapses in 14 patients who were regular aspirin users (Figs 2A and 2B, Table 3). The univariate HR for RFS with regular low-dose aspirin compared with no aspirin use was 0.92 (95% CI, 0.60 to 1.42; $P = .71$) in the *PIK3CA* wild-type group and 0.11 (95% CI, 0.001 to 0.805; $P = .023$) in the *PIK3CA*-mutant cohort. After Cox multivariate regression, the adjusted HR for RFS was 0.94 (95% CI, 0.59 to 1.49; $P = .786$) in the group with no aspirin use and 0.11 (95% CI, 0.001 to 0.832; $P = .027$) in aspirin users. Analysis confirmed a significant interaction between tumor *PIK3CA* mutation and aspirin use on RFS ($P = .024$). There was no evidence of an alteration in OS with aspirin use in patients with *PIK3CA* wild-type cancers (HR, 1.05; 95% CI, 0.62 to 1.77; $P = .85$). In the *PIK3CA*-mutant group, aspirin users had better survival, but this did not reach significance in univariate or multivariate analyses (HR, 0.44; 95% CI, 0.13 to 1.48; $P = .19$; and HR, 0.29; 95% CI, 0.04 to 2.33; $P = .245$, respectively; Figs 2C and 2D; Table 3). Similarly, formal testing for an interaction between *PIK3CA* mutation and aspirin use on OS was not significant ($P = .25$).

Clinical Outcome by Tumor COX-2 Expression, Rofecoxib Treatment, and Aspirin Use

It has been reported that the benefits of aspirin use in patients with CRC are greater in patients with high levels of tumor COX-2 expression,[19] and previous analysis of the VICTOR study suggested similar findings with rofecoxib therapy, although no statistically

