RESEARCH PAPER

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Immunogenicity and safety of a NeuGcGM3 based cancer vaccine

Results from a controlled study in metastatic breast cancer patients

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Increased levels of NeuGc-containing gangliosides have been described in human breast cancer. A controlled Phase II clinical trial was conducted in patients with metastatic breast cancer to evaluate immunogenicity, safety and to identify evidences of biological activity of a cancer vaccine composed by NeuGcGM3 in a proteoliposome of *Neisseria meningitidis* together with Montanide ISA 51 as adjuvant. After first line chemotherapy, 79 women were randomized 1:1 to receive the vaccine candidate or best supportive care. All patients achieved at least stable disease to the first line therapy for the metastatic condition. Treatment consisted on 5 vaccine doses every 2 weeks and then, monthly re-immunization to complete 15 doses. Vaccination with the NeuGcGM3 based vaccine was safe and the most frequent adverse events consisted on injection site reactions, fever, arthralgia and chills. The vaccine was immunogenic and a sustained increase of both IgG and IgM antibody titters against NGcGM3 was observed after the second vaccination month. Antibodies were able to recognize the NeuGcGM3$^+$ murine tumor cell line L1210 and the myeloma cell line P3X63. Humoral response was specific since vaccination did not result in Neu-Acetyl GM3 or GM2-antibody response. Hyperimmune sera from vaccinated patients were able to prevent the NeuGcGM3 mediated CD4 down-modulation on T lymphocytes. In the intent to treat analysis, there was a trend toward a survival advantage for the vaccine group and this effect was significant for women bearing non-visceral metastasis. Two phase III clinical studies with this vaccine candidate are ongoing.

Introduction

Breast cancer is one of the leading causes of death from cancer among women, worldwide.[1] Despite the availability of hormonal, chemotherapeutic and biologic agents, metastatic breast cancer (MBC) remains essentially incurable, with less than 5% of patients being disease free beyond 5 years.[2] Therefore, the search for new therapeutic options to prolong survival constitutes the main goal of breast cancer research.

Gangliosides are membrane glycoesfingolipids that contain two variants of sialic acid, the *N*-acetylated (NeuAc) and the *N*-glycolylated (NeuGc) variant. The high expression of this antigen specific molecule has been associated with malignant tumor progression and immunosuppressive mechanisms.[3,5,6,30] Increased levels of NeuGc-containing gangliosides have been described in human breast cancer.[4,31] The presence of large amounts of these tumor associated carbohydrate antigens on cancer cells, as compared to normal cells, opens the possibilities to use them in immunotherapeutic approaches which engage the immune system to fight against a tumor.[5]

Although in humans the N-glycolylated variant of GM3 ganglioside is almost exclusively expressed in tumor tissues, the significance of this glycolipid for malignant cell biology remained obscure. In in vitro models, it has demonstrated the capacity of NeuGcGM3 ganglioside to down-modulate CD4 expression in murine and human T lymphocytes, especially in non-activated T cells.[3] The CD4 complete recovery in these cells occurred after 48 h of ganglioside removal, due to neo-synthesis. Restored T cells kept similar sensitivity to ganglioside-induced CD4 down-modulation after a new challenge. Recent results indicated that NeuGcGM3 contribute to cancer progression mainly by influencing DC and CD4$^+$CD25$^-$ T lymphocyte functions, rather than increasing the inhibitory capacity of naturally occurring regulatory T cells.[6] The relevance of these findings, contributed to the ganglioside validation as target for cancer immunotherapy

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Table 1. Patient characteristics by group

Characteristic	Control group	Vaccine group	Total
Age, years			
Median	54.97	56.72	
Range	28–70	28–80	
Stage at diagnosis			
I	9 (24.3%)	6 (15.8%)	15 (20%)
II	19 (51.4%)	20 (52.6%)	39 (52%)
III	5 (13.5%)	8 (21.1%)	13 (17.3%)
IV	4 (10.8%)	4 (10.5%)	8 (10.7%)
Dominant metastatic site			
Visceral	10 (25%)	13 (33.3%)	23 (29.1%)
Bone + Soft Tissue	28 (70%)	25 (64.1%)	53 (67.1%)
ER/PgR status			
ER/PgR positive	3 (7.5%)	7 (17.9%)	10 (12.7%)
ER/PgR negative	2 (5.0%)	3 (7.7%)	5 (6.3%)
Unknown	35 (87.5%)	29 (74.4%)	64 (81.0%)
Prior Surgery			
	38 (95%)	36 (92.3%)	74 (93.7%)
Prior Radiotherapy			
	32 (80%)	29 (74.4%)	61 (77.2%)
Previous Chemotherapy (Metastatic Disease)			
CMF	4 (11.4%)	3 (8.8%)	7 (10.1%)
AC	15 (42.9%)	19 (55.9%)	34 (49.3%)
Taxane	14 (40%)	11 (32.3%)	25 (36.2%)
Endocrine therapy (tamoxifen)			
	15 (39.5%)	15 (41.7%)	30 (40.5%)
Response to first line therapy for metastatic disease			
CR	5 (13.9%)	8 (20.5%)	13 (17.3%)
PR	5 (13.9%)	3 (7.7%)	8 (10.7%)
SD	26 (72.2%)	28 (71.8%)	54 (72%)
Second Line Chemotherapy			
	13 (32.5%)	8 (20.5%)	21 (26.6%)

In a previous report from a phase I trial, our vaccine, composed by NeuGc-containing gangliosides and the outer membrane protein complex of *Neisseria meningitidis* to form very small size proteoliposomes (VSSP)[7] showed to be immunogenic and safe in advance breast cancer patients. Twenty one stage III or IV breast cancer patients received up to 15 doses of the vaccine by intramuscular injection. Main toxicities included erythema and induration at the injection site. All treated patients who completed the induction phase developed IgG and IgM anti-NeuGcGM3 antibody titers.[7]

A Phase I/II clinical trial was also carried out in patients with advanced cutaneous and ocular malignant melanomas, to evaluate immunogenicity and toxicity of the same vaccine. Twenty-two patients were included at 2 different dose levels. Toxicities were mostly grade 1 or 2, according NCI CTC criteria. Interestingly, 3 patients developed vitiligo at the lower dose, although the nominal antigen NeuGcGM3 was not present in melanocytes. Safety and immunogenicity with NeuGcGM3 vaccine treatment in advanced melanoma patients were established.[10]

In the present paper, we show the result of the first randomized, multicenter, phase II trial in MBC patients. Here we evaluated the safety and immunogenicity of vaccination with NeuGcGM3/VSSP/Montanide ISA 51 in MBC women after finishing first line chemotherapy. Secondary objectives included the evaluation of the biological role of the antibodies induced after vaccination as well as the preliminary evidences of survival benefit. The trial was designed as a proof-of-concept, to better understand the activity of the vaccine relative to what is currently available for the purported indication prior to the transition to randomized late phase clinical trial designed to establish efficacy and confirm safety.

