RESEARCH PAPER

Evaluation of Vascular Endothelial Growth Factor (VEGF) and Thrombospondin-1 as Biomarkers of Metronomic Chemotherapy in Progressive Pediatric Solid Malignancies

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Objectives: We compared Vascular Endothelial Growth factor (VEGF) and Thrombospondin-1 between patients with progressive paediatric malignancies randomized to metronomic chemotherapy *versus* placebo to determine their role as biomarker. **Methods:** In this double-blinded, placebo-controlled randomized study of 108 progressive pediatric malignancies, serum VEGF and Thrombospondin-1 levels were evaluated using ELISA at baseline, A2 (week-9 or earlier if progressed) and A3 (week-18 or earlier if progressed). **Results:** Mean VEGF and Thrombospondin-1 at baseline, A2 and A3 and the change from baseline to A2 were not different between two groups. In metronomic arm, responders (those completing 3 cycles) had significantly lower mean (SD) baseline VEGF levels [659.7(362.1) *vs* 1143.9 (622.0) µg/mL] (*P*=0.002) and significant decrease in thrombospondin-1 from baseline to A2 [-4.43(8.0) µg/mL *vs* 1.7(11.3) µg/mL] (*P*=0.04), as compared to non-responders. Similar changes were not observed in responders on placebo arm. No consistent trend of these biomarkers was observed. **Conclusions:** VEGF and Thrombospondin-1 are not reliable biomarkers for response to metronomic chemotherapy.

Keywords: *Anti-tumor activity, Anti-angiogenic, Outcome*.

Trial registration: *Clinicaltrial.gov, NCT01858571.*

PII: S097475591600146

Metronomic chemotherapy is the frequent administration of chemotherapeutic drugs at doses significantly below the 'maximum tolerated dose' with no prolonged drug-free breaks; it has carved a niche in modern paediatric oncology practice, especially in the recurrent metastatic or progressive disease settings [1,2]. Powerful and reliable biomarkers are yet to be identified and validated for the selection of a metronomic regimen for a given patient, in a given clinical setting. Vascular endothelial growth factor (VEGF) is an *in vivo* proangiogenic cytokine while Thrombospondin – 1 (TSP-1) is an intrinsic anti-angiogenic cytokine. Studies have shown increase in VEGF levels during successful therapy with anti-VEGF monoclonal antibodies and tyrosine kinase inhibitors (TKI) [3,4].

Even though evidence for these cytokines is contradictory [5-8], these angiogenic peptides are attractive bio-markers because of their ease of sampling and estimation in clinics. We previously published the first randomized trial in pediatric metronomics comparing metronomic chemotherapy with placebo in progressive pediatric malignancies [9]. In this report, we present the planned secondary objective of the study wherein we did a comparative analysis of two angiogenic peptides between these two groups of patients at different time-points.

Accompanying Editorial in print issue.

METHODS

The design, setting, participants and methodology of the clinical study have been described elsewhere [9]. Eligible patients (*n*=108) underwent 1:1 simple centralised randomization to metronomic chemotherapy (4-drug regimen of daily celecoxib and thalidomide with alternating periods of etoposide and cyclophosphamide) and placebo groups Institute Ethics Committee.

After informed consent, blood samples were taken for biomarker evaluation at baseline (A1) and interim assessments (A2 = 9 weeks or earlier if progressed, A3 = 18 weeks or earlier if progressed) (***Fig.* 1**).