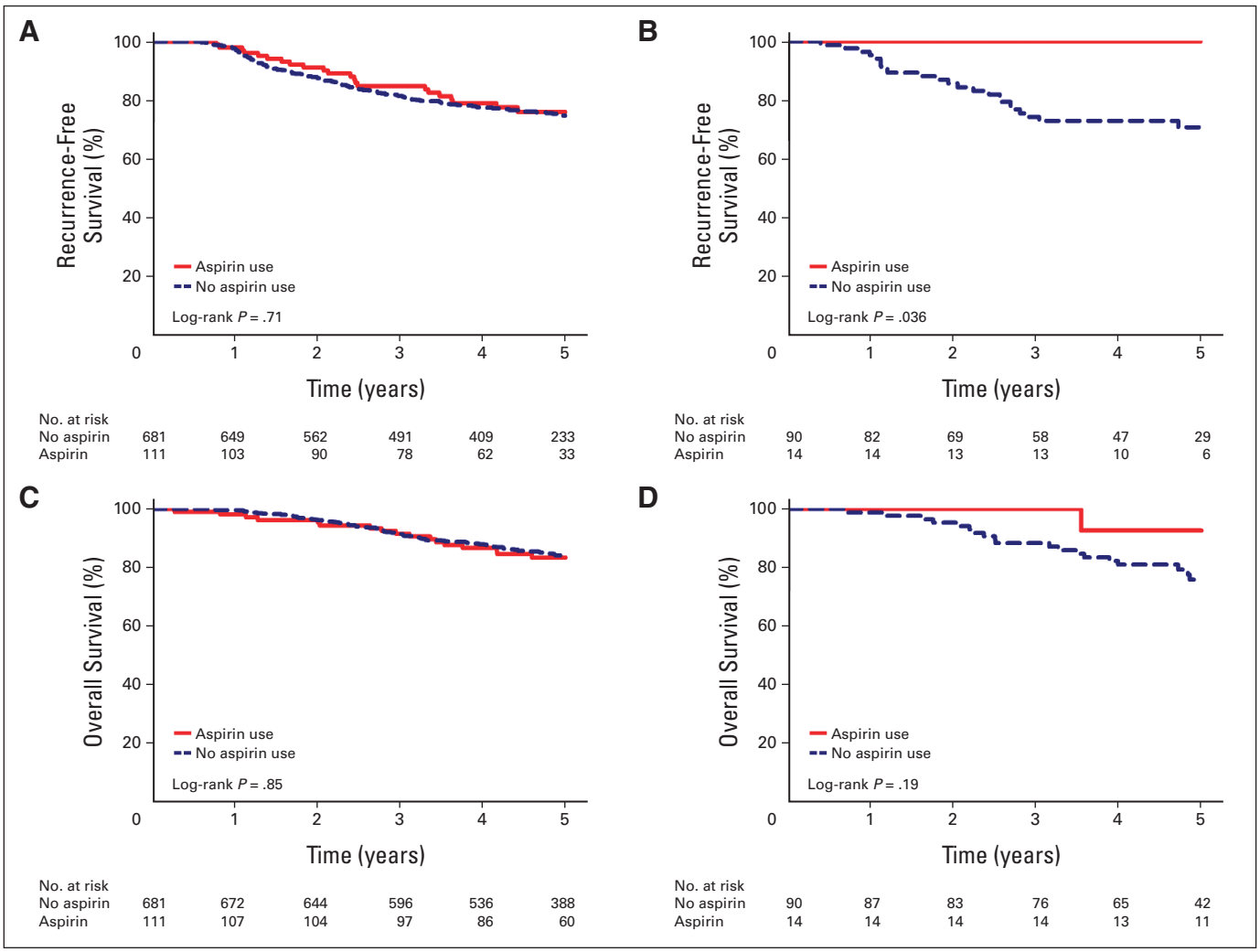

Fig 2. Recurrence-free survival of regular low-dose aspirin users and nonusers according to the (A) absence or (B) presence of tumor *PIK3CA* mutation. Overall survival of regular low-dose aspirin users and nonusers according to the (C) absence or (D) presence of tumor *PIK3CA* mutation. The number of patients at risk is indicated below each panel. Comparison between groups was made using the log-rank test.

Table 3. RFS and OS by Tumor *PIK3CA* Mutation and Aspirin Use

Group	No. of Patients	RFS							OS					
		3-Year RFS (%)	95% CI (%)	Univariate HR	95% CI	Multivariate Adjusted HR	95% CI	*P**	3-Year OS (%)	95% CI (%)	Univariate HR	95% CI	Multivariate Adjusted HR	95% CI	*P**
Wild-type *PIK3CA*															
Aspirin use	111	86.5	78.8 to 91.8	0.92	0.60 to 1.42	0.94	0.59 to 1.49	.024	91.9	85.1 to 95.9	1.05	0.62 to 1.77	0.95	0.56 to 1.61	.260
No aspirin use	681	82.8	79.8 to 85.4						91.9	89.6 to 93.7					
Mutant *PIK3CA*															
Aspirin use	14	100	74.9 to 100	0.11	0.001 to 0.805	0.11	0.001 to 0.832		100	74.9 to 100	0.44	0.13 to 1.48	0.29	0.04 to 2.33	
No aspirin use	90	76.7	68.9 to 84.3						88.9	80.6 to 94.0					

Abbreviations: HR, hazard ratio; OS, overall survival; RFS, recurrence-free survival.
*P value for interaction of tumor *PIK3CA* mutation and aspirin use after multivariate analysis by Cox proportional hazards with Firth's correction.

significant interaction was proven.[23] Exploratory analysis of outcome by COX-2 expression (Appendix Table A5, online only) in our cohort demonstrated similar trends for both rofecoxib therapy (adjusted HR, 0.74; 95% CI, 0.36 to 1.51; $P = .40$; Appendix Figs A1A and A1B and Appendix Table A6, online only) and aspirin use (adjusted HR, 0.55; 95% CI, 0.16 to 1.91; $P = .35$; Appendix Figs A1C and A1D and Appendix Table A7, online only). However, these did not reach significance in either unadjusted or adjusted analyses, and no evidence of significant treatment interaction was found ($P = .251$ and $P = .349$, respectively). The small number of patients with tumors demonstrating both *PIK3CA* mutation and COX-2 overexpression precluded meaningful analysis of outcome.