Results

Patient characteristic. Seventy-nine MBC patients were recruited in 5 hospitals from August 2002 to August 2006. Patients were randomized 1:1 to NeuGcGM3/VSSP/Montanide ISA 51 vaccine or best supportive care. Patients' demographic characteristics are shown in **Table 1**. Almost all women entered the study with relapsing disease while 10.7% of patients presented initially with metastatic disease. All women finished first-line chemotherapy for the metastatic condition, at least 4 weeks before entering the trial: 68.4% showed disease stabilization, 15.1% had partial response and 16.5% attained complete response to first line therapy. The most common site of metastasis was non visceral, accounting for 67.1% of all patients. Both groups were not significantly different as regards the demographic and tumor characteristics, but noticeably, visceral metastases were more common in the vaccinated group (33.3%) than in the control group (25%). A drawback of the trial is that 81% of the patients had unknown hormonal receptors status; however 40.5% of patients received Tamoxifen. The lack of information on endocrine receptors is associated with the time interval between the first diagnose and the relapse and the availability of the paraffin-embedded blocks from the primary tumor. Metastatic lesions were not biopsied to evaluate the presence of endocrine receptors.

The most frequent chemotherapy regimen used as first line treatment for metastatic disease before randomization was doxorubicin and cyclophosphamide (49.3%), taxane based combinations were used in 36.2% of the patients while cyclophophamide-methotrexate-5 Fluoro-uracilo was used in 10.1% of the women. Only 26.6% of patients received second line chemotherapy, once they entered the study, due to progressive disease. In general, both groups were also statistically comparable regarding first or second line chemotherapy, even though second line chemotherapy was more frequently administered to patients randomized to the control group (32.5% vs. 20.5%).

The average vaccine dose per patient was 10. Thirty-seven percent (37.5%) of patients completed 1 year treatment schedule, consisting of 15 immunizations, in total.

Safety. The NeuGcGM3/VSSP/Montanide ISA 51 vaccine was safe. The most frequent adverse events related to vaccination were mild to moderate injection site reactions such as pain

(68.2%) and local erythema (12.2 %). Two patients developed skin abscess at the injection site, which were classified as severe. Other frequent adverse events consisted on grade 1 or 2 fever (61.5 %), arthralgia (48.71 %), chills (43.5 %) and headache (35.89 %). There were no differences between the vaccinated and control arms regarding hematological and biochemical parameters.

NeuGcGM3-specific antibody response in patient's sera after vaccination with NeuGcGM3/VSSP/Montanide ISA 51 vaccine. A complete set of serum samples was available for 30 vaccinated patients. Median specific antibody titters against NGcGM3 showed a significant and sustained increase of both IgG and IgM immunoglobulin subclasses after the second month of immunization (**Fig. 1A and Table 2**).

After the induction phase (5 doses of the vaccine), median antibody titters ranged between 1:500 and 1:2,000 sera dilution. Ninety percent (90%) of the evaluated patients (27 out of 30) showed at least a 2 fold increase in the peak antibody titers as compared to baseline for both IgG and IgM isotypes.

On the other hand, humoral response was specific against the immunizing antigen, since vaccination did not result in Neu-Acetyl GM3 or GM2-antibody response (Data not shown). Interestingly, the NeuGcGM3-specific IgG response mainly comprised IgG1 and IgG3 antibody subclasses (**Fig. 1B**), although IgG3 predominated.