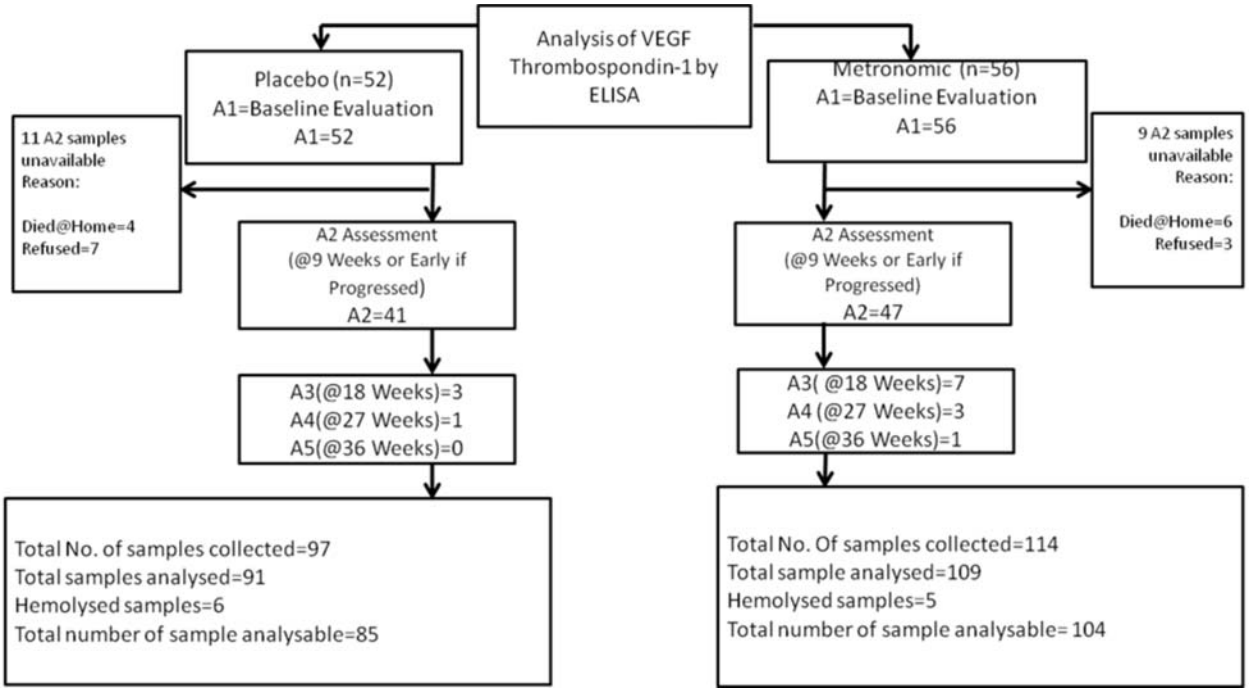

FIG. 1 *Study flow diagram.*

Serum was separated and centrifuged at 1000 g for 10 min within 30 min from collection. Serum was aliquoted and stored at –80°C. ELISA for VEGF and TSP-1 levels were evaluated from these samples of serum using Quantikine Human VEGF Immunoassay DVE00 and Quantikine Human Thrombospondin-1 Immunoassay DTSP10, respectively (R&D Systems, Inc, Minneapolis, MN 5541 USA).

We analyzed pattern of VEGF and TSP-1 in both study arms, comparing them at baseline, at second assessment (A2) and at third assessment (A3) as well as the change in their levels at A2. The clinical assessment during the study had shown no significant difference in Progression free survival (PFS) or Overall survival (OS) between the two arms [9]. However, in post hoc subgroup analysis, those who had completed more than 3 cycles (*i.e.* 9 weeks) and those who did not have a bone sarcoma benefitted from metronomic chemotherapy [9]. Hence, we also analyzed the patients as responders versus non-responders, defining responders as those who had completed 9 weeks of therapy.

RESULTS

The baseline characteristics of the 108 recruited subjects are presented in ***Table* I**. Baseline levels of VEGF greater than mean value of 1135.45 pg/mL was found to adversely affect OS with hazard ratio of 1.77 (1.18-2.65) (P=0.006). Baseline TSP-1 did not affect OS [HR (95% CI) =0.99 (0.99-1.00) (P=0.92)].