DISCUSSION

By molecular analysis of CRCs from patients enrolled onto a large randomized study of COX-2 inhibition, we have shown that although tumor *PIK3CA* mutation does not predict benefit from rofecoxib therapy, it does predict reduced CRC recurrence in regular users of low-dose aspirin after diagnosis. This is unlikely to be a result of modification of disease phenotype by aspirin use before CRC diagnosis because only three of 14 patients in this group were taking aspirin at the time of random assignment. Our findings are consistent with those recently reported[21] and suggest that prospective evaluation of aspirin as adjuvant therapy in this tumor molecular subtype is merited.

The advantages of evaluating prognostic and predictive biomarkers within clinical trials are well recognized,[26] and our study has several strengths in this regard. Comprehensive data on baseline demographics, clinicopathologic characteristics, and antineoplastic therapy were available for all patients, and the majority of tumors underwent rigorous molecular analysis. Details of aspirin use at random assignment and during follow-up were available for all patients, and clinical follow-up including clinical examination, imaging, and carcinoembryonic antigen measurement was mandated. Prognostic variables were matched between rofecoxib and placebo groups in the overall study population by minimization, and this balance was largely maintained in the *PIK3CA*-mutant subgroups when categorized by either rofecoxib/placebo treatment or aspirin use/nonuse and when adjusted for in multivariate analyses, making it unlikely that our results are a result of a disparity in prognostic factors between groups. Our analysis was restricted to the prespecified examination of the effect of *PIK3CA* mutation and COX-2 expression on rofecoxib benefit and a confirmatory analysis of the effect of these biomarkers on outcome in aspirin users, limiting the potential for confounding by multiple comparisons and subgroup analyses. However, in common with many retrospective biomarker studies, our work has limitations. First, it is possible that the attenuated duration of rofecoxib therapy was insufficient to demonstrate a differential effect in *PIK3CA*-mutant tumors. Although we cannot exclude this possibility, most CRC recurrences occur within 1 year of surgery,[27] and the absence of a trend toward rofecoxib benefit in *PIK3CA*-mutant cases during this period suggests that tumor *PIK3CA* mutation is unlikely to be a strong predictor for benefit from COX-2–specific inhibition. Second, there was a limited number of patients with *PIK3CA*-mutant tumors who were regular aspirin users, reflecting the relatively modest frequency of *PIK3CA* mutation in CRC and the absence of prospective allocation to aspirin within the study. In this regard, it is worth noting that in an unselected trial, to obtain 100 patients with CRC with *PIK3CA* mutations in both the treatment and control arms, the study would have to include more than 1,500 patients.

The mechanistic basis of our findings awaits clarification. Preclinical data indicate that PI3K-AKT pathway activation induces COX-2 mRNA,[22] and it has been postulated that this may explain the differential effect of aspirin use in *PIK3CA*-mutant CRC. However, in keeping with recent data,[21] we found no increase in the frequency of COX-2 protein overexpression in tumors with *PIK3CA* mutations. It is notable that our limited analysis of rofecoxib and aspirin effect in tumors with COX-2 overexpression demonstrated similar trends to increased benefit, consistent with previous reports[23] and in contrast to the discordant results when analyzed by *PIK3CA* mutation. Whether this disparity results from COX-2–independent actions of aspirin[28,29] is currently unclear. Unfortunately, limited numbers of tumors with both *PIK3CA* mutation and COX-2 overexpression precluded evaluation of the aspirin effect in this group.

