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Association between baseline VEGF/sVEGFR-2 and VEGF/TSP-1 ratios and response to metronomic chemotherapy using cyclophosphamide and celecoxib in patients with advanced breast cancer

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Abstract

BACKGROUND: Metronomic chemotherapy (MCT) with cyclophosphamide (Cy) and celecoxib (Cel) has therapeutic efficacy and low toxicity profile in advanced breast cancer patients (ABCP), but no reliable biomarkers of response have been found yet that allow patient selection for treatment. **AIM:** To investigate the potential role as biomarkers of pro- and antiangiogenic parameters and evaluate their response in ABCP receiving metronomic Cy 50 mg p.o./day + Cel 400 mg p.o./day. **MATERIALS AND METHODS:** Serum levels of vascular endothelial growth factor-C (VEGF-C), soluble VEGF receptors 2 and 3 (sVEGFR-2, sVEGFR-3), were measured at different time points in 13/15 patients included in a phase II trial of MCT with Cy+Cel. **RESULTS:** Serum levels of sVEGFR-2 and sVEGFR-3 increased significantly during treatment ($P = 0.0392$; $P = 0.0066$, respectively). VEGF-C showed no significant modifications. Previous determinations of VEGF and TSP-1 in the same patients were utilized. VEGF/sVEGFR-2, VEGF/TSP-1, and VEGF-C/sVEGFR-3 ratios decreased significantly along the treatment ($P = 0.0092$; $P = 0.0072$; $P = 0.0141$, respectively). Nonsignificant variations were observed for VEGF-C/sVEGFR-2 ratio. Baseline values of VEGF/sVEGFR-2 and VEGF/TSP-1 ratios were associated with time to progression (TTP) ($P = 0.0407$; $P = 0.0394$, respectively) meanwhile baseline VEGF was marginally significant ($P = 0.0716$). Patients with values lower than the 50th percentile for both ratios showed longer TTP. **CONCLUSIONS:** We have identified the baseline VEGF/sVEGFR-2 and VEGF/TSP-1 ratios as potential biomarkers of response in ABCP treated metronomically with Cy+Cel. This finding warrants its confirmation in a higher number of patients.

Key Words: Biomarkers, breast cancer, celecoxib, cyclophosphamide, metronomic chemotherapy

Introduction

Cancer is one of the main causes of morbidity and mortality among men and women. In Argentina, during 2008 there were 104.859 new cases of malignant tumors, being breast cancer the malignant illness with higher incidence in women.[1]

The identification of predictive biomarkers is one of the main goals in cancer research. The advantages of reaching such an objective are the reduction of both the time in selecting the best therapy for each individual patient and the public health costs.[2]

Metronomic chemotherapy (MCT) offers a new concept in cancer treatment based on continuous administrations for a long period of time and at much lower doses of the maximum tolerated dose regimen of chemotherapeutic and nonchemotherapeutic agents,

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targeting to the tumor vasculature or to the process of tumor angiogenesis. Also it has been suggested that MCT effect can be related to the restoration of the antitumor immune response.[3] The therapeutic effect of MCT was probed in several preclinical[4,5] and clinical trials[6-8] for different kinds of tumors and with diverse drug combinations, under the legend that "less is more,"[9] but no reliable biomarkers or predictors of response have yet been found.

The fact that the drugs administered in MCT are already approved and used in general practice, makes it even more interesting their proposal for the development of scheme treatments that could not only have therapeutic effect, but also lower the public expenditure on health, avoiding toxicities and hospitalizations and improving the quality of life of patients. Moreover, the use of "old and cheap" nononcologic molecules with antitumor properties, such as metformin,[10,11] propranolol,[12] celecoxib (Cel),[13] in metronomic schemes has widened the therapeutic opportunities of treating cancer successfully.

The aim of this study was to analyze several pro- and antiangiogenic molecules, to evaluate their potential role as predictors of response duration in patients with advanced breast cancer receiving MCT with cyclophosphamide (Cy) and Cel.