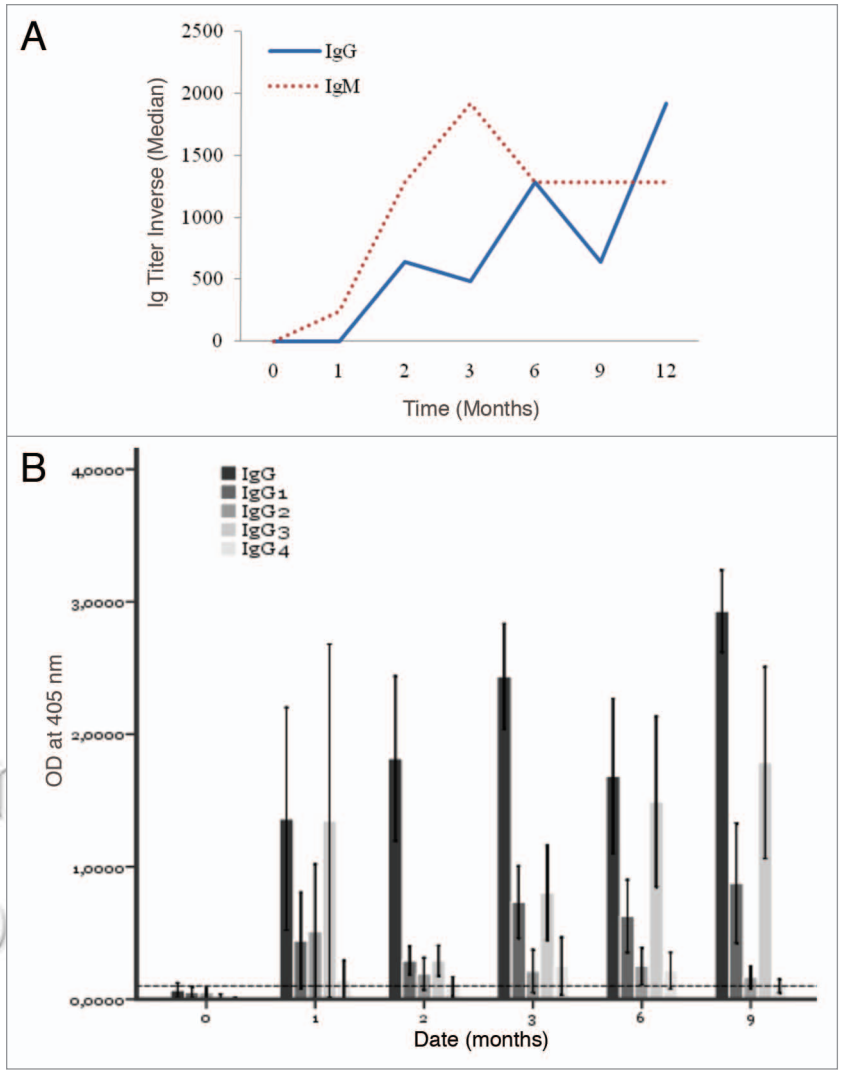

Figure 1. Characterization of humoral response. (A) Kinetic of NeuGcGM3-specific antibody response. Median titers are plotted versus time points for 30 evaluable vaccinated patients. One month after first vaccination, there were significant differences between post-treatment IgG (dotted line) or IgM titers (solid line) and pre-tretament response.(B) IgG response consisted on IgG1 and IgG3 subclasses. Optical Density (OD) at 405 nm for a serum dilution of 1:80 is plotted over time.

The biological role of the anti-NeuGcGM3 antibodies in vaccinated patients was assessed by FACS using the NeuGcGM3 antigen positive mouse myeloma cell line P3X63. Hyper-immune sera from 8 patients showed a strong recognition of P3X63 cells, compared to their baseline serum (data not shown). Likewise, the NeuGcGM3$^+$ mouse tumor cell line, L1210, was strongly recognized by sera (**Fig. 2A–C**), showing a significant correlation with the amount of either IgG or IgM NeuGcGM3-response (**Fig. 2D**).

NeuGcGM3-specific antibody response didn't seem to influence clinical outcome as analyzed on the 30 evaluable patients (Data not shown). Nevertheless, when analyzed on non-visceral metastatic patients we saw a marginal benefit for those patients who reached a maximal NeuGcGM3-specific IgG higher than 1,280 (*LogRank,* p = 0.058) (Data not shown).

Hyper-immune sera interfere with the NeuGcGM3-induced CD4 down modulation on T cells. The inhibitory effect of the vaccine-generated anti-NeuGcGM3 antibodies on ganglioside induced CD4 down-modulation[6] was conducted. The CD4 expression recovery ratio (R_{CD4}) on T cells was estimated (see Materials and Methods) (**Fig. 3A**) and considered as recovery of CD4 expression when $R_{CD4} \geqslant 1$. In this sense a CD4 expression recovery on T cells was observed in 9 out of the 12 evaluated patients (**Table 3**). Strikingly, once dichotomised by a R_{CD4} of 1.5, we observed a survival benefit for those patients whose R_{CD4} is higher than the mean value of 1.5 (**Fig. 3B**) suggesting a functional capacity of anti-NGcGM3 antibodies for immunerestoration in patients-bearing tumors. To confirm this finding, large amounts of patients should be tested.

Preliminary efficacy analysis. In the intent to treat analysis (ITT), there was a trend toward a survival benefit for the vaccine group, which was not significant at this sample size. This trend became more marked when patients were analyzed by primary site of metastasis (visceral vs. non visceral metastases). For patients with non visceral metastases, the median overall survival

Table 2. Patient NeuGcGM3-specific IgG & IgM titers over time

Patients inclusion number	Date (IgG/IgM)						
	0	1	2	3	6	9	12
1	0/0	0/0	0/0			2560/640	160/160
2	0/0	0/0	2560/1280		1280/1280	5120/1280	2560/1280
4	0/320	0/160			1280/1280	640/1280	
7	320/320		2560/2560	2560/2560			
8	0/0	0/1280	640/1280	160/640	160/640	160/1280	
9	0/0	0/0	2560/10240	5120/2560	2560/1280	2560/2560	
14	0/0	0/0	5120/320	80/10240	320/1280		
16	0/0			160/2560	2560/1280	640/640	
18	0/0	320/2560	320/1280				
19	0/640		0/0	0/0			
20	0/0	0/320	0/320	1280/5120	1280/1280		
25	0/0	0/0	320/2560	0/1280		320/2560	
26	0/0	0/0		320/0			320/320
28	0/0	1280/640					
29	160/0		160/640	1280/640	320/1280		1280/1280
31	0/0	0/0	1280/2560				
35	0/0	0/0	640/1280				
36	0/160		640/1280		1280/2560	320/1280	10240/1280
37	320/0		1280/2560		640/1280		
41	160/80	160/640		1280/2560	80/20480		
48	0/0	1280/1280	1280/2560				
51	80/320		2560/10240	2560/10240	2560/2560		
54	0/0		160/1280		40960/20480	163840/10240	10240/1280
57	0/0	640/640	640/1280	640/1280	20480/5120		
58	80/0	320/640	320/640	320/640			
61	0/0	0/0					
63	0/0	0/1280	320/2560				
67	0/320	0/160					
64	0/0		0/640	160/2560			
77	0/320			640/1280			

Unfilled spaces correspond to unavailable samples.

was 26.17 months for the vaccinated group and 12.17 months for the control group (Breslow p value 0.0493).

Discussion

Metastatic breast cancer remains incurable despite the introduction of new therapeutic agents in the last few years. Many researchers argue that those results reflect the heterogeneity of this disease which explains its unpredictable clinical behavior.[8,12,13,15,16] The constant search for new and improved treatments with real impact on the overall survival of metastatic breast cancer patients is guaranteed.[11,17-20]

Gangliosides have been involved in multiple cellular processes such as growth, differentiation and adhesion, and more recently as regulators of cell death signaling pathways. Some of these molecules can be considered as tumor-associated antigens, in particular, N-glycolyl sialic acid-containing gangliosides, which are promising candidates for cancer-targeted therapy because of their low expression in normal human tissue.[23]

Here we present the results of a proof of concept study intended to evaluate immunogenicity, safety and evidences of biological activity of a cancer vaccine composed by NeuGcGM3 in a proteoliposome of *Neisseria meningitidis* together with Montanide ISA 51 as adjuvant.