Although multiple lines of evidence indicate a benefit from aspirin use after CRC diagnosis, the toxicities of therapy, particularly in older patients, have limited its use in routine clinical practice. Although several biomarkers have been suggested to predict benefit from NSAID/aspirin therapy,[19,30] to the best of our knowledge, our study is the first to provide external corroboration of predictive biomarker utility. Combined with recent data from Liao et al,[21] our data support the prospective evaluation of adjuvant low-dose aspirin in patients with tumor *PIK3CA* mutation.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) and/or an author's immediate family member(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.
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Appendix

Methods

Polymerase chain reaction (PCR) primers and target amplicons for *PIK3CA* exon 9 and 20 are listed in Appendix Table A1 (online only). PCR was performed using 25 ng of template DNA with Qiagen multiplex PCR kit (Qiagen, Hilden, Germany) with Q solution in a 15-μL reaction volume, with primers at 0.4 μmol/L final concentration and proportions of other reagents according to the manufacturer's recommendations. Reaction conditions were as follows: 95°C denaturation for 15 minutes; 94°C melt for 45 seconds; 55°C annealing for 90 seconds and 72°C extension for 45 seconds repeated for 38 cycles; and final 72°C extension for 10 minutes. PCR products were confirmed by gel electrophoresis and cleaned up with Exosap-IT (Affymetrix, Santa Clara, CA) as per manufacturer's instructions. BDT (Life Technologies, Carlsbad, CA) sequencing was performed according to manufacturer's recommendations using the forward primer in both cases.

Statistical Methods

All informative patients were included in the statistical analyses, and patients for whom data were absent were excluded. Comparison between baseline clinicopathologic and molecular variables was made using the Fisher's exact test or the χ^2 test for categorical variables. The *t* test or analysis of variance was used to compare continuous variables. Subgroup analyses performed in this biomarker study are listed Appendix Table A1. To control confounding from prognostic factor imbalance between subgroups, we used multivariate Cox proportional hazards models. Multivariate models included age, sex, tumor grade, tumor location, microsatellite instability, and previous radiotherapy/chemotherapy treatment irrespective of whether these differed significantly between subgroups. Patients with missing categorical data (tumor grade, 2.2%; tumor location, 2.2%; microsatellite instability, 1.1%; prior chemotherapy, 0.1%) were excluded from the model. For assessment of the interaction between rofecoxib or aspirin and tumor *PIK3CA* mutation, Cox regression was performed including all prognostic covariates. For analysis of aspirin subgroups, Firth's correction (Heinze G, Schemper M: Biometrics 57:114-119, 2001; Heinze G, Dunkler D: Stat Med 27:6455-6469, 2008) was used in view of the lack of events in the aspirin/*PIK3CA* mutant cohort. We confirmed that use of the Firth correction resulted in minimal modification of results compared with the uncorrected Cox model in the rofecoxib subgroup (not shown). The significance of interaction between *PIK3CA* mutation and cyclooxygenase-2 inhibition was assessed by the Wald test on the cross product term of the rofecoxib/aspirin and *PIK3CA* variables. Proportionality of hazards was confirmed for the aspirin subgroups by determination of Schoenfeld residuals. For the rofecoxib analysis, suggestion of greater effect of treatment in the period 6 to 18 months after random assignment did not affect the study findings.

Table A1. *PIK3CA* Sequencing Polymerase Chain Reaction Primer Details

Target (residues) and Primer Name	Primer Sequence	Codons Sequenced
Exon 9 (513-554)		513-554
PIK3CA-Ex9Fw	5′-TTGAAAATGTATTTGCTTTTTC-3′	
PIK3CA-Ex9Rv	5′-TCCATTTTAGCACTTACCTGTGAC-3′	
Exon 20 (979-1,068)		992-1,068
PIK3CA-Ex20Fw	5′-CAGCATGCCAATCTCTTCAT-3′	
PIK3CA-Ex20Rv	5′-TTTTCAGTTCAATGCATGCTG-3′	

Table A2. Subgroup Analyses Performed and Reported in This Study

Exposure	Subgroups	Primary Outcome	Secondary Outcome	Analysis	Reported
Rofecoxib use *v* placebo	Tumor *PIK3CA* mutation *v* *PIK3CA* wild type	RFS	OS	Univariate and multivariate adjusted HR, *P* interaction (rofecoxib × *PIK3CA* mutation)	Table 1, Fig 1
Aspirin use *v* no aspirin use	Tumor *PIK3CA* mutation *v* *PIK3CA* wild type	RFS	OS	Univariate and multivariate adjusted HR, *P* interaction (aspirin × *PIK3CA* mutation)	Table 2, Fig 2
Rofecoxib use *v* placebo	Tumor COX-2 positive *v* negative	RFS	N/A	Univariate and multivariate adjusted HR, *P* interaction (rofecoxib × COX-2 overexpression)	Table A6, Fig A1
Aspirin use *v* no aspirin use	Tumor COX-2 positive *v* negative	RFS	N/A	Univariate and multivariate adjusted HR, *P* interaction (aspirin × COX-2 overexpression)	Table A7, Fig A1

