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A Randomized, Multicenter, Placebo-Controlled Clinical Trial of Racotumomab-Alum Vaccine as Switch Maintenance Therapy in Advanced Non–Small Cell Lung Cancer Patients

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Abstract

Purpose: Racotumomab-alum is an anti-idiotype vaccine targeting the NeuGcGM3 tumor-associated ganglioside. This clinical trial was conducted to provide a preliminary estimate of efficacy and safety of racotumomab as switch maintenance for patients with advanced non–small cell lung cancer (NSCLC).

Experimental design: Patients with stage IIIb/IV NSCLC who have at least stable disease after first-line chemotherapy were randomized 1:1 to racotumomab-alum (5 immunizations every 2 weeks and re-immunizations every 4 weeks) or placebo. Treatment was administered beyond progressive disease, until severe performance status worsening or toxicity. At progression, only five patients per group received further anticancer therapy. The primary endpoint was overall survival (OS).

Results: One-hundred and seventy-six patients were randomized to racotumomab-alum ($n = 87$) and placebo ($n = 89$). Median OS was 8.23 and 6.80 months, respectively [HR, 0.63; 95% confidence interval (CI), 0.46–0.87; $P = 0.004$]. Median progression-free survival (PFS) in vaccinated patients was 5.33 versus 3.90 months for placebo (HR, 0.73; 95% CI 0.53–0.99; $P = 0.039$). The most common adverse events in the racotumomab-alum arm were burning and pain at the injection site, bone pain, and asthenia. A high antibody response of IgM and IgG isotype against the NeuGcGM3 ganglioside was obtained. Hyperimmune sera were able to specifically recognize and kill the NeuGcGM3-expressing L1210 cell line. Patients who developed anti-NeuGcGM3 antibodies capable to bind and kill ≥30% L1210 cells showed longer median survival times.

Conclusions: Switch maintenance with racotumomab-alum is an effective and a well-tolerated treatment option for patients with advanced NSCLC. *Clin Cancer Res; 20(14); 3660–71. ©2014 AACR.*

Introduction

Lung cancer is the most common cause of cancer-related death worldwide (1). Non–small cell lung cancer (NSCLC) constitutes the predominant histologic type among the lung neoplasias, representing more than 75% of all lung tumors. A high percentage of the patients are diagnosed at locally advanced stage or with metastatic disease (2). Unfortunately, little progress has been achieved in the overall survival (OS) of patients with advanced NSCLC after first-line chemotherapy, as various chemotherapy combinations have not improved the results obtained with platinum doublets (3).

Recently, the role of maintenance treatment for patients with advanced NSCLC with favorable response [complete response (CR), partial response (PR), or stable disease (SD)] after first-line chemotherapy has been evaluated by an international group of experts. On the basis of the available evidence, the expert panel agreed that maintenance therapy represents a treatment option in advanced NSCLC, in any of its modalities: continuation or switch, the latter with a different chemotherapy or targeted agent (4). Most trials showed significant prolongation of progression-free survival (PFS; refs. 5–14), whereas some studies also showed extension of the OS, particularly after the use of pemetrexed and erlotinib (6, 7, 9, 14, 15).

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Translational Relevance

In advanced non–small cell lung cancer (NSCLC), there have been few improvements in outcome after first-line chemotherapy as new combinations of cytotoxic drugs have not enhanced the results obtained just with platinum doublets. Thus, the development of new more effective targeted strategies continues to be a priority. Here, we present the clinical and immunologic results from a randomized study of racotumomab-alum vaccine, targeting NeuGcGM3 ganglioside, as switch maintenance therapy in patients with advanced NSCLC. A clinical meaningful improvement in overall survival (OS) and progression-free survival for racotumomab-alum versus placebo is showed. The capacity of IgM anti-NeuGcGM3 antibodies to bind 30% or more of NeuGcGM3-expressing L1210 cells and to kill that percentage of cells was associated with longer OS. To our knowledge, this is the first report showing an association between the active induction of cytotoxic NeuGcGM3-specific antibodies and the clinical outcome of patients with NSCLCs treated with racotumomab-alum vaccine.

Active immunotherapy is one of the most promising fields in cancer research and several strategies have been pursued to design cancer vaccines. One approach to generate an effective immune response against tumor-associated antigens involves the use of anti-idiotype monoclonal antibodies (Ab2 mAb). Anti-idiotypic antibodies have proved to mimic the nominal antigens and induce antigen-specific responses, even against non-protein tumor-associated antigens like gangliosides (16, 17).

Neu glycolyl (NeuGc)—containing gangliosides are attractive targets for immunotherapy. These glycolipids are not present in the cytoplasmic membrane of humans (18), after a 92-bp deletion in the gene that encodes the cytidine-monophosphate-*N*-acetylneuraminc acid hydroxylase (*cmah*), which catalyzes the conversion of *N*-acetyl to NeuGc sialic acid (19, 20). However, NeuGc—containing gangliosides have been detected in several human tumors, including NSCLC (21–24). NeuGcGM3 is besides a potent immunosuppressive molecule (25).

We previously reported a vaccine preparation composed of a murine anti-idiotype mAb that mimics NeuGcGM3 ganglioside. This Ab2, named 1E10 and later racotumomab (26), was generated from immunizing BALB/c mice with P3 mAb, an Ab1 antibody that recognizes NeuGc gangliosides (27–30). Racotumomab vaccine was able to generate a strong antitumor activity in mice bearing melanoma or breast carcinomas (26) and to induce antimetastatic effect in 3LL-D122 Lewis Lung carcinoma, a poorly immunogenic and highly metastatic murine model in C57BL/6 mice. Therapeutic effect was associated with an increase of T cells infiltrating the metastases, a reduction of blood vessels, and an increase of apoptotic cells in the lung metastases (31). Three different toxicology studies evaluating acute toxicity, repeated toxicity, and local tolerance were done. A mutagenic study was also performed. Racotumomab was demonstrated to be safe and non-mutagenic.