The vaccine was very well tolerated. Most common adverse events consisted on injection site reactions and general symptoms. We speculate that injection site reactions and particularly, abscess formation in 2 patients can be attributed to the use of an oily adjuvant such as Montanide ISA 51. This adverse event has been described previously for the referred adjuvant.[9,21,22] In general,

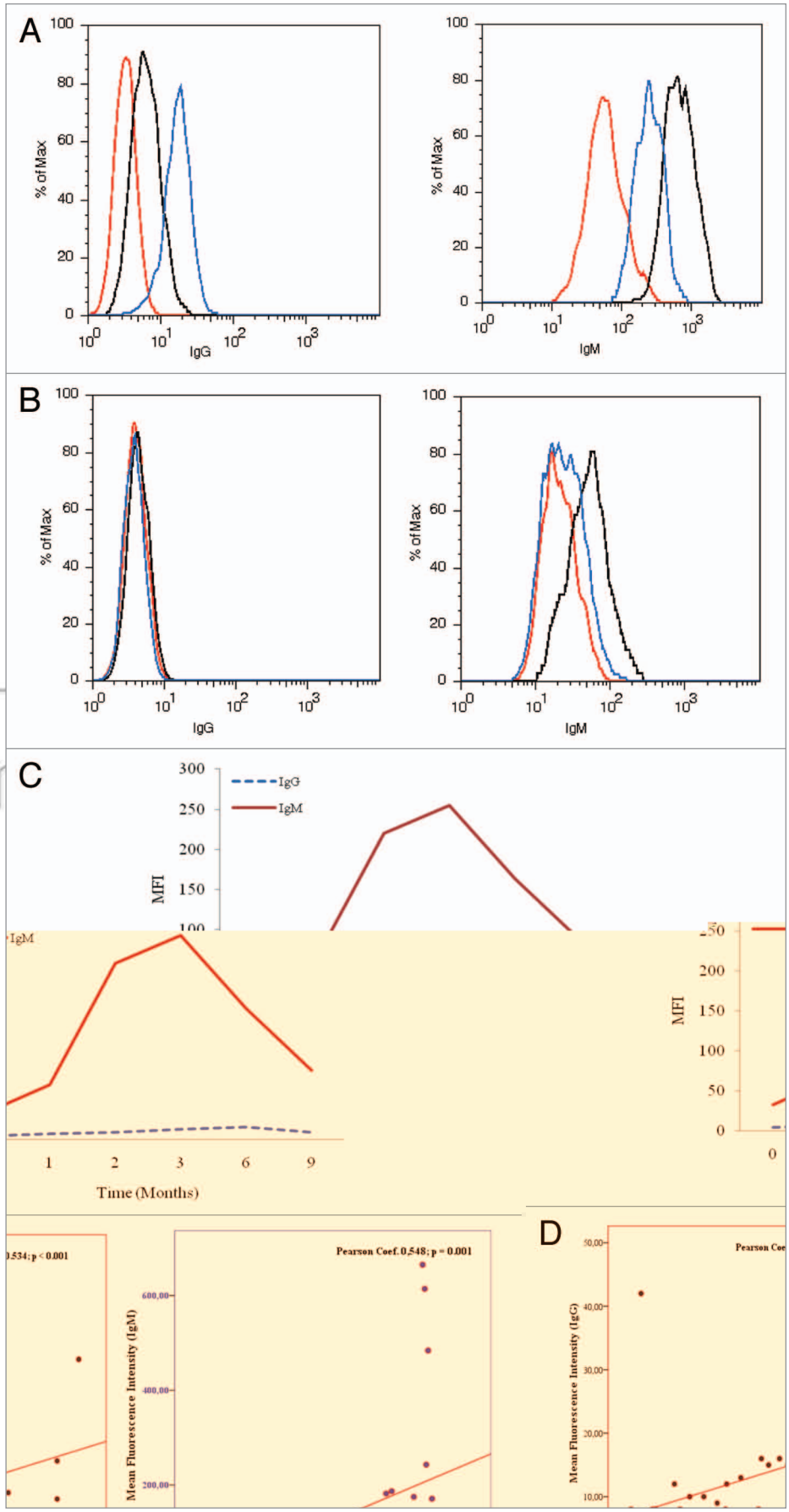

Figure 2. Sera recognized mouse NeuGcGM3$^+$ tumor cell line, L1210. Cytometric assay was carried on by incubating L1210 cells with serum, diluted 1:5, and the cells-attached Igs were detected by incubating with FITC-anti human IgG or FITC-anti human IgM antibody. Representative patients are depicted. (A) Patient 7, who showed a good antibody response, pre-treatment (red), 2 months (black) and 3 months (blue) later. (B) Patient 75, who showed poor NeuGcGM3$^+$-specific antibody response, pre-treatment (red), 2 months (black) and 3 months (blue) later. X-axis represents the mean fluorescence intensity of immunoglobulins atached to cells; and, y-axis represents the percentage of cell count. (C) Summary of all tested patients. (D) Immunoreactivity against L1210 cells correlates with the level of NeuGcGM3-specific antibody response. The mean fluorescence intensity (MFI)-y-axis-is plotted versus its respective optical density (OD) at 405 nm for a serum dilution of 1:80-x-axis.

the vaccine safety profile is in agreement with earlier reports in advanced cancer patients.[7,24]

The vaccine was immunogenic with 90% of patients showing seroconversion for both IgM and IgG immunoglobulin classes. Interesting, antibody response was observed after the second month of immunization. It seems to be critical for our vaccine that patients receive at least 5 immunization doses in order to generate an effective humoral response that could later be translated into a clinical benefit. In agreement with the aforementioned, in a previous clinical trial, the vaccine proved to be immunogenic in stage III & IV breast cancer patients who completed the induction phase.[7]

On the other hand, NeuGcGM3-specific IgG response mostly consisted on IgG1 and IgG3 subclasses, the typical protein-induced IgG profile. Furthermore, we assessed if sera from immunized patients was able to recognize other structurally related gangliosides, i.e., NeuAcGM3 and GM2. As expected, patients' sera did not recognize any of the latter gangliosides suggesting that vaccination induced not only a consistent but also a specific humoral response against the immunizing antigen.