Abbreviations: COX-2, cyclooxygenase-2; HR, hazard ratio; N/A, not applicable; OS, overall survival; RFS, recurrence-free survival.

Table A3. Demographics and Clinicopathologic Characteristics of Patients According to *PIK3CA* Mutation Status and Rofecoxib Therapy

Demographic or Clinicopathologic Characteristic	No *PIK3CA* Mutation				*PIK3CA* Mutation				
	Placebo (n = 395)		Rofecoxib (n = 397)		Placebo (n = 50)		Rofecoxib (n = 54)		
	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%	*P*
Age, years									.611
Median	64.3		65.0		63.5		65.8		
Range	24.6-89.1		26.9-84.1		37.1-84.6		38.7-86.3		
Sex									.350
Male	249	62.8	253	63.7	35	70	40	74.1	
Female	146	37.0	144	36.3	15	30	14	25.9	
Stage									.559
II	203	51.4	186	46.9	25	50	29	53.7	
III	192	48.6	211	53.1	25	50	25	46.3	
Location									.459
Right	134	33.9	133	33.5	22	44.0	16	29.6	
Left	251	63.5	254	64.0	27	54.0	36	66.7	
Not known	10	2.5	10	2.5	1	2.0	2	3.7	
Grade									.077
1	35	8.9	30	7.6	2	4.0	5	9.3	
2	320	81.0	308	77.6	43	86.0	46	85.2	
3	29	7.3	52	13.1	4	8.0	2	3.7	
Not known	11	2.8	7	1.8	1	2.0	1	1.9	
Chemotherapy									.934
No	153	38.8	146	36.8	18	36.0	20	37.0	
Yes	241	61.2	251	63.2	32	64.0	34	63.0	
Not known	1	0.3	0	0	0	0	0	0	
Radiotherapy									.373
No	361	91.4	355	89.4	46	92.0	52	96.3	
Yes	34	8.6	42	10.6	4	8.0	2	3.7	
COX-2 expression									.293
No (0-2+)	259/341*	76.0	293/364	80.5	37/44	84.1	41/49	83.7	
Yes (3+)	82/341	24.0	71/364	19.5	7/44	15.9	8/49	16.3	
CIN									.421
No CIN	120/372	32.3	116/381	30.4	21/50	42.0	18/53	34.0	
CIN	252/372	67.7	265/381	69.6	29/50	58.0	35	66.0	
MSI									.055
MSS	345/386	89.4	338/393	86.0	38/50	76.0	46/53	86.8	
MSI	41/386	10.6	55/393	14.0	12/50	24.0	7/53	13.2	
KRAS									< .001
Wild type	269/392	68.3	269/394	68.3	21/50	42.0	29/54	53.7	
Mutant	123/392	31.7	125/394	31.7	29/50	38.0	25/54	46.3	
BRAF									.359
Wild type	346/393	88.0	363/397	91.4	46/50	92.0	50/54	92.6	
Mutant	47/393	12.0	34/397	8.6	4/50	8.0	4/54	7.4	
TP53									.201
Wild type	183/326	56.1	183/339	54.0	26/40	65.0	32/47	68.1	
Mutant	143/326	43.9	156/339	46.0	14/40	35.0	15/47	31.9	
FBXW7									.276
Wild type	304/324	93.8	326/337	96.7	39/40	97.5	44/47	93.6	
Mutant	20/324	6.2	11/337	3.3	1/40	2.5	3/47	6.4	

Abbreviations: CIN, chromosomal instability; COX-2, cyclooxygenase-2; MSI, microsatellite instability; MSS, microsatellite stable.
*No. of patients/total No. of patients.

