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# Elevated plasma levels of transforming growth factor-$\beta_1$ (TGF-$\beta_1$) in patients with advanced breast cancer: association with disease progression

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**Abstract**

We examined the association between an elevated plasma TGF-$\beta_1$ level and the disease progression of advanced breast cancer (BC) patients ($n=44$). TGF-$\beta_1$ levels were detected by an enzyme-linked immunosorbent assay (ELISA). Platelet carryover and *in vitro* platelet activation in our plasma samples was assessed and found to be insignificant. Plasma TGF-$\beta_1$ values were significantly elevated ($P<0.05$) in stage IIIB/IV patients (median value: 2.40 ng/ml, range: 0.13–8.48 ng/ml, $n=44$) compared with healthy donors (median value: 1.30 ng/ml, range: 0.41–4.93 ng/ml, $n=36$). Although pronounced in metastatic patients, especially those who had been newly diagnosed, TGF-$\beta_1$ elevation was independent of tumour mass, site of distant metastases, histopathological type, steroid receptor (SR) content and age of the BC patients. Follow-up of 6 patients indicated a relationship between the plasma TGF-$\beta_1$ and the patient's response. This suggests that TGF-$\beta_1$, may be a promising prognostic marker for breast cancer patients with advanced disease. Confirmatory large-scale studies are needed, particularly given the overlap of values between our different subgroups analysed.


*Keywords:* TGF-$\beta_1$; Platelet factor 4; Human plasma; Prognostic biomarker(s); Breast cancer progression

## 1. Introduction

Despite an enlarged repertoire of anti-cancer agents, breast cancer (BC) is still the leading cause of cancer-related death in Western women and the primary cause of death in Serbia [1]. The lack of good response rates in locally advanced BC or metastatic disease is particularly problematic. In Serbia more than 30% of BC patients are diagnosed at advanced stages [2], when the five-year survival rate is very low and there is no cure. Early prognosis is therefore essential to identify BC patients at risk of disease relapse and mortality. A marker that could correlate with a biological behaviour such as invasiveness or metastasis would be of significant value in determining prognosis and targeted therapy. The prognostic role of the Tumour-Node-Metastasis (TNM) staging system has been well documented and is independent of treatment modalities [3]. However, it is clear that even within TNM groups, there are subsets of patients with a better or worse prognosis than the group as a whole [4]. The need to identify these subsets of patients has led to the exploration of molecular biomarkers [4,5]. Historically, tissue biomarkers have been viewed as prognostic and predictive tumour indicators, and circulating markers have been predominantly used for the monitoring of the disease course [6]. However, this tendency has shifted and considerable research efforts are now focused on the prognostic/predictive role of circulating tumour-associated markers [6,7]. These biomarkers do not require surgical intervention and provide relatively cheap monitoring at any time during the disease course.

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Current evidence [8] suggests a role in malignant progression for the circulating polypeptide Transforming Growth Factor-β1 (TGF-β1). The overexpression of this biomarker *in vitro* can enhance cell invasion of fibrosarcoma [9], prostatic carcinoma [10], and mammary adenocarinoma cells, with a consequent increase in the metastatic potential of the tumour [11]. *In vivo* studies have demonstrated a positive correlation between an elevated plasma TGF-$\beta_1$ level and malignant progression in colorectal [12,13], prostate [14,15], bladder [16] and liver [17] cancers. However, overlapping of the plasma TGF-$\beta_1$ values occurs between controls and cancers of different clinical stages [12–17], thus affecting the potential usefulness of this biomarker.

In the plasma of breast cancer patients, on the other hand, controversial results have been reported. One line of evidence has demonstrated positive correlation of elevated plasma TGF-$\beta_1$ with progression [18,19], and with post-therapeutic BC complications including liver and lung fibrosis after high-dose chemotherapy [20] and breast fibrosis after radiotherapy [21]. Therefore, it has been proposed that this biomarker has prognostic validity in BC. However, Wakefield and colleagues (22), have observed unchanged plasma TGF-$\beta_1$ values in advanced BC patients relative to healthy donors (HD), therefore disputing its prognostic validity. The discrepancy between these reports has been attributed to false-positives in the former case, due to *in vitro* platelet activation, and resulting in an overestimation of the plasma TGF-$\beta_1$ concentration [22]. To clarify this area, we have previously developed a TGF-$\beta_1$ specific protocol for the preparation of platelet-poor/protease-inhibited human plasma as monitored by an antibody-based TGF-$\beta_1$ enzyme-linked immunosorbent assay (ELISA) [23]. Recently, the use of receptor-based TGF-$\beta_1$ ELISA [24], and the correction for spurious marker release by platelets using platelet and granule marker platelet factor 4 factor (PF4) [7,22], have been recommended.

The main objective of the present study was to evaluate the putative role of plasma TGF-$\beta_1$ as a prognostic biomarker in breast cancer progression, by examining the true value of plasma TGF-$\beta_1$ levels in advanced BC patients (clinical stages IIIB/IV). Specific goals included validation of the plasma preparation protocol to reduce false-positive results, analysis of plasma TGF-$\beta_1$ with respect to classical tumour/host factors, and analysis of the relationship of TGF-$\beta_1$ levels with the patient's therapeutic response.

## 2. Patients and methods

### 2.1. Subjects and treatment

The present study included 44 breast cancer patients, described in Table 1, attending the Institute of Oncology and Radiology of Serbia in Belgrade from 2000 to 2001. Healthy women donors ($n=36$) of a similar age were used as controls (HD). None of these women were on any drugs/systemic treatment or oestrogen replacement therapy (Table 1). The study received Institutional Review Board approval and informed consent was obtained from each woman according to the National Health Regulations.

### 2.2. Materials

The Quantikine TGF-$\beta_1$ ELISA kit was obtained from R&D Systems Inc. (Minneapolis, MN, USA) and apyrase, Phenylmethylsulphonyl Fluoride (PMSF), and Ethylenediamine tetraacetic Acid (EDTA) from the Sigma Chemical Co. (St. Louis, MO, USA). The PF4 ELISA kit and aprotinin were purchased from Behring (Marburg, Germany).

### 2.3. Methods

#### 2.3.1. Collection of blood and preparation of plasma and serum

Phlebotomy and plasma preparation (anticoagulant mixture: EDTA, apyrase, PMSF and aprotinin) were performed as previously described in Ref. [23] except for the addition of apyrase. The venous blood (4 ml) was drawn into a test-tube containing a final concentration of 0.25% EDTA, 1mM PMSF, 250 Kallikrein Inaktivator Enhetier KIE/ml aprotinin, and 2.5 units/ml apyrase. The sample was mixed, centrifuged (20 min, at 800 g, +4 °C) and the supernatant recentrifuged (10 min, at 9400 g, +4 °C), to quantitatively remove residual platelets and thus prevent platelet carryover. The re-centrifuged supernatant was aliquoted (150 μl/aliquot, +4 °C), and stored frozen at −100 °C until analysis, within the shelf life of 6 months. For serum preparation, autologous blood (2 ml) was left to coagulate (30 min, room temperature), centrifuged (15 min at 540 g, room temperature), aliquoted (150 μl/aliquot) and stored frozen at −100 °C until analysis, within the shelf life of 1 month.

#### 2.3.2. TGF-$\beta_1$ and PF4 detection

TGF-$\beta_1$ was analysed by the receptor-based Quantikine TGF-$\beta_1$ ELISA kit according to the manufacturer's instructions.The absorbance at 450