Likewise, these antibodies were able to recognize NeuGcGM3 in its natural

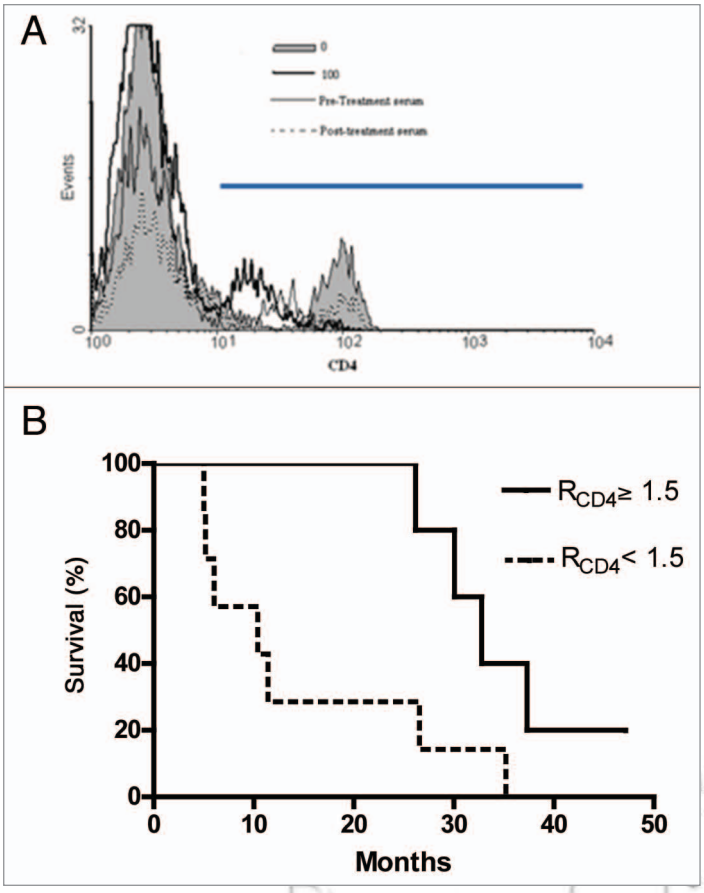

Figure 3. Hyperimmune sera prevent NeuGcGM3-induced down-modulation of CD4 molecules on T cells. (A) PBMC from healthy donors were co-incubated with 100 μg of NeuGcGM3 and selected sera diluted 1:500. After 1 hour, cell samples were analyzed for CD4 expression by flow cytometry. CD4 expression recovery by a representative hyperinmune patient's serum. Filled histogram corresponds to normal CD4 expression on PBMC. CD4 molecule down-modulation on PBMC induced by 100 μg of NGcGM3 (black line). CD4 expression after pre-immune serum incubation (gray line) or post-immune serum treatment (dashed line). (B) The serum ability for CD4 rescue on NeuGcGM3-treated human T cells correlates with better overall survival (LogRank, p = 0,0323, 5 vs. 7 patients).

Table 3. CD4 expression recovery on T cells by sera from vaccinated cancer patients

Patient	Pre-treatment serum (%)[a]	Hyperimmune serum (%)[a]	CD4 recovery ratio[b]
36	44	46	1
47	75	95	1:3
24	48	93	1:9
7	38	49	1:3
4	36	69	1:9
25	40	39	0:9
2	41	62	1:5
18	80	86	1:1
20	27	46	1:7
8	21	33	1:6
19	17	16	0:9
14	17	16	0:9

[a]Percent of positive CD4 T cells as compared to the CD4 expression of normal PBMC (results from a single assay are shown). [b]CD4 expression recovery ratio (R_{CD4}) was estimated by fold increase of CD4 expression recovery between hyperimmune and pre-treatment serum.

microenvironment at the cell membrane, as suggested by the immunoreactivity against the NeuGcGM3$^+$ murine tumor cell line, L1210 and the myeloma cell line P3X63. This recognition appears to depend on the magnitude of the antibody response, since there was a direct correlation between the mean fluorescence intensity of either IgG or IgM attached to L1210 cells and the antibody titers.

Presumably, these polyclonal NeuGcGM3-specifc antibodies are able to mediate either complement (CDC) or antibody-dependent cytotoxicity (ADCC) since they mostly comprise IgM isotype and IgG1 & IgG3. For another ganglioside-based vaccine strategy, CDC and ADCC have been ascertained as one of the mechanism by which vaccine might induce a direct impact on tumor shrinkage, and, consequently, a clinical benefit.[28,29] Whether for our vaccine this is a plausible mechanism or not, is partially demonstrated by the cell death induced by sera from breast cancer patients vaccinated with NeuGcGM3/VSSP plus Montanide ISA51 in a previous clinical trial.[6] Nonetheless, the molecular pathway that is activated under polyclonal NeuGcGM3-specific antibodies effect remains unclear.

Although, as analyzed in all 30 evaluable vaccinated patients, the magnitude of NeuGcGM3-specific antibody response doesn't seem to be related to a better clinical outcome, when analyzed in non visceral metastasis-bearing patient subset, maximal NeuGcGM3-specific IgG response marginally influences clinical benefit. It's well documented that non-visceral metastatic breast cancer patients have a better prognosis.[8] The fact that NeuGcGM3-specific IgG antibody response tends to benefit vaccinated patients might be related to the cytotoxic properties of this antibody isotype as compared to IgM isotype. Indeed, some reports have shown the relevance of ganglioside-specific antibody response, including that elicited against NeuGcGM3.[27] In twenty NSCLC patients treated with the anti-idiotype 1E10 antibody, that mimics NeuGcGM3, the induction of either NeuGcGM3-specific IgG or IgM antibody correlate with a better overall survival. This highlights the suitable clinical relevance that the presence of these antibodies might have on patients' clinical benefit. Nevertheless, a higher number of patients should be analyzed in order to prove any further relevance of the humoral response induced by this vaccine in breast cancer patients.