Materials and Methods

Clinical trial design and treatments

The advanced breast cancer patients included in this study were those enrolled in the first stage of a nonrandomized, monoinstitutional, Phase II Clinical Trial of MCT using Cy and Cel. Details about the clinical study have been described elsewhere.[7] This study was approved by the School of Medicine Bioethics Committee and by A.N.M.A.T. (Argentine Regulatory Authority). Written informed consent was required. In brief, patients (age, 18–80 years) with histologically confirmed advanced breast cancer progressing after three, and no more than four, chemotherapy schemes were enrolled. All patients received Cy 50 mg p.o. daily, plus Cel 400 mg (200 mg p.o. bid). Clinical response and toxicity were evaluated every two months or earlier if it was necessary. Patients were followed until progression or death. All patients who have received at least two months of treatment and have, at least, one tumor assessment, were considered evaluable for response. Time to progression (TTP) was defined as the period of time going from the beginning of the treatment until the progression of disease. TTP was censored at the date the patients exit the protocol because of disease progression or at the last visit when patients are in treatment.

Biomarker evaluations

Serum levels of soluble VEGF receptors 2 and 3 (sVEGFR-2, sVEGFR-3) and vascular endothelial growth factor-C (VEGF-C) were measured at baseline, during follow-up, and on the day of clinical progression. The same evaluation had been previously reported for VEGF and TSP-1 serum concentrations.[7] In brief, ELISA (Enzyme-Linked ImmunoSorbent Assay) test was performed for sVEGFR-2, sVEGFR-3, and VEGF-C quantification, according to manufacturer's instructions (Quantikine© ELISA kit, R and D Systems Inc, Minneapolis, MN, USA). Blood samples were allowed to clot for 2 h at room temperature. After centrifugation, the serum was removed and stored at − 20°C until used. Determinations were done in duplicates.

Statistical analysis

Spearman correlation coefficients were utilized for assessing whether there was a relationship between the different biomarkers evaluated and time during treatment for the whole group of patients.

With the purpose to construct a reliable biomarker, several ratios were calculated according the biologic function of each molecule, and their modifications during treatment were analyzed. The association between TTP with baseline values of VEGF, sVEGFR-2, sVEGFR-3 and VEGF/sVEGFR-2, VEGF/TSP-1, VEGF-C/sVEGFR-3, and VEGF-C/sVEGFR-2 ratios were investigated using linear regression models. A multiple regression analysis was also applied including VEGF, VEGF/sVEGFR-2, and VEGF/TSP-1 as covariates to evaluate the goodness of prediction.

Group-specific survival curves were generated according to the Kaplan–Meier product-limit method and were compared using the Log-rank test. To analyze the percentage of progression-free survival (PFS), the cutoff value was set at the 50th percentile of the baseline value.

All statistical tests were one sided with significance defined as a $P < 0.05$. STATA was used for the analysis (Statacorp. Stata Statistical Software: Release 6.0., College Station, TX, USA, 1999), and GraphPad Prism® version 3.0 (GraphPad Software, San Diego, CA, USA) was used for the graphics.

Results

For the analysis of the biomarkers herein described, serum samples of 13 over 15 patients included in the first stage of the protocol were evaluable. All patients were heavily pretreated and had advanced disease. More details of patients' characteristics were described by Perroud *et al*.[7]

Serum biomarkers

Concentrations of sVEGFR-2 and sVEGFR-3 increased during the treatment (r = 0.2815, P = 0.0392; r = 0.3837, P = 0.0066, respectively) [Figure 1a and b], whereas nonsignificant variations were detected for VEGF-C (r = −0.1151, P = 0.2398) [Figure 1c]. Previously, it had been determined, in the same group of patients, VEGF serum concentrations which decreased significantly during treatment (P = 0.004), and TSP-1 levels that showed nonsignificant modifications.[7]

The VEGF/sVEGFR-2 ratio [Figure 1d] decreased significantly along the treatment (r = −0.3712, P = 0.0092). The same behavior was observed with the ratios VEGF/TSP-1 [Figure 1e] (r = −0.2936, P = 0.0072) and VEGF-C/sVEGFR-3 [Figure 1g] (r = −0.3853, P = 0.0141). On the other hand, nonsignificant variations for VEGF-C/sVEGFR-2 ratio [Figure 1f] were observed (r = −0.2834, P = 0.1741).