Several phase I trials using racotumomab-alum vaccine were conducted in patients with advanced melanoma, breast, and lung cancer. Vaccine was safe and immunogenic as high antibody response to NeuGcGM3 was detected in vaccinated patients (28–30, 32, 33). In addition, NeuGcGM3-specific IFNγ-secreting T cells were detected by ELISPOT in patients with breast cancer vaccinated with racotumomab (34). Although these studies were not designed to evaluate the therapeutic efficacy of the vaccine, a prolonged survival was observed in several patients. In particular, encouraging results were obtained when racotumomab-alum vaccine was evaluated in patients with NSCLC (30). A strong IgM and IgG antibody response against NeuGcGM3 was elicited in patients with advanced NSCLC. Patients' hyperimmune sera were able to specifically recognize and induce cell death of the NeuGcGM3-expressing X63 myeloma cells. Remarkably, patients who developed IgM or IgG antibodies against NeuGcGM3 showed longer median survival time (33).

To assess the efficacy and tolerability of switch maintenance therapy with racotumomab-alum in patients with advanced NSCLC, we conducted this randomized, double-blind, placebo-controlled phase II/III clinical trial.

Patients and Methods

Patient's selection

Eligible patients were 18 years or older, with histologically or cytologically confirmed stage IIIB–IV NSCLC (6th edition AJCC staging system), who had achieved CR, PR, or SD after the standard first-line therapy. Random assignment, 87 patients in the vaccine group and 89 in the placebo group, was performed at least 28 days after completing first-line treatment, but within 2 months. All patients had measurable disease, an Eastern Cooperative Oncology Group performance status (ECOG PS) ≤ 2 (35), adequate renal, hepatic, and hematologic functions.

Exclusion criteria included patients who had received immunotherapy or other investigational drugs; patients with known hypersensitivity to any component of the formulation; patients who were pregnant or lactating; patients with uncontrolled chronic diseases, history of severe allergic reactions; patients with brain metastases or other primary neoplastic lesion; patients with active infections, symptomatic congestive heart failure, unstable angina, cardiac arrhythmia or psychiatric disorders; and patients receiving systemic corticosteroids at the time of inclusion and patients with positive serology for hepatitis B and C or HIV.

Study design

This double-blinded, placebo-controlled clinical trial was conducted at 3 hospitals from Villa Clara, Matanzas, and Havana, Cuba. Sample size was estimated considering that 160 patients, or 103 events, would achieve 81% power to

detect a hazard rate of 0.56 when the proportions of survivors in each group are 0.25 and 0.46 at a significance level of 0.05, using a 2-sided log-rank test. About 10% patients' loss was anticipated, and accordingly, the final sample size was 176 patients. The projected accrual period was 3 years. An interim analysis on futility was planned when 52 events occurred. The primary objective was to evaluate the effect of racotumomab-alum vaccine in OS of patients with advanced NSCLC. The secondary objectives were to evaluate PFS, immunologic response, and safety.

Eligible patients were randomized 1:1 to receive racotumomab-alum (group A) or placebo (group B). Randomization was not stratified but it was balanced according to gender, clinical stage, performance status, race, and response to first-line treatment.

Patients were injected intradermally and received 15 doses of 1 mg of racotumomab-alum or placebo. The induction phase consisted of 5 doses administered every 2 weeks. After induction, patients were vaccinated every 4 weeks, for 1 year (10 doses). After treatment was completed (15 doses of vaccine or placebo), the blinding was opened, and only patients in the racotumomab arm continued vaccination every 4 weeks, even beyond progression. Criteria for stopping vaccination included voluntary withdrawal, unacceptable toxicity, or severe worsening of the patient's PS. Patients who discontinued study treatment were followed until death or study termination. Concurrent antitumor therapy was not permitted.

An independent CRO (CENCEC) was responsible for conducting the study. Treatment allocation was done using the automatic MINIM system. To balance both study groups, the investigator provided the information on patient sex, race, clinical stage, PS, and response to first-line treatment before randomization. The trial was blinded and the investigational drug and placebo had the same organoleptic characteristics and were presented in the same primary and secondary containers. A sealed letter enclosing the information of the treatment group was sent together with the investigational product. Information on treatment was disclosed only after completing 15 doses or in case of severe or serious adverse events.

The trial was approved by the local ethic review boards and the Cuban Regulatory Agency. All patients provided written informed consent before study participation. The trial was conducted in accordance to the principles of the Declaration of Helsinki and Good Clinical Practice guidelines. It is registered at the Cuban Registry of Clinical Trials (www.registroclinico.sld.cu; ID: RPCEC00000009).

Efficacy assessments

OS was defined as the time elapsed from randomization to death from any cause. PFS was calculated from the date of randomization up to the date of documented progression or death from any cause.

Patient assessment was performed at baseline and every 4 weeks. Baseline evaluation included medical and smoking history, physical examination, vital signs, PS, complete blood cell count, and blood biochemistry. Toxicity was evaluated according to the Common Terminology Criteria for Adverse Events (version 3.0), by physical examination and clinical laboratory tests, performed during treatment and follow-up. Tumors were measured by X-rays and CT scans at baseline and then every 3 months. The RECIST 1.0 guideline (36) was used to classify tumor response. After disease progression, patients could receive any subsequent therapy at the discretion of the treating physician.

Anti-idiotype racotumomab mAb

Active drug was composed of the anti-idiotype antibody racotumomab, alum hydroxyl gel, mono- and dibasic sodium phosphatase salt, sodium chloride, and water for injection. The placebo had the same composition excluding racotumomab. Racotumomab mAb was purified from mouse ascites, and the aluminum hydroxide–precipitated vaccine was produced according the Good Manufacturing Practice guidelines. The manufacturing process was certified by the Quality Control Department of the Center of Molecular Immunology (CIM).

Ganglioside and tumor cell lines

NeuGcGM3 ganglioside was purified from horse erythrocytes as described previously (37) and was provided by Dr. Carr (CIM, Havana, Cuba). The L1210 murine lymphocytic leukemia cell line, expressing high levels of NeuGcGM3 ganglioside (38, 39), was obtained from the ATCC. L1210 cells deficient of NeuGc sialoconjugates (L1210 *cmah-kd*), were generated at CIM, as previously explained (40). Cells were grown in DMEM/F12 medium (Gibco-BRL) supplemented with 10% of heat-inactivated fetal bovine serum (FBS) (Gibco-BRL), 2 mmol/L L-glutamine, 25 mmol/L HEPES, penicillin (100 μg/mL), and 100 μg/mL of streptomycin (Invitrogen) and maintained at 37°C with 5% CO_2.

Measurement of antibody response

The trial goal was to evaluate the immune response in 30% of the subjects (53 vaccinated and placebo patients). However, 6 of the 53 patients did not complete the vaccine induction period (5 doses) or did not have at least 3 serial serum samples. Consequently, the immunologic assessment was done in a cohort of 47 patients, 24 vaccinated and 23 controls.