Mean level of VEGF and TSP-1 in patients at baseline, at A2 and at A3 were not different in the placebo and metronomic groups (***Web Table* I**). The difference from baseline values to second assessment (A2) for both these biomarkers in each group was also not significantly different.

In the metronomic arm, responders (*i.e.* those who completed at least 9 weeks of chemotherapy) had a significantly lower baseline VEGF levels as compared to non-responders (P=0.002). However, there was no difference in TSP-1 levels between them. The mean difference from baseline to the second assessment (A2-A1) for TSP-1 was significantly different (P=0.04); while TSP-1 decreased in the responders, it increased in the non-responders. Such a difference was not noted for VEGF (***Table* II**). There was no significant difference in the baseline levels of VEGF and TSP-1 between responders and non-responders of placebo arm. Neither was there any significant difference in the mean change of both VEGF and TSP-1 from baseline to A2 (***Table* II**).

DISCUSSION

Our study showed that baseline VEGF predicted OS for the entire study population, whereas baseline TSP-1 did

Table I Comparison of baseline characteristics of the two study groups

	Placebo (n=52)	*Metronomic (n=56)*
Age, y	15 (5-18)	13 (5-18)
Male: female	3.3:1	3:1
ECOG-PS		
0	1 (1.9)	3 (5.3)
1	19 (36.5)	18 (32.1)
2	21 (40.3)	25 (44.6)
3	11 (21.1)	10 (17.8)
Diagnosis		
Bone	32 (61.4)	40 (71.3)
Sarcoma(PNET/Osteosarcoma)		
Neuroblastoma	5 (9.6)	5 (8.9)
RMS	6 (11.5)	3 (5.3)
Esthesioneuroblastoma	1 (1.9)	1 (1.7)
STS	4 (7.6)	2 (3.8)
Others	3 (5.7)	3 (5.3)
Retinoblastoma	1 (1.9)	2 (3.8)
Previous lines		
2	48 (92.3)	53 (94.6)
3	4 (7.7)	2 (3.6)
4	0	1 (1.8)

*All P values >0.05; PNET: primitive neuroectodermal tumours; RMS: rhabdomyosarcoma; STS: soft tissue sarcoma; ANC= absolute neutrophil count; ECOG-PS: Eastern Cooperative Oncology Group- Performance Status. All values in no. (%) except *median (range).*

not predict the same. In the total study sample, there was no difference in the levels of VEGF or TSP1, neither at baseline, nor at any other time-point, between the placebo and metronomic arms. The magnitude of change from baseline to A2 was also not different significantly different between the two arms. But then, there was no difference in survival as well between the two arms.

While our findings are in contrast to studies on other solid tumours treated with anti-angiogenic agents, *eg*. metastatic colorectal cancer treated with bevacizumab [10], it corroborates with the findings in metastatic breast cancer [11]. When we focussed our analysis on the metronomic arm, we found that responders had significantly lower baseline levels of VEGF but no difference was noted in TSP-1. This is consistent with previous studies that have noted an aggressive tumor progression with injection of TSP-1 in preclinical models [12]. Although, our study demonstrated some trends, we could not provide proof of the principle that the 4-drug anti- angiogenic chemotherapy actually acts by altering the cytokine milieu of pro and anti angiogenic factors, and inhibiting angiogenesis *in vivo*. Our results are consistent with the results Stempak, *et al*. [5] and Kesari, *et al*. [8] who found that none of the four tested markers (VEGF, bFGF, endostatin, and TSP-1) were of prognostic significance.

In a previous study, baseline TSP-1 levels appeared to correlate with prolonged response; this conclusion was based on just three patients who had a baseline high TSP-1 level and did not progress for more than a year [6]. In another study of 100 patients treated with metronomic chemotherapy, 52 baseline patient samples were available and herein serum TSP-1 levels increased in patients who completed therapy than in non- completers [7]. Our study is a larger study with a placebo arm, but still we could not replicate those findings. The reason why we could not demonstrate a trend in these cytokines may probably be the fact that the small subset of proteins that we selected is unlikely to be representative of the overall effect of all of the regulators of angiogenesis. Angiogenesis is a complex interacting cascade of pathways with an interplay of a large number of proteins inside and outside of the cell and we cannot gauge them by relying on only one or two of these proteins. The strengths of our study are its randomized nature and comparison with placebo.