Predictors of response

The baseline values of the different biomarkers and ratios that varied significantly along the treatment were tested with linear regression analyses to know their capacity to predict time to progression [Figure 2].

VEGF/sVEGFR-2 and VEGF/TSP-1 ratios [Figure 2d and e, respectively] were good predictors of time to progression (r^2 = 0.3283, P = 0.0407; r^2 = 0.3318, P = 0.0394, respectively), whereas the ability of VEGF baseline value [Figure 2a] to anticipate the response was marginally significant (r^2 = 0.2655, P = 0.0716). In contrast, sVEGFR-2 [Figure 2b], sVEGFR-3 [Figure 2c], VEGF-C/sVEGFR-2 [Figure 2f], and VEGF-C/sVEGFR-3 [Figure 2g] baseline values were not able to predict TTP (r^2 = 0.0134, P = 0.7059; r^2 = 0.0032, P = 0.8544; r^2 = 0.0557, P = 0.4376; r^2 = 0.0840, P = 0.3367, respectively).

When considering VEGF, VEGF/VEGFR-2 and VEGF/TSP-1 or VEGF/VEGFR-2, and VEGF/TSP-1 in a multiple regression analysis, the goodness of prediction was not improved with respect to that obtained with each putative predictor (data not shown).

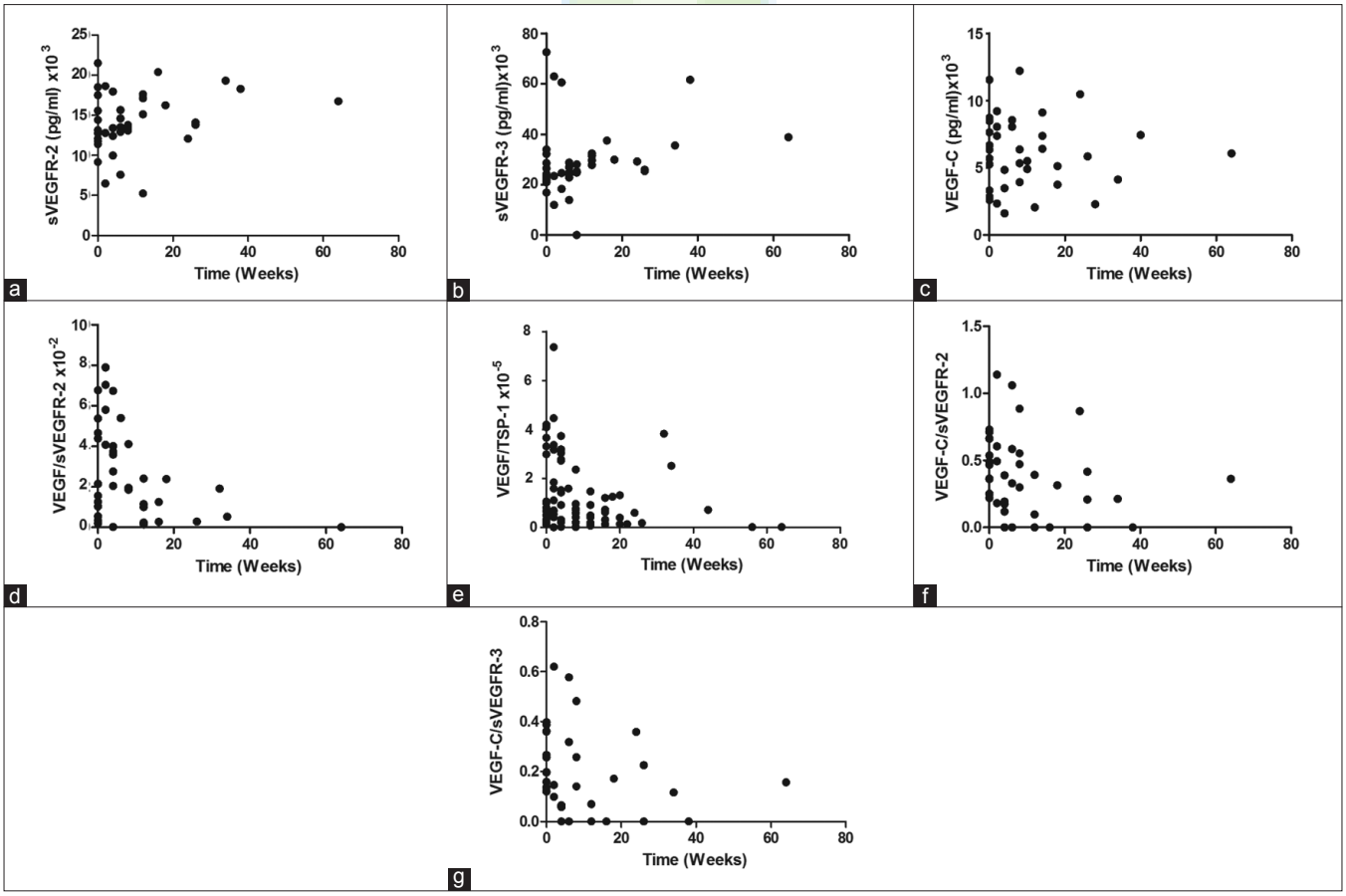

Figure 1: Correlation of serum markers with time. (a) sVEGFR-2: r = 0.2815, P = 0.0392; (b) sVEGFR-3: r = 0.3837, P = 0.0066; (c) VEGF-C: r = –0.1151, P = 0.2398; (d) VEGF/sVEGFR-2: r = –0.3712, P = 0.0092; (e) VEGF/TSP-1: r = –0.2936, P = 0.0072; (f) VEGF-C/sVEGFR-2: r = –0.2834, P = 0.1741; (g) VEGF-C/sVEGFR-3: r = –0.3853, P = 0.0141; Spearman correlation