The antibody response against racotumomab mAb and the purified ganglioside NeuGcGM3 was determined by a solid-phase ELISA, as formerly reported (28). The inverse of the highest serum dilution giving optical density (OD) values ≥ 0.25 and twice the value of the pre-immune serum was considered as the antibody titer. Assays were performed in triplicate for each sample and the coefficient of variation (CV) was less than 15%. The ODs of the blanks were less than 0.1.

Antibody-binding assay by flow cytometry

The detection of antibodies binding to wild-type and mutated L1210 in patients' sera was performed as reported before (41). L1210 cells were blocked in PBS containing 1% BSA for 20 minutes on ice. Patients' serum samples,

corresponding to pre-immune and hyperimmune sera from 2 to 6 months of treatment, were diluted 1:10 and incubated with 10^5 cells for 30 minutes on ice. After washing with cold PBS, the cells were incubated for 30 minutes on ice, with FITC-conjugated goat anti human IgM or FITC-conjugated goat anti-human IgG antibodies (Jackson ImmunoResearch Laboratories). Percentage of positive stained cells was determined in a FACScan flow cytometer (Becton Dickinson). The FlowJo program (version 5.7.2) was used to analyze a total of 10^4 cells acquired on every FACS assay. Positive patients had a binding percent $\geq$20%, after subtracting the pre-immune to the hyperimmune recognition percent. The L1210 *cmah-kd* cell line, deficient of NeuGc sialoconjugates (40), was used as control of the antibody specificity for the NeuGcGM3 ganglioside.

Induction of cell death

The lytic capacity of patients' sera was tested by incubating the 1:5 diluted sera with 10^5 tumor cells in DMEM/F12 medium supplemented with 1% FBS in 5% CO_2 atmosphere at 37°C, for 3 hours (33, 39, 41). Cell death percent was determined by flow cytometry after the addition of propidium iodide (PI; Sigma-Aldrich) at a final concentration of 10 μg/mL. The cytotoxicity was considered positive when the percentage of dead cells was $\geq$20%, after subtracting the pre-immune to hyperimmune percent.

To determine whether the binding and cytotoxic effects of serum were specifically mediated by anti-NeuGcGM3 antibodies, L1210 *cmah-kd* cells and L1210 cells cultured with 10 μmol/L of D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-threo-PDMP; Matreya, LLC), an inhibitor of glucosylceramide synthetase (40), were used.

Statistical analyses

For the analyses of OS and PFS, median values and 95% confidence intervals (CI) were estimated by Kaplan–Meier methodology, and 2-sided log-rank tests were used to compare treatment groups. A secondary analysis of OS was performed using the Cox proportional hazards regression model to determine the effect of treatment in different subgroups. This analysis included age, sex, ethnic origin, tumor stage, histology, smoking history, race, ECOG, response to first-line treatment, and the immunologic variables. The χ^2 and Fisher tests were used for evaluating distribution differences between groups. Wilcoxon test was used as a nonparametric test for matched-pairs signed-ranks test. Statistical analyses were done with SPSS program (version 17.0).

Results

Patient characteristics

From September 27, 2006, to June 28, 2010, 176 patients were randomized: 87 to racotumomab-alum and 89 to placebo (Supplementary Fig. S1). Thus, 176 patients were included in the intent-to-treat efficacy analysis. One patient with histologically unconfirmed NSCLC randomized to racotumomab-alum group did not receive treatment and was excluded from the safety analysis. Demographic and baseline characteristics were similar in both groups (Table 1).

All patients received platinum-based regimens as first-line chemotherapy. These regimens included vinblastine or etoposide combined with cisplatin or carboplatin. One hundred and sixty-nine patients (98.3%) received 4 to 6

Table 1. Demographic and baseline characteristics

	Racotumomab-alum (*n* = 87)	Placebo (*n* = 89)
Age, y		
≤60	38 (43.7%)	41 (46.1%)
≥60	49 (56.3%)	48 (53.9%)
Gender		
Female	28 (32.2%)	30 (33.7%)
Male	59 (67.8%)	59 (66.3%)
ECOG PS		
0	40 (46.0%)	40 (44.9%)
1	45 (51.7%)	45 (50.6%)
2	2 (2.3%)	4 (4.5%)
Race		
White	70 (80.5%)	71 (79.8%)
Afro-Caribbean	11 (12.6%)	13 (14.6%)
Other	6 (6.9%)	5 (5.6%)
Smoking history		
Current smoker	16 (18.45%)	19 (21.3%)
Former smoker	67 (77.0%)	65 (73.0%)
Nonsmoker	4 (4.6%)	5 (5.6%)
Tumor histology		
Squamous cell carcinoma	33 (37.9%)	33 (37.1%)
Adenocarcinoma	25 (28.7%)	31 (34.8%)
Large cell carcinoma	18 (20.7%)	14 (15.7%)
NSCLC NOS	11 (12.6%)	11 (12.4%)
Disease stage		
IIIB	48 (55.2%)	51 (57.3%)
IV	39 (44.8%)	38 (42.7%)
First-line treatment		
Chemotherapy	87 (100%)	89 (100%)
Radiotherapy	50 (57.5%)	51 (57.3%)
First-line chemotherapy		
Platinum compounds	87 (100%)	89 (100%)
Cisplatin/Vinblastine	28 (32.2%)	18 (20.2%)
Cisplatin/Etoposide	6 (6.9%)	5 (5.6%)
Carboplatin/Vinblastine	39 (44.8%)	50 (56.2%)
Carboplatin/Etoposide	11 (12.6%)	13 (14.6%)
Other Platinum doublets	3 (3.5%)	3 (3.4%)
Response to first-line treatment		
CR	2 (2.3%)	5 (5.6%)
PR	38 (43.7%)	51 (57.3%)
SD	47 (54.0%)	33. (37.1%)

NOTE: Data are *n* (%).
Abbreviation: NOS, not otherwise specified.

chemotherapy cycles as first-line treatment and only 3 patients (1.7%) received less than 4 cycles (1 patient from the vaccine group and 2 from the placebo arm). As part of the first-line treatment, 50 vaccinated patients (57.5%) and 51 patients (57.3%) from the placebo group received radiotherapy.