Table A4. *PIK3CA* Mutations Detected in the Study

Exon	Domain	Codon	Nucleotide Substitution	Amino Acid Substitution	No. of Patients
9	Helical	542	c.1624G>C	p.E542Q	2
9	Helical	542	c.1624G>A	p.E542K	15
9	Helical	543	c.1628T>A	p.I543N	1
9	Helical	545	c.1635G>C	p.E545D	1
9	Helical	545	c.1633G>A	p.E545K	37
9	Helical	545	c.1634A>G	p.E545G	4
9	Helical	546	c.1636C>A	p.Q546K	8
9	Helical	546	c.1638G>T	p.Q546H	1
9	Helical	546	c.1637A>G	p.Q546R	4
20	Kinase	1043	c.3127A>G	p.M1043V	1
20	Kinase	1045	c.3133G>A	p.D1045N	1
20	Kinase	1047	c.3140A>G	p.H1047R	19
20	Kinase	1047	c.3139C>T	p.H1047Y	3
20	Kinase	1048	c.3142C>T	p.H1048T	1
20	Kinase	1049	c.3145G>C	p.G1049R	2
20	Kinase	1049	c.3145G>A	p.G1049S	1
20	Kinase	1049	c.3146G>C	p.G1049A	2
20	Kinase	1049	c.3146G>A	p.G1049D	1

Table A5. Distribution of COX-2 Staining Intensity in Tumors

COX-2 Staining Intensity	No. of Patients	%
0 (absent)	28	3.5
1+ (weak expression)	269	33.5
2+ (moderate expression)	333	41.7
3+ (strong expression)	168	21.1

Abbreviation: COX-2, cyclooxygenase-2.

Table A6. RFS by Tumor COX-2 Expression and Rofecoxib Treatment

		RFS						
Group	No. of Patients	3-Year RFS (%)	95% CI (%)	Univariate HR	95% CI	Multivariate Adjusted HR	95% CI	*P**
COX-2 negative								
Rofecoxib	339	84.4	80.1 to 87.9	0.91	0.65 to 1.25	0.82	0.59 to 1.14	.251
Placebo	299	80.7	76.9 to 84.9					
COX-2 positive								
Rofecoxib	79	89.9	81.0 to 95.0	0.56	0.28 to 1.10	0.74	0.36 to 1.51	
Placebo	91	75.3	65.3 to 83.1					

Abbreviations: COX-2, cyclooxygenase-2; HR, hazard ratio; RFS, recurrence-free survival.
**P* value for interaction of tumor COX-2 expression and aspirin use after multivariate analysis by Cox proportional hazards.

Table A7. RFS by Tumor COX-2 Expression and Aspirin Use

Group	No. of Patients	RFS						
		3-Year RFS (%)	95% CI (%)	Univariate HR	95% CI	Multivariate Adjusted HR	95% CI	*P**
COX-2 negative								
Aspirin use	89	89.7	81.3 to 94.7	0.77	0.46 to 1.30	0.76	0.45 to 1.29	.528
No aspirin use	549	81.6	78.1 to 84.6					
COX-2 positive								
Aspirin use	26	92.0	73.9 to 98.9	0.46	0.14 to 1.51	0.55	0.16 to 1.91	
No aspirin use	144	80.4	73.1 to 86.1					

Abbreviations: COX-2, cyclooxygenase-2; HR, hazard ratio; RFS, recurrence-free survival.
**P* value for interaction of tumor COX-2 expression and aspirin use after multivariate analysis by Cox proportional hazards.