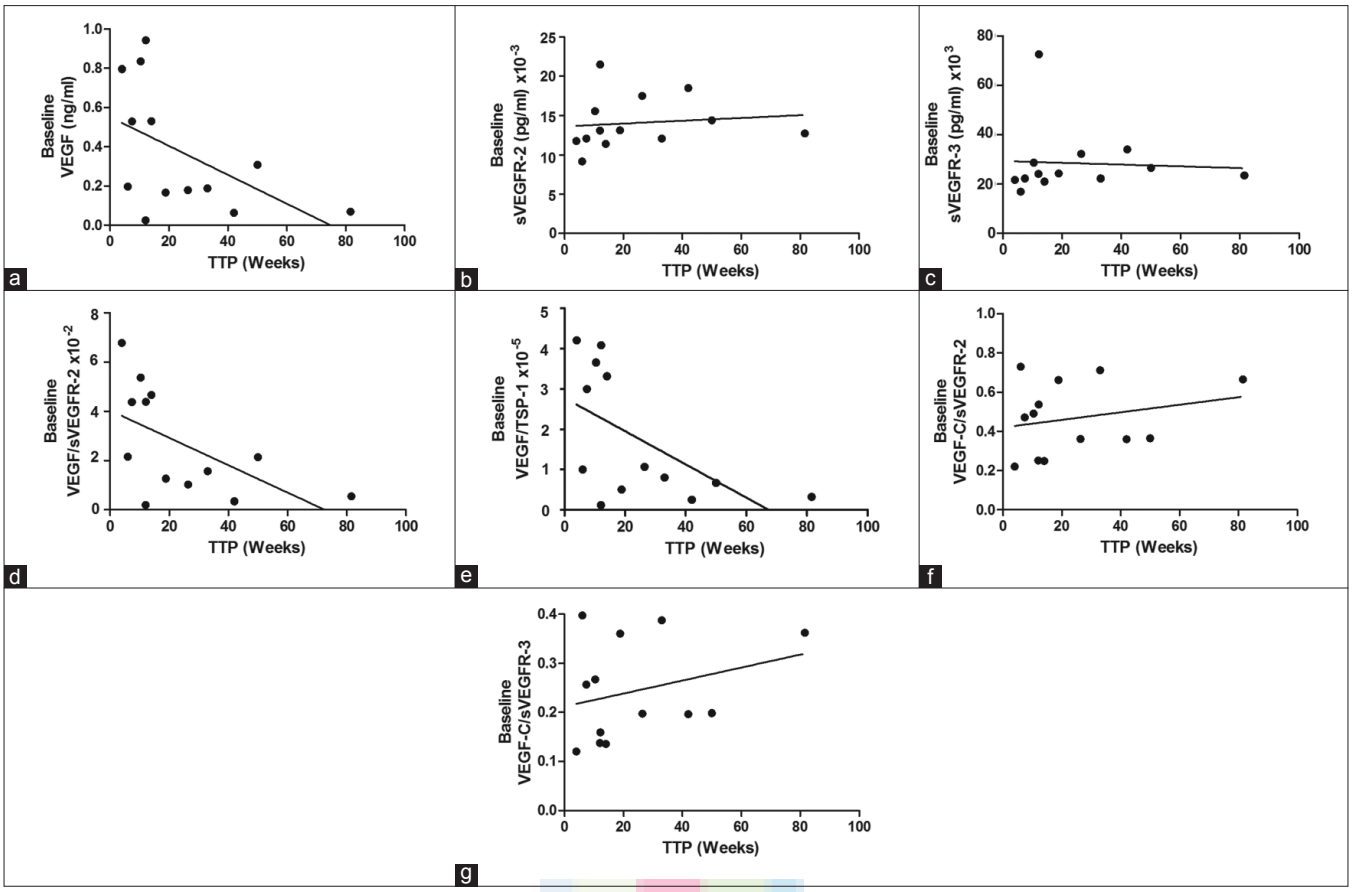

Figure 2: Association between baseline values of biomarkers and TTP. (a) VEGF: $r^2 = 0.2655$, $P = 0.0716$. The data to construct this graph was taken from Perroud *et al*.;[7] (b) sVEGFR-2: $r^2 = 0.0134$, $P = 0.7059$; (c) sVEGFR-3: $r^2 = 0.0032$, $P = 0.8544$; (d) VEGF/sVEGFR-2: $r^2 = 0.3283$, $P = 0.0407$; (e) VEGF/TSP-1: $r^2 = 0.3318$, $P = 0.0394$; (f) VEGF-C/sVEGFR-2: $r^2 = 0.0557$, $P = 0.4376$; (g) VEGF-C/sVEGFR-3: $r^2 = 0.0840$, $P = 0.3367$; linear regression

Survival analysis

To confirm the values of baseline VEGF/sVEGFR-2 and VEGF/TSP-1 ratios as predictors of response, we used the 50th percentile as a cutoff value to analyze the percentage of PFS. Patients who showed VEGF/sVEGFR-2 values equal or lower than the cutoff were those who had longer TTP, differing from those who had values above the cutoff (median survival = 33 and 8.93 weeks, respectively; $P = 0.0012$) [Figure 3a]. A similar result was obtained when studying baseline VEGF/TSP-1 ratio (median survival = 33 and 11.29 weeks, respectively; $P = 0.0369$) [Figure 3b].

Discussion

Tumor angiogenesis is a process that takes place in the microenvironment of the tumor, promoting not only the survival and proliferation of tumor cells but also invasion and metastasis.[14] There are several molecules that stimulate angiogenesis among which, VEGF, is one of the most important. Its expression is tightly regulated in normal angiogenesis; on the contrary, it is upregulated in different kinds of tumors favoring blood vessels growth and, hence, tumor growth.[15] Also, VEGF-C, one of the members of the VEGF family is overexpressed in diverse types of tumors and it was associated with lymphangiogenesis which, in turn, provides a pathway for tumor dissemination and metastasis.[16,17] Going to the antiangiogenic side of the scale that regulates the process of angiogenesis, several factors that counteract the angiogenic ones can be found. Soluble VEGF receptor 2 (sVEGFR-2), a splice variant of VEGFR-2, is an endogenous protein that is able to inhibit lymphangiogenesis.[18,19] Moreover, soluble VEGF receptor 3 (sVEGFR-3) can suppress both angiogenesis and lymphangiogenesis and lymphatic metastasis.[20-22] Another antiangiogenic factor like TSP-1 is considered responsible, in part of the antiangiogenic effect of MCT.[23,24]