All patients included in the trial achieved SD, PR, or CR after first-line therapy and were randomized between 4 and 8 weeks after completing treatment, with a median of 1.51 months (vaccine) and 1.43 months (placebo). Median time from randomization to start racotumomab or placebo was 0.46 and 0.43 months, respectively.

After interrupting vaccine or placebo, 5 patients per group (5.6% of all enrolled patients), received further anticancer therapy. Because of treatment unavailability, only 1 patient from the placebo arm received docetaxel, one of the recommended drugs for second-line therapy in NSCLC. The remaining 4 patients in the placebo cohort received carboplatin (2 patients) or etoposide (2 patients). On the other hand, 4 patients from the racotumomab arm received new cycles of carboplatin, whereas 1 subject was treated with cisplatin/vinblastine.

Efficacy

The intent-to-treat median OS was 8.23 months (95% CI, 5.59–10.87) for the racotumomab-alum group versus 6.80 months (95% CI, 5.77–7.83) for the placebo group. This difference was statistically significant (HR, 0.63; 95% CI, 0.46–0.87; $P = 0.004$; Fig. 1A). Ten patients from the racotumomab arm and 2 patients from the placebo cohort were alive at the moment of the analysis and were consequently censored. The 1- and 2-year survival rates were 40.2% and 18.4% for the racotumomab-alum group compared with 22.5% and 6.7% for the placebo group.

Almost all patient subgroups benefited from therapy. Remarkably patients with stage IIIB NSCLC derived a larger benefit than those with stage IV (Supplementary Fig. S2).

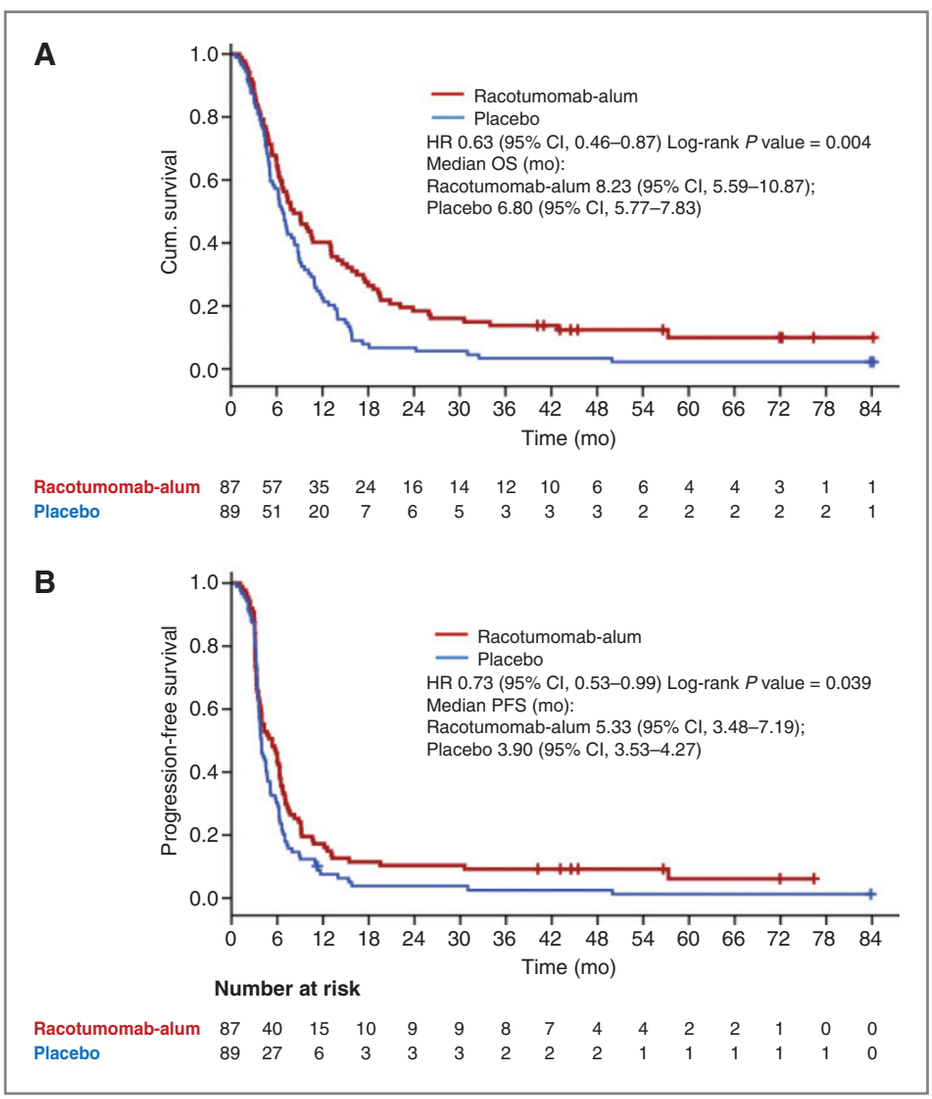

Figure 1. Intent-to-treat OS (A) and PFS (B) in patients with advanced NSCLC receiving racotumomab-alum vaccine or placebo.

Vaccinated patients also had a statistically significant advantage in PFS (HR, 0.73; 95% CI, 0.53–0.99; $P = 0.039$; Fig. 1B). Median intent-to-treat PFS was 5.33 months (95% CI, 3.48–7.19) in the racotumomab-alum group versus 3.90 months (95% CI, 3.53–4.27) in the placebo group. Seven patients from the racotumomab arm and 2 from the placebo arm have not progressed at the moment of this report.

Safety

The distribution of treatment-related adverse events in the 2 arms is shown in Table 2. The most common racotumomab-related adverse events (all grades) were burning (41.9%) and pain at the injection site (33.7%), followed by bone pain (18.6%) and asthenia (16.3%). In the placebo group, the most frequently reported adverse events were burning (39.3%) and pain at the injection site (24.7%), as well as bone pain (19.1%).

All but 4 treatment-related adverse events were classified as mild or moderate. Two racotumomab-attributed adverse events consisting of asthenia and bone pain were classified as grade III–IV, whereas 2 patients in the placebo arm developed grade III–IV bone pain and dyspnea. Even though these adverse events were categorized as related with the investigational drug, they might also be associated with the natural course of the lung disease and its metastasis. This needs to be confirmed in a larger study in patients with advanced NSCLCs.

Overall, racotumomab-alum was very well-tolerated and its safety profile was consistent with the previously described (28–30).