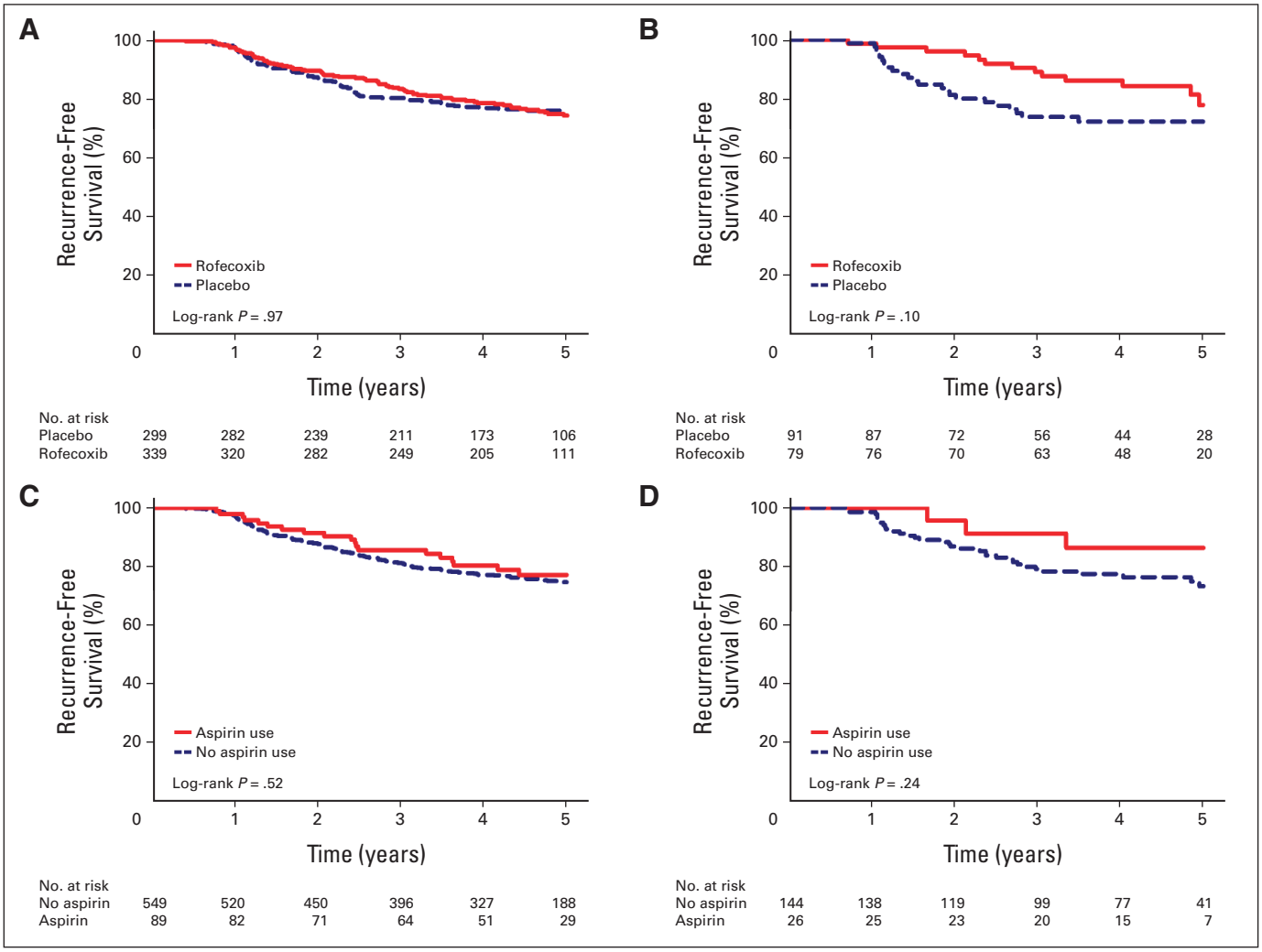

Fig A1. Recurrence-free survival according to tumor cyclooxygenase (COX-2) expression and COX-2 inhibitor therapy. Recurrence-free survival of patients treated with rofecoxib or placebo in subgroups with (A) negative or low tumor level of COX-2 expression and (B) high COX-2 expression. Recurrence-free survival of patients using aspirin or not using aspirin in subgroups with (C) negative or low tumor level of COX-2 expression and (D) high COX-2 expression. The number of patients at risk is indicated below each panel. Comparison between groups was made using the log-rank test.