The results of the first stage of a clinical trial for advanced breast cancer patient treated with MCT combining Cy and Cel has been recently published. Among those results, it was found a significant decrease of VEGF serum concentration during treatment, whereas TSP-1 did not show significant modifications.[7] In the same group of patients we have now determined the modifications of sVEGFR-2, sVEGFR-3, and

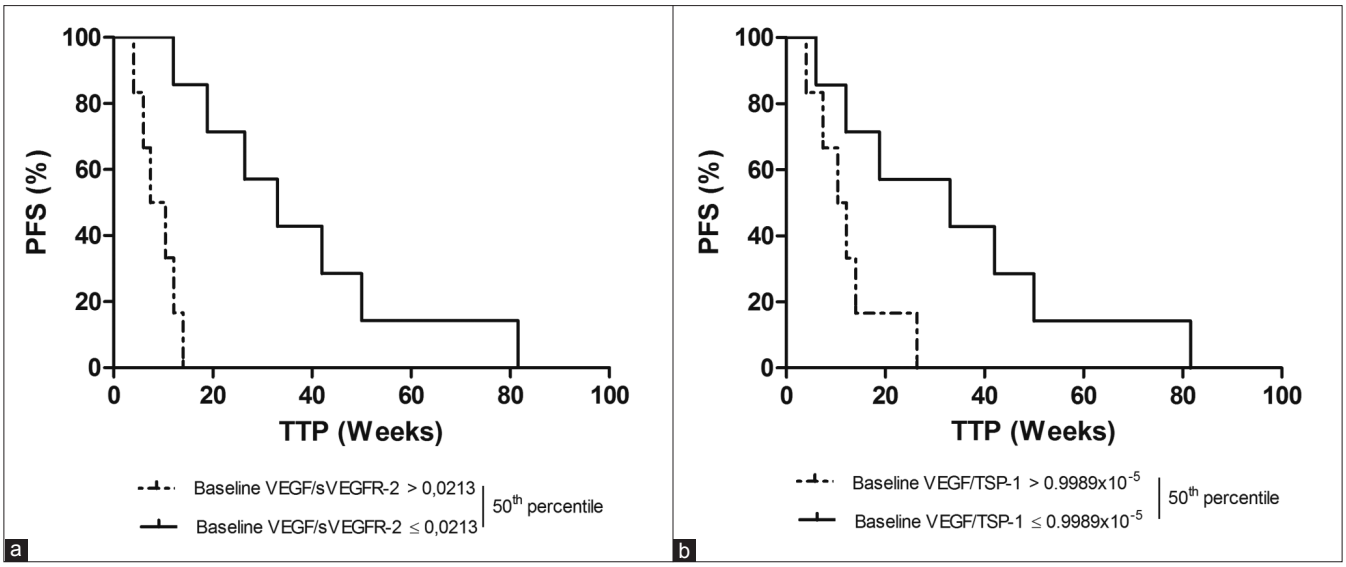

Figure 3: PFS stratified by baseline VEGF/sVEGFR-2 (a) and VEGF/TSP-1 (b) ratios. (a) *P* = 0.0012, (b) *P* = 0.0369; Kaplan–Meier survival curves and log-rank test. PFS, progression-free survival; TTP, time to progression

VEGF-C serum concentration in the same period of time, to know if they were correlated with treatment duration. Interestingly, sVEGFR-2 correlated positively and significantly with time. This result agrees with that obtained in breast cancer patients treated metronomically with daily dalteparin and cyclophosphamide, twice-weekly methotrexate, and daily prednisone,[25] or with a standard regimen of bevacizumab.[26] At variance, a decrease of sVEGFR-2 over time was informed for breast and renal cell carcinoma patients treated with a standard schedule of sunitinib.[27,28]

The concentration of sVEGFR-3 also increased significantly with time. To our knowledge there are no previous antecedents on such a determination in cancer patients treated with MCT. Conversely, standard therapy with sunitinib of breast[27] and renal cell carcinoma patients[28,29] yielded its decrease during treatment. Whether or not the contrasting results for sVEGFR-2 and -3 are caused by the different drugs or schedules employed is a subject that must be clarified in the future.