The proportion of all adverse events (regardless causality) was not different between treatment groups (Supplementary Table S1). One-hundred and fifty-eight (90.3%) patients had at least 1 adverse event of any grade (Supplementary Table S2). Thirty-four patients (39.5%) in the racotumomab group and 31 (34.8%) in the placebo arm experienced serious adverse events (SAE). Four events from each group consisting of dyspnea, asthenia, bone pain, and abscess in right upper limb were classified as related to the investigational drug. No patient in the vaccine group and only one (1.1%) patient in the placebo group was withdrawn from treatment because of an adverse event. The proportion of SAEs was not different between both groups. No adverse events leading to death were related to the treatment.

Immune response induced by racotumomab-alum vaccine

All vaccinated patients developed a high antibody response against racotumomab of IgG isotype. The titers ranged from 1:200 to 1:51,200, with a median of 1:6,400 (Supplementary Table S3). No reactivity to racotumomab mAb was detected in the patient's sera from the placebo group, at the lowest serum dilution tested (1:50; data not shown).

To determine whether antibodies induced after vaccination had the same specificity of P3 mAb (Ab1), pre- and hyperimmune serum samples were tested for the recognition of NeuGcGM3 ganglioside. An antibody response against NeuGcGM3 was generated in all vaccinated patients (Supplementary Table S3). Twenty-three patients (95.8%) developed antibodies of both IgG and IgM isotypes that bind to NeuGcGM3 by ELISA, whereas 1 subject (4.2%) elicited only IgM antibodies. None of the patients developed IgG subclass antibodies alone. Titers of up to 1:51,200 and 1:6,400 for IgM and IgG isotypes, respectively, were

Table 2. Treatment-related adverse events recorded in at least 1.5% in either group

	Racotumomab-alum (n = 86)		Placebo (n = 89)	
Adverse event	**All grades**	**Grade III–IV**	**All grades**	**Grade III–IV**
Burning in injection site	36 (41.9%)	0 (0%)	35 (39.3%)	0 (0%)
Pain in injection site	29 (33.7%)	0 (0%)	22 (24.7%)	0 (0%)
Bone pain	16 (18.6%)	1 (1.1%)	17 (19.1%)	1 (1.1%)
Cough	7 (8.1%)	0 (0%)	11 (12.4%)	0 (0%)
Dyspnea	5 (5.8%)	0 (0%)	5 (5.6%)	1 (1.2%)
Asthenia	14 (16.3%)	1 (1.2%)	10 (11.2%)	0 (0%)
Anorexia	6 (7.0%)	0 (0%)	7 (7.9%)	0 (0%)
Expectoration	1 (1.2%)	0 (0%)	3 (3.4%)	0 (0%)
Induration	10 (11.6%)	0 (0%)	9 (10.1%)	0 (0%)
Headache	8 (9.3%)	0 (0%)	9 (10.1%)	0 (0%)
Pruritus	9 (10.5%)	0 (0%)	5 (5.6%)	0 (0%)
Fever	8 (9.3%)	0 (0%)	12 (13.5%)	0 (0%)
Increased volume in injection site	9 (10.5%)	0 (0%)	3 (3.4%)	0 (0%)
Local erythema	11 (12.8%)	0 (0%)	11 (12.4%)	0 (0%)
Myalgia	5 (5.8%)	0 (0%)	7 (7.9%)	0 (0%)
Arthralgia	5 (5.8%)	0 (0%)	5 (5.6%)	0 (0%)

NOTE: Data are number of patients (%). Adverse events graded by CTCAE version 3.0.

obtained. No reaction was detected when pre-immune patients' sera were tested.

Recognition of tumor cells expressing NeuGcGM3 ganglioside

Next, we evaluated the ability of patient's sera to recognize the NeuGcGM3 ganglioside in the natural context of tumor cell membranes. Hyperimmune sera from 11 of 24 patients (45.8%) showed significant binding of antibodies of both isotypes (IgM and IgG) to L1210 cells, compared with the pre-immune sera ($P < 0.0001$; Wilcoxon test; Supplementary Table S3 and Fig. 2A and B). Seven (29.2%) and 4 patients (16.7%) showed IgM- or IgG-binding antibodies to L1210 cells. Only the hyperimmune sera from 2 patients (8.3%) were not able to recognize the NeuGcGM3-expressing tumor cell line (Supplementary Table S3). To confirm that antibodies specifically recognized NeuGcGM3 ganglioside on the cell surface, the binding of patients' serum samples to L1210 *cmah-kd* cells, devoid of Neu5Gc-sialoconjugates, was assessed. As is shown in Fig. 2A, sera that bind the native L1210 cells were not able to recognize L1210 *cmah-kd* cells, suggesting that the antigen recognized is presumably a Neu-glycolylated molecule. Binding of IgM and IgG antibodies to L1210 cells, corresponding to 2 representative patients, is shown in Fig. 2C and D.

Cytotoxic effect of anti-NeuGcGM3 antibodies induced by vaccination with racotumomab-alum

To study whether the antibodies induced in vaccinated patients were capable not only to recognize but also to kill