The role of antibodies in the antitumor effect of ganglioside vaccines has been considered to be predominant, if not exclusive.[34] Among the mechanisms of antibody action against circulating tumor cells and micrometastases, complement-mediated lysis and antibody-dependent cell-mediated cytotoxicity have been highlighted.[35] On the other hand, ganglioside-specific antibodies role (either induced or passively administered) during the

natural development of cancer has been thoroughly documented.[36] Compelling evidences derived from clinical studies have shown that the frequency of induction of ganglioside-specific antibodies correlates with survival.[37] This notion was supported by the in vitro cytotoxic activity of the induced serum antibodies.

The capacity of the tumor cells to shed gangliosides toward tumor microenviroment and systemic circulation has been described.[32] These tumor-derived molecules impair immune system functions and have been considered as immunosuppressor factors. The acetylated variant of ganglioside (NAcGM3) has a significant effect over CD4 expression.[33] It was published that NGcGM3 induced a significant and concentration-dependent CD4 down-modulation on mouse and human T lymphocytes affecting their functionality.[3] In addition, recent studies demonstrated a reduction in proliferative capacity and secretion of anti-inflammatory cytokines when $CD4^+CD25^-$ T cells were cultured in the presence of NGcGM3.[7]

As breast cancer cells *express* high quantities of NGcGM3, T lymphocyte from those patients might be suppressed by the tumor-derived NeuGcGM3 ganglioside. We hypothesized that a potent humoral immune response against NeuGcGM3 could avoid its effect on T lymphocytes. In this report we showed that sera from vaccinated breast cancer patients were able to prevent the NeuGcGM3 effect over CD4 expression in vitro. This effect on the NeuGcGM3-induced CD4 downmodulation could be translated into "T lymphocytes protection" from tumor-induced immunosuppression, allowing an effective immune response.

Even though the trial was not designed to establish the vaccine efficacy, there was a trend towards survival benefit for all vaccinated women. This effect was more remarkable for women bearing non-visceral metastasis, but these results need to be confirmed in a larger series.

Vaccine therapy in the metastatic setting has yet to demonstrate clinical significance in a phase III testing. Data of clinical activity have been observed by using other cancer vaccines targeting HER-2/neu protein, human telomerase reverse transcriptase, carcinoembryonic antigen (CEA), and carbohydrate antigens, but so far, there are no positive results from Phase III trials.[25] Theratope, consisting of a synthetic mimic of the tumor-associated *O*-linked epitope of MUC-1 (STn-serine), tethered to an immunostimulatory protein (KLH) and delivered along with an oil droplet emulsion containing monophosphoryl lipid A, showed no improvement in either time to progression or overall survival in a big phase III trial in 1,028 women with metastatic breast cancer.[26]

In summary, our vaccine was well tolerated and immunogenic. Two controlled, double blinded, Phase III clinical studies with this vaccine candidate are ongoing in breast cancer patients, in the adjuvant and the advanced setting. Biomarker studies to identify the predictive factors of vaccine efficacy as well as new studies on humoral and cellular response are in progress.

Patients and methods. *Eligibility criteria.* Female patients aged ⩾18 years old with a life expectancy of ⩾6 month and a diagnosis of histological or cytological confirmed MBC (stage IV or recurrent) were eligible. Only patients showing complete, partial or stable disease after chemotherapy were selected. Random assignment was performed at least 28 days after completing first-line chemotherapy for their metastatic disease. Patients were stratified according to their response to first line therapy.

Patients were required to have a Performance Status (PS) ⩽2, according to the World Health Organization (WHO), neutrophil count ⩾ 1,000/mm^3, platelet count ⩾100 x 10^9 cells/L, haemoglobin ⩾9 g/dl and normal creatinine, bilirubin and transaminase values according to each institutional standards.

Pregnancy or lactation, secondary malignancies, brain metastases, progressive disease, contra-lateral breast metastases as unique site of recurrence and history of severe allergic reactions rendered patients ineligible.

The trial was approved by Institutional Review Boards of all participating institutions and by the State Centre of Drug Quality Control. All patients signed informed consent before inclusion.

Treatment schedule. Twenty-eight days after finishing first-line chemotherapy, patients were randomly assigned to enrolment in the vaccine or control group. Random assignment was performed centrally through a validated minimisation program for allocating patients to treatment in clinical trials (MINIM) version 1.5.

Patients in the vaccine group received 200 μg of NeuGcGM3/VSSP/Montanide ISA 51 vaccine every 14 days for the first 5 immunizations and every 28 days afterwards, until disease progression or a total of 15 doses. Criteria for premature vaccine discontinuation included voluntary withdrawal, unmanageable toxicity, worsening of the patient general conditions or irregularities on the treatment schedule. Vaccinated patients received supportive care if required. Patients in the control group received best supportive care alone. At the moment of progressive disease, patients received second line chemotherapy and immunization was interrupted in the vaccine arm.

The NeuGcGM3/VSSP/Montanide ISA 51 vaccine was produced at the Center of Molecular Immunology (Havana, Cuba). The vaccine is composed by the NGcGM3 ganglioside bound to the outer membrane vesicles of *N. meningitidis* in the form of very small sized proteoliposomes (VSSP). The conjugate is then mixed with Montanide ISA 51 (Seppic, Paris, France) to form water in oil emulsion immediately before injection. The vaccine was administered intramuscularly. Each vaccine dose contained 200 μg of NeuGcGM3/VSSP. The product was released by the Quality Control Direction at the Center of Molecular Immunology according to the manufacturer specifications.

Patient assessment. Patient assessment was performed at baseline, with each immunization and every 4 weeks during the study period and included physical exam and complete blood count for clinical laboratory test. Additionally, chest radiography, computerized tomography (CT) scan and abdominal ultrasound were done at baseline and every 3 months to assess clinical response. Bone scintigraphies were performed to evaluate bone metastases every 6 months.