Table II Comparison of VEGF and TSP-1 levels among responders and non-responders of metronomic and placebo arms of the study

	Metronomic (n=56)		*Placebo (n=52)*	
	Responders (N=21) [Mean (SD)]	*Non-Responders (N=35) [Mean (SD)]*	*Responders (N=19) [Mean (SD)]*	*Non-Responders (N=33) [Mean (SD)]*
Baseline TSP-1 (A1)	19.4 (7.0)	21.3(10.6)	24.3(13.4)	20.9(12.5)
Baseline VEGF (A1)	659.7(362.1)*	1143.9(622.0)*	961.9(496.3)	1238.5(770.1)
Difference from baseline A1 TO A2 (TSP-1): (A2-A1)	-4.43(8.0)$^{\#}$	1.7(11.3)$^{\#}$	-4.90(16.4)	-6.2(12.2)
Difference from baseline to A2 (VEGF): (A2-A1)	173.1(618.2)	90.8(706.1)	224.9(615.7)	-87.0(535.9)

VEGF: Vascular Endothelial Growth Factor, TSP-1: Thrombospondin-1, A2= second assessment at 9 weeks or earlier if progression of disease. Values of VEGF are in pg/ml and the values of TSP-1 are in µg/ml respectively, P value of *=0.02 and $^{\#}$=0.04.

WHAT THIS STUDY ADDS?

- We found no consistent trend of these peptides among the responders of metronomic chemotherapy and conclude that these are not reliable biomarkers for metronomic chemotherapy thus settling a long-standing debate in medical literature.

Identifying reliable predictive and/or prognostic biomarkers for anti-angiogenic therapies has been unsuccessful to date. Looking for a biomarker for a therapy can be a realistic objective only if that therapy targets the tumor cells of interest, but when we are using metronomic chemotherapy, we are actually targeting the host endothelial cells and not directly the tumor. So, it is unlikely that universal mechanistically-driven markers will ever be unveiled for metronomic chemotherapy, especially given its varied mechanisms of action, multiple drug combinations and many clinical settings. We suggest that other biomarkers be explored for measuring the efficacy of metronomic chemotherapy like circulating cell free DNA, circulating endothelial cells, and circulating endothelial precursor cells and micro-particles.

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Webtable I VEGF and TSP-1 in Study Participants at Different Time-points

Time point	*Placebo*	*Metronomic*	*P value*
VEGF (pg/mL)			
Baseline (*n*=107)	1135.4 (688.9)	962.4 (585.5)	0.16
A2 (≤9 weeks) (*n*=88)	1103.4 (712.9)	1032.9 (708.7)	0.64
A3 (≤18 weeks) (*n*=7)	1471.7 (954.3)	1412.2 (687.3)	0.92
Change from baseline at A2 (*n*=88)	-57.5 (588.4)	-125.8 (664.4)	0.61
Thrombospondin-1 (µg/mL)			
Baseline (*n*=107)	22.2 (12.8)	20.6 (9.4)	0.54
A2 (≤9 wk) (*n*=87)	17.3 (9.4)	19.1 (11.2)	0.42
A3 (≤18 wk (*n*=9)	17.1(5.9)	19.2 (8.3)	0.76
Change from baseline at A2	-5.6 (14.1)	-0.9 (10.4)	0.07

VEGF: Vascular endothelial growth factor, A2: Assessment 2 (at 9 wk or earlier if disease progression); A3: Assessment 3 (at 18 wk or earlier if disease progression); all value in mean (SD).