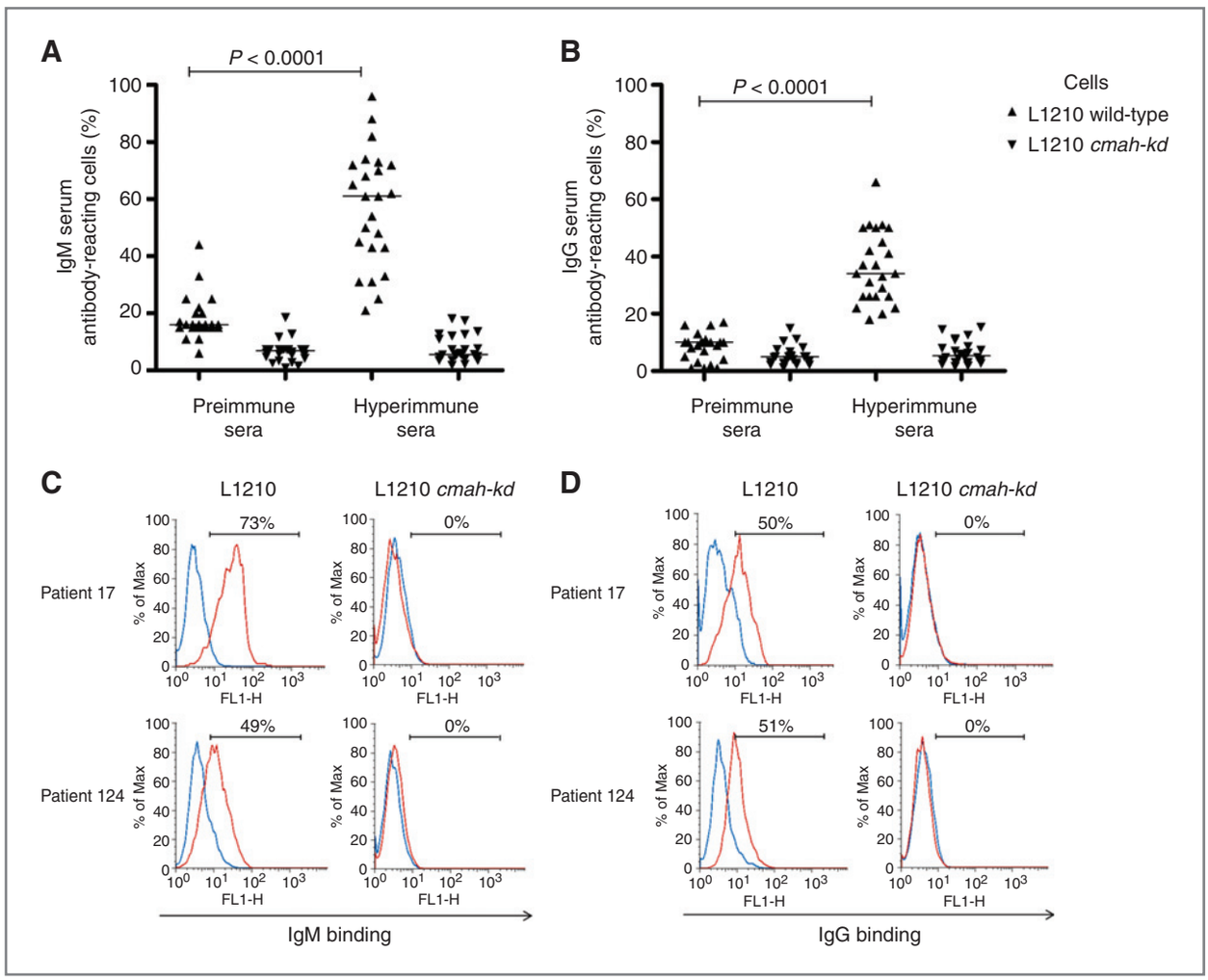

Figure 2. Binding of pre-immune and hyperimmune sera from 24 NSCLC vaccinated patients to the NeuGcGM3-expressing cell line L1210. Recognition assay was performed by incubating the 1:10 diluted sera with L1210 cells or with L1210 *cmah-kd* cells, devoid of NeuGc sialoconjugates. The reaction was developed with FITC-conjugated anti-human IgM (A) or IgG (B). Bars indicate median values. A significant increment in the median binding to L1210 of IgM and IgG hyperimmune antibodies as compared with the pre-immune sera was observed ($P < 0.001$; Wilcoxon test). Binding was affected when sera were incubated with L1210 *cmah-kd* cells, deficient of NeuGc sialoconjugates, confirming that the recognized antigen is presumably a NeuGc glycosphingolipid. C and D, flow cytometric assays from 2 representative patients, showing both isotypes' binding to L1210. The numbers represent the percentage of positive cells after subtracting the binding of pre-immune sera.

the NeuGcGM3-expressing cells, patients' sera were incubated with L1210 cells. Cytotoxicity was evaluated by flow cytometry using the PI exclusion assay. In 18 of 24 patients (75%), a significant increase in PI incorporation was observed ($P < 0.0001$; Wilcoxon test; Supplementary Table S3 and Fig. 3A). Sera cytotoxicity was significantly reduced when hyperimmune sera were incubated with L1210 *cmah-kd* or with L1210 cells treated with D-threo-PDMP, suggesting that the antigen recognized in the tumor cell membrane is a Neu-glycolylated glycosphingolipid (Fig. 3A). Histograms of PI-stained cells from 2 representative patients are shown in Fig. 3B.

Immunological response and clinical outcomes of patients

The individual survival data from the 24 vaccinated patients that were sampled for immune response evaluation are shown in Supplementary Table S3. Interestingly, we found that the percentage of IgM recognition (HR, 0.97; 95% CI, 0.95–0.99; $P = 0.040$) and the cytotoxicity against L1210 cells (HR, 0.98; 95% CI, 0.96–0.99; $P = 0.019$) were statistically associated with survival (Fig. 4). No association was observed when other immunologic variables were analyzed. Then, aiming to find a surrogate of patients' outcome, we looked for the lower recognition and cell death percentage associated with patients' survival. Better survival was found in patients with IgM antibodies able to recognize at least 20% of L1210 cells (19.30 vs. 7.83 months; HR, 0.30; 95% CI, 0.11–0.83; $P = 0.014$) or kill at least 25% of the same NeuGcGM3-positive cells (19.50 vs. 8.23 months; HR, 0.26; 95% CI, 0.10–0.70; $P = 0.005$). The highest differences were detected using 30% recognition (19.50 vs. 8.23 months; HR, 0.26; 95% CI, 0.10–0.73; $P = 0.006$) and 30% cell death cut-off (19.63 vs. 8.23 months; HR, 0.21; 95% CI, 0.08–0.58; $P = 0.003$; Supplementary Table S4).

Discussion

In this randomized, placebo-controlled study, patients with advanced NSCLC who received racotumomab-alum