Toxicity was assessed according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC; version 3) for all patients during the study period.

Serum sample collection and evaluation. Patient's blood was collected before initiating vaccination and 1, 2, 3, 6, 9, and 12 months after the first vaccination. Ganglioside-specific antibodies

were analyzed as previously described.[5] Briefly, gangliosides (200 ng/well) in 50 μL of methanol were dried in 96-well polystyrene plates (PolySorp, Nunc, Denmark) for 90 minutes at 37°C. Then the plates were washed with phosphate-buffered saline containing Tween 20 (0.05% vol/vol). Serum samples dilutions were incubated overnight at room temperature. Plates were washed with phosphate-buffered saline containing Tween 20 and biotinylated conjugated goat antihuman immunoglobulin M (IgM) or antihuman IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) was added and incubated for 1.5 hours at 37°C. After a new wash, streptavidin-alkaline phosphatase conjugate (Jackson Immunoresearch Laboratories) was used at the same incubating conditions. The plates were washed again and *p*-nitrophenylphosphate (Sigma Chemical Co., St. Louis, MO) in diethanolamine buffer (pH 9.8) solution was added. After 30 minutes, absorbance was measured at 405 nm with an ELISA reader (Organon Teknika, Salzburg, Austria). Absorbance was plotted against sera dilution and endpoint titer was determined at an OD value of 0.1 after methanol blank OD value was subtracted. Maximal IgG and IgM titer were calculated.

For the immunofluorescence assay, murine tumor cell lines (X63 and L1210, ATCC), were incubated at 10^5 cells/ml with sera diluted at 1:20 for 30 minutes in ice. After wash steps, cell samples were incubated with a goat anti-human IgG and/or IgM conjugated with biotin (Serotec) conjugated to FITC. For positive controls, the NeuGcGM3-specific monoclonal antibody 14F7 (specific for NeuGcGM3) was used. Cells were washed 3 times before they were analyzed with a FACScalibur cytometer (Becton-Dickinson).

Down-modulation assay. CD4 down-modulation assay was performed as previously described.[3] Briefly, sera of healthy donors and patients diluted at 1:500 were incubated with normal human peripheral lymphocytes (10^6 per ml) and NeuGcGM3 at 100 μg/ml. After 1 hour of incubation, cells were washed and incubated with mouse anti-human CD4 conjugated with FITC (DakoCytomation) for 30 minutes. Cells were washed 3 times and median fluorescent intensity (MFI) was analyzed with a FACScalibur cytometer (Becton-Dickinson). The MFI was determined after the incubation of lymphocytes with 100 μg/ml of NeuGcGM3 in the presence of patient or healthy serum at 1:500, respectively. Post-treatment sera correspond to the maximal immunoglobulin titer. Percentage of CD4 expression was determined as follow:

$$\%CD4 = (MFI_{\text{patient serum}} - MFI_{\text{NeuGcGM3 + healthy serum}})/(MFI_{\text{Healthy serum}} - MFI_{\text{NeuGcGM3 + healthy serum}}) \times 100$$

Statistical analysis. Safety and efficacy analyses were performed on intention to treat basis. Survival estimations were performed according to the Kaplan-Meier method and the log-rank and Breslow estimates. Pearson correlation coefficient and Fisher exact test were used to compare different variables between groups. The statistical significance of comparisons in median titer values between IgG and IgM at each time point was determined with the Mann-Whitney test. Pre-treatment and post-treatment comparisons regarding IgG or IgM were done with the Wilcoxon Sign test. For the referred analysis, SPSS software was used.