Therefore, MCT with Cy+Cel induced an increase in both the soluble VEGF receptors studied, which have the potentiality of inhibiting angiogenesis and lymphangiogenesis and therefore, could be responsible, at least in part, of the therapeutic effect obtained.

The importance of VEGF-C in lymphangiogenesis and lymphatic metastasis in breast cancer has been already adressed.[30] Nevertheless, there were no reports related to its modulation during MCT or to the likeliness of being considered a therapeutic target and/or a putative biomarker of response. The evaluation of VEGF-C levels along treatment showed no significant modifications, avoiding its use, as an individual molecule, for calculations of its power as a biomarker. In another study, standard treatment with sunitinib caused its decrease in renal cell carcinoma patients.[29]

Because all biologic processes depend on the balance between those molecules that stimulate and inhibit it, our next goal was to obtain several ratios between pro- and antiangiogenic molecules that, if significant, could then be tested, along with the individual molecules, for its value as predictors of response to MCT. Therefore, utilizing the previous and the present results obtained, the VEGF/sVEGFR-2, VEGF/TSP-1, VEGF-C/sVEGFR-3, and VEGF-C/sVEGFR-2 ratios were calculated and correlated with time. Negative and significant correlations were found for the first three ratios calculated, pointing to their potential value as biomarkers. On the contrary, the modifications of VEGF-C/sVEGFR-2 did not correlate with time.

Our main objective was to distinguish biologic predictors that will enable us to identify those patients who are prone to be benefited by MCT. Consequently, the next step we carried out was to analyze the association, if any, between baseline values of every molecule or ratio that showed a significant modification during treatment, and time to progression. The association between baseline VEGF and TTP was marginally significant, and a higher number of patients is needed to validate, or eliminate it as a candidate of predictor of response. In the metronomic setting, VEGF serum concentration was found a good prognostic, but not predictive, marker of response,[31-33] whereas in standard chemotherapy schedules the baseline VEGF

level was related to response in ovarian,[34] gastric,[35] and hepatic[36] cancers, among others.

The baseline values of soluble VEGF receptors 2 and 3, along with the VEGF-C/sVEGFR-3 ratio did not correlate with TTP. Similar results were obtained for sVEGFR-2 and sVEGFR-3 in other protocols.[27,37,38] However, in breast cancer patients treated with standard chemotherapy plus bevacizumab, baseline sVEGFR-2 levels were associated with clinical response.[26]

Interestingly, baseline values of VEGF/sVEGFR-2 and VEGF/TSP-1 ratios were significantly associated with TTP, suggesting their probable use as predictors of the duration of response in advanced breast cancer patients treated with MCT. Moreover, using the 50th percentile as a cutoff value, it was found that lower values of both ratios were associated with higher TTP, being the significance of the association for VEGF/sVEGFR-2 higher than that for VEGF/TSP-1. These results support the predictor value of those markers. However, larger studies are needed for establishing the veracity of this proposal. As far as we know, this is the first time that such ratios are proposed as biomarkers. Thus, it would be interesting that those groups that have worked, or are presently working, in MCT protocols for breast cancer patients and stored baseline serum samples, could determine VEGF, sVEGFR-2, and/or TSP-1 concentrations. The data obtained would allow them to calculate the VEGF/sVEGFR-2 and VEGF/TSP-1 ratios and to analyze its association with therapeutic response. Widening the number of breast cancer patients tested and the diversity of MCT drug combinations administered may give the scientific support required to accept or reject those variables as anticipators of patient's responses which, in turn, would allow administering to each one of them the treatment that will give the best therapeutic benefit.

In summary, we have identified potential predictive biomarkers of response in breast cancer patients treated metronomically with Cy and Cel. Should this finding be confirmed by other groups with a higher number of patients will be of importance, because that would allow us to apply more personalized treatments.

The need for identifying noninvasive biomarkers that could enable us to predict the response to cancer therapies in general and, particularly, to antiangiogenic cancer therapies, has not yet been fulfilled. Hence, we believe that the results herein described may help to achieve such a goal.

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