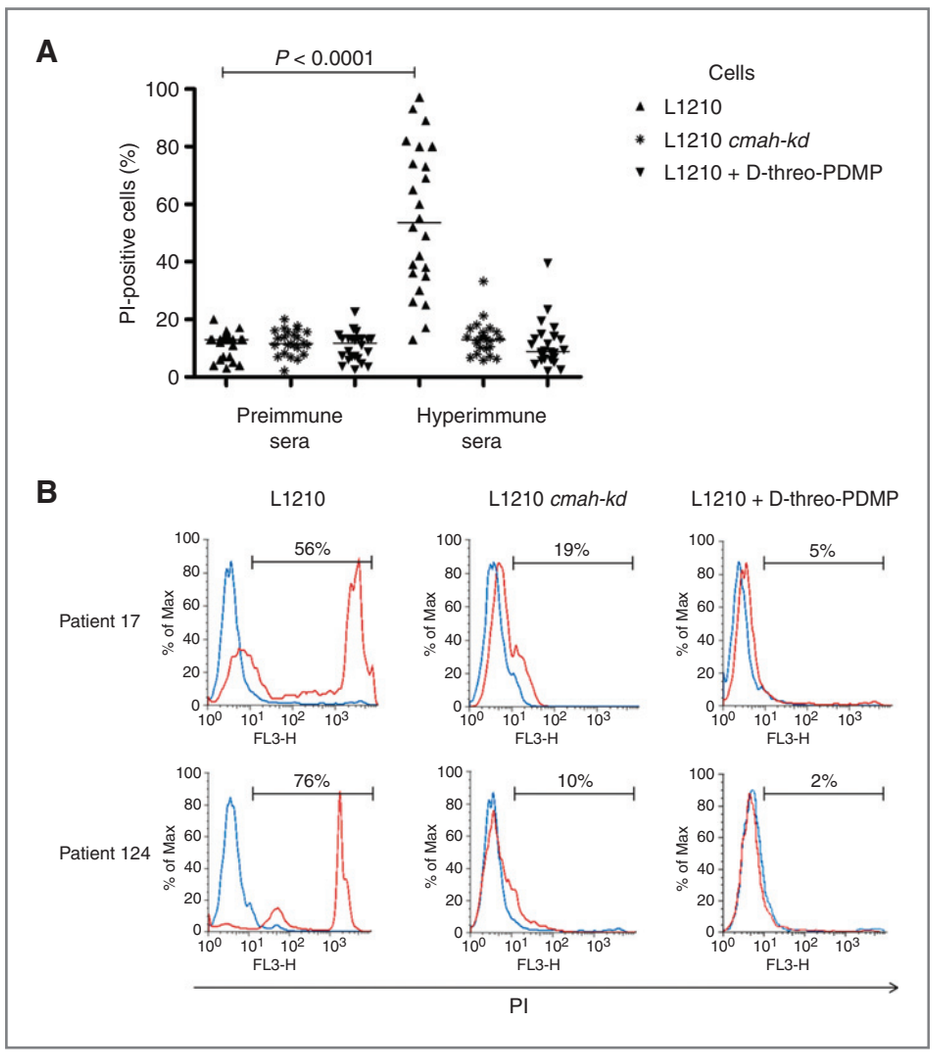

Figure 3. Cytotoxicity induced by sera from 24 NSCLC vaccinated patients on the NeuGcGM3-expressing L1210 cell line. Cells were incubated for 3 hours at 37°C and 5% CO_2 atmosphere with 1:5 diluted pre-immune (blue line) and hyperimmune (red line) sera. The percentage of cell death was measured by PI incorporation assay. A, percentage of cell death after incubation of pre-immune and hyperimmune sera with the NeuGcGM3-expressing L1210 cells, with L1210 cells transduced with a CMP-Neu5Ac-neuraminic acid hydroxylase gene–specific shRNA (L1210 *cmah-kd*) or with L1210 cells treated with 10 μmol/L D-threo-PDMP, an inhibitor of glycosphingolipid biosynthesis. Bars indicate median values. A significant increase in the cytotoxic activity of hyperimmune sera over L1210 cells was observed. $P < 0.001$; Wilcoxon test. Loss of NeuGc sialoconjugates or treatment with the inhibitor of glucosylceramide synthetase inhibited cytotoxicity, confirming that the antigen recognized is a *N*-glycolylated glycosphingolipid. B, histograms of PI-stained cells from 2 representative patients. The numbers represent the percentage of positive cells after subtracting the lysis of pre-immune sera.

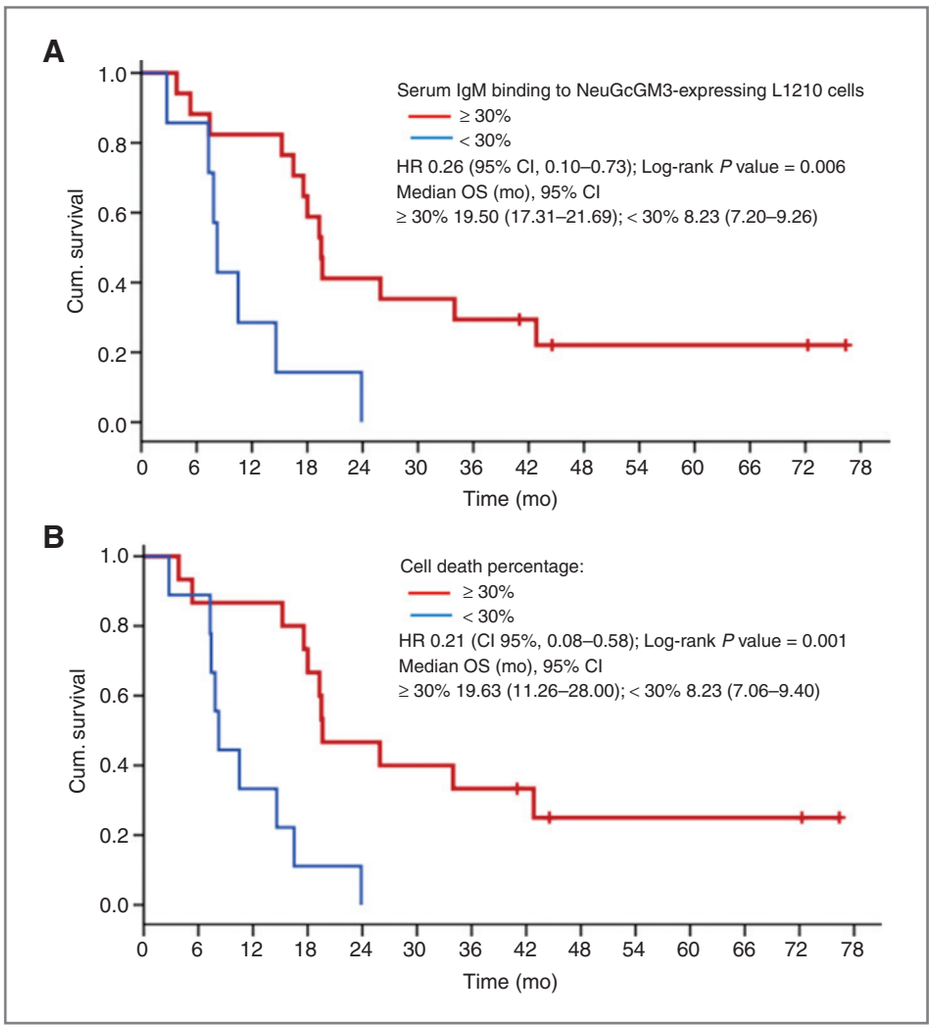

Figure 4. OS according to functional capacity of the anti-NeuGcGM3 antibodies elicited after racotumomab-alum vaccination. A, survival of patients with IgM antibodies binding ≥30% (red line) versus <30% (blue line) to L1210 cells. B, survival of patients with cytotoxic antibodies killing 30% or more (red line) L1210 cells versus patients with less than 30% lysis (blue line).

vaccine as switch maintenance after first-line treatment had a significant survival improvement compared with those who received a placebo.

This study is the first to demonstrate the efficacy of a NeuGc-containing ganglioside anti-idiotype vaccine as switch maintenance therapy for advanced NSCLC. It is noteworthy that most studies designed to find the role of maintenance treatment in patients with advanced NSCLC have shown improvement in PFS (5–14), but only a few demonstrated an impact in OS (6, 7, 9, 14, 15). Our vaccine improved both PFS and OS.

The shorter OS seen in our trial can be attributed to the lack of second-line therapy and the time elapsed between completing first-line chemotherapy and trial enrollment. In addition, apart from other studies on maintenance (6, 9), our trial involved patients with a worse PS (≤2) and carrying any NSCLC histology.

Several cancer vaccines have been tested for NSCLC, without great success. Belagenpumatucel-L (Lucanix) is an allogeneic whole-cell vaccine with a TGF-β2 antisense. Results from a phase III, placebo-controlled clinical trial demonstrated that vaccination did not improve OS. Some patients, mainly those randomized within 12 weeks of the completion of chemotherapy, did derive some survival benefit (42). L-BLP25 (Stimuvax) is a second cancer vaccine in development for advanced NSCLC, which targets aberrantly glycosylated MUC1. Phase III START study recently failed to reach its primary endpoint of improving OS of patients with stage III NSCLC. Only patients who received concurrent chemoradiotherapy before vaccination had a significant survival increase (43). In both cases, new studies should be done to demonstrate vaccine efficacy. On the other hand, preliminary data from a phase III trial evaluating an EGF-based vaccine suggested that vaccination significantly improved survival of patients with advanced NSCLC. Subjects with higher baseline EGF concentration in serum, derived the greatest benefit (44). Final results from this phase III are still pending.

Better survival with racotumomab-alum was not associated with an increase in adverse events as compared with

placebo. Although patients received repeated injections with racotumomab-alum vaccine, the most common adverse events were classified as mild or moderate, confirming our former results on safety (28–30, 32).

Our group has previously reported the induction of an antibody response against NeuGcGM3 ganglioside in patients with breast, melanoma, SCLC, and NSCLC treated with the racotumomab-alum vaccine (28, 29, 32, 33). In the present study, we confirmed the immunogenicity of this anti-idiotype vaccine. Racotumomab-alum elicited antibodies specific to the NeuGcGM3 ganglioside and most patients developed high titers of both IgM and IgG isotypes. This is a very relevant result taking into account the general lower immunogenicity of carbohydrates (45, 46). This high antibody response against NeuGcGM3 can be explained by the unique properties of NeuGc-containing gangliosides, which are non–self-antigens in humans, as the result of the inactivation of the gene encoding the enzyme responsible for NeuGc conjugate synthesis (19, 20). The selective expression of NeuGc-gangliosides in tumors appears to originate from exogenous sources (47, 48), and various authors have proposed that tumor hypoxia induces the transcription of a sialin transporter that facilitates the incorporation of NeuGc units into tumor gangliosides (48). Recent reports described the unexpected presence of naturally occurring anti-NeuGcGM3 antibodies, with antitumor potential in healthy donors (39). However, the same team found reduced levels of circulating anti-NeuGcGM3 antibodies in nontreated patients with NSCLC, attributed to the inhibition of the specific antibody-secreting B-cell population, or the formation of immune complexes with the NeuGcGM3 released from tumor cells (39). It has been proposed that idiotypic vaccination is able to boost this natural immune response in patients with cancer.

The current clinical study was designed to evaluate the clinical effect of racotumomab vaccine in NSCLC and to define the surrogate value of the antibodies induced after vaccination. In our set, anti-NeuGGM3 antibodies were able to recognize and kill the NeuGcGM3-expressing cell line L1210, confirming the previously reported results (33). More recently, it was proposed that anti-NeuGcGM3 antibodies elicited by racotumomab induced death by an oncotic–necrosis mechanism, independent of complement activation (41). In the current study, we confirmed that the binding and cytotoxicity on L1210 cells was dependent on the presence of NeuGcGM3 in the cell membrane because the hyperimmune sera did not recognize or kill L1210 cells lacking NeuGcGM3 expression.

Several clinical trials using anti-idiotype vaccines in patients with cancer have reported a correlation between survival and the immune response against the nominal antigen (33, 49). To our knowledge, this is the first report showing an association between the functional capacity of the anti-NeuGcGM3 antibodies elicited after vaccination and the survival. These preliminary results need to be validated in a larger series of patients. Although the mechanism of action of racotumomab seems to be associated with the induction of cytotoxic antibodies capable to kill NeuGcGM3-expressing cells, others mechanisms cannot be ruled out. In fact, there are preclinical evidences of the link between tumor-infiltrating T cells and the antimetastatic and pro-apoptotic effects of the vaccine (31). A specific NeuGcGM3 IFNγ response was also found in patients with metastatic breast cancer treated with racotumomab (34). In the forthcoming trials, immunologic studies should be done to define the capacity of the racotumomab to activate the cellular immunity.

In conclusion, the results of the present study support the safety and efficacy of racotumomab-alum as switch maintenance for patients with advanced NSCLC. This study suggests the value of anti-idiotype immunization to generate vigorous serologic responses in situations where tumor-associated antigens are poorly immunogenic. The impact of using racotumomab as switch maintenance followed by second-line therapy is currently being explored in a new randomized, multinational phase III study (ClinicalTrials.gov identifier: NCT01460472). In this trial, patients with advanced NSCLC who progress on racotumomab may receive pemetrexed, docetaxel, or erlotinib at physician prescription.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Senior medical research designed, conducted, and interpreted all the clinical and experimental data of the study: A.E. Macías

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