References

1. Gray KL. The Leading Cause of Cancer Death in Women. The Internet Journal of Academic Physician Assistants 2007; 6:1.
2. Colozza M. Achievements in systemic therapies in the pregenomic era in metastatic breast cancer. The Oncologist 2007; 12:253-70.
3. De Leon J, Fernandez A, Mesa C, Clavel M, Fernández LE: Role of tumour associated N. glycolylated variant of GM3 ganglioside in cancer progression: effect over CD4 expression on T cells. Cancer Immunol Immunother 2006; 55:443-50.
4. Marquina G, Waki H, Fernandez LE, Kon K, Carr A, Valiente O, et al. Gangliosides expressed in human breast cancer. Cancer Res 1996; 56:5165-71.
5. Horwacik I. Gangliosides as carbohydrate antigens associated with cancer and their possible use in tumor immunotherapy. Przegl Lek 2004; 61:14-9.
6. de León J, Fernández A, Clavell M, Labrada M, Bebelagua Y, Mesa C, et al. Differential influence of the tumour-specific non-human sialic acid containing GM3 ganglioside on $CD4^+$ $CD25^-$ effector and naturally occurring $CD4^+CD25^+$ regulatory T cells function. Int Immunol 2008; 20:591-600. [Epub 2008 Feb 29].
7. Carr A, Rodriguez E, Arango MC, Camacho R, Osorio M, Gabri M, et al. Immunotherapy of advance breast cancer with a heterophilic ganglioside (NeuGcGM3) cancer vaccine. J Clin Oncol 2003; 21:1015-21.
8. Andreopoulou E, Hortobagy GN. Prognostic factors in metastatic breast cancer: successes and challenges toward individualized therapy. J Clin Oncol 2008; 22:3660-2.
9. Aucouturier J, Ascarateil S, Dupuis L. The use of oil adjuvants in therapeutic vaccines. 8-Aucouturier J, Dupuis L, et al. Montanide ISA 720 and 51: a new generation of water in oil emulsions as adjuvants for human vaccines. Expert Rev Vaccines 2002; 1:111-8.
10. Osorio M, Gracia E, Rodriguez C, Suarez G, Arango Mdel C, Noris E, et al. Heterophilic NeuGcGM3 ganglioside cancer vaccine in advanced melanoma patients: results of a Phase Ib/IIa study. Cancer Biol Ther 2008; 7:488-95.
11. Di Leo A, Gomez HL, Aziz Z, Zvirbule Z, Bines J, Arbushites MC, et al. Phase III, Double-Blind, randomized study comparing lapatinib plus paclitaxel with placebo plus paclitaxel as first-line treatment for metastatic breast cancer. J Clin Oncol 2008; 26:5544-52.
12. Guarneri V. Comparison of HER-2 and hormone receptor expression in primary breast cancers and asynchronous paired metastases: Impact on patient management. The Oncologist 2008; 13:838-44.
13. NCCN Clinical Practice Guidelines in Oncology. Breast Cancer 2008; 2.
14. Wilking U, Skoog L, Elmberger G, Wilking N, Bergh J. HER2 gene amplification (HER2) and hormone receptor expression (ER/PR) in early (EBC) and metastatic breast cancer (MBC) in the same patients. J Clin Oncol 2007; 25:1023.
15. Broom RJ, Tang P, Simmons C, Bordeleau L, O'Malley FP, Miller N, et al. Changes in estrogen receptor (ER), progesterone receptor (PR) and HER2/neu status with time: Discordance rates between primary and metastatic breast pathology samples. J Clin Oncol 2007; 25:1024.
16. Lower EE, Glass EL, Bradley DA, Blau R, Heffelfinger S. Impact of metastatic estrogen receptor and progesterone receptor status on survival. Breast Cancer Res Treat 2005; 90:65-70.
17. Piccart-Gebhart MJ, Burzykowski T, Buyse M, Sledge G, Carmichael J, Lück HJ, et al. Taxanes alone or in combination with anthracyclines as first-line therapy of patients with metastatic breast cancer. J Clin Oncol 2008; 26:1980-6.
18. Beslija S, Bonneterre J, Burstein H, Cocquyt V, Gnant M, Goodwin P, et al. Second consensus on medical treatment of metastatic breast cancer. Ann Oncol 2007; 18:215-25.
19. Verma S, Clemons M. First-Line treatment options for patients with HER-2-Negative metastatic breast cancer: The impact of modern adjuvant chemotherapy. The Oncologist 2007; 12:785-97.
20. Al-Batran SE, Bischoff J, von Minckwitz G, Atmaca A, Kleeberg U, Meuthen I, et al. The clinical benefit of pegylated liposomal doxorubicin in patients with metastatic breast cancer previously treated with conventional anthracyclines: Amulticentre phase II trial. Br J Cancer 2006; 94:1615-20.
21. Aucouturier J, Ascarateil S, Dupuis L. The use of oil adjuvants in therapeutic vaccines. Vaccine 2006; 24:2-44.
22. Aucouturier J, Dupuis L, Deville S, Ascarateil S, Ganne V. Montanide ISA 720 and 51: a new generation of water in oil emulsions as adjuvants for human vaccines. Expert Rev Vaccines 2002; 1:111-8.
23. Roque-Navarro L, Chakrabandhu K, de León J, Rodríguez S, Toledo C, Carr A, et al. Anti-ganglioside antibody-induced tumor cell death by loss of membrane integrity. Mol Cancer Ther 2008; 7:2033-41.
24. Shumway NM, Ibrahim N, Ponniah S, Peoples GE, Murray JL. Therapeutic breast cancer vaccines: a new strategy for early-stage disease. BioDrugs 2009; 23:277-87.
25. Curigliano G, Locatelli M, Fumagalli L, Goldhirsch A. Immunizing against breast cancer: a new swing for an old sword. Breast 2009; 18:51-4.
26. Holmberg LA, Sandmaier BM. Vaccination with Theratope (STn-KLH) as treatment for breast cancer. Expert Rev Vaccines 2004; 3:655-63.

27. Hernández AM, Toledo D, Martínez D, Griñán T, Brito V, Macías A, et al. Characterization of the antibody response against NeuGcGM3 ganglioside elicited in non-small cell lung cancer patients immunized with an anti-idiotype antibody. J Immunol 2008; 181:6625-34.
28. Ragupathi G, Livingston PO, Hood C, Gathuru J, Krown SE, Chapman PB, et al. Consistent antibody response against ganglioside GD2 induced in patients with melanoma by a GD2 lactone-keyhole limpet hemocyanin conjugate vaccine plus immunological adjuvant QS-21. Clin Cancer Res 2003; 9:5214-20.
29. Krug LM, Ragupathi G, Hood C, Kris MG, Miller VA, Allen JR, et al. Vaccination of patients with small-cell lung cancer with synthetic fucosyl GM-1 conjugated to keyhole limpet hemocyanin. Clin Cancer Res 2004; 10:6094-100.
30. Vangsted A, Drivsholm L, Andersen E, Pallesen T, Zeuthen J, Wallin H. New serum markers for small-cell lung cancer. The ganglioside fucosyl-GM1. Cancer Detect Prev 1994; 18:221-9.
31. Birklé S, Zeng G, Gao L, Yu RK, Aubry J. Role of tumour-associated gangliosides in cancer progression. Biochimie 2003; 85:455.
32. Wiesner DA, Sweeley CC. Circulating gangliosides of breast-cancer patients. Int J Cancer 1995; 3:294.
33. Sorice M, Pavan A, Misasi R, Sansolini T, Garofalo T, Lenti L, et al. Monosialoganglioside GM3 induces CD4 internalization in human peripheral blood T lymphocytes. Scand J Immunol 1995; 41:148.
34. Livingston PO, Ragupati G. Carbohydrate vaccines that induce antibodies against cancer. Previous experience and future plans. Cancer Immunol Immunother 1997; 45:10-9.
35. Livingston PO, Zhang S, Old LJ. Carbohydrate vaccines that induce antibodies against cancer. Rationale. Cancer Immunol Immunother 1997; 45:1-9.
36. Gelderman KA, Tomlinson S, Ross GD, Gorter A. Complement function in mAb-mediated cancer immunotherapy. Trends Immunol 2004; 25:158-64.
37. Takahashi T, Johnson TD, Nishinaka Y, Morton DL, Irie RF. IgM anti-ganglioside antibodies induced by melanoma cell vaccine correlate with survival of melanoma patients. J Invest Dermatol 1999; 112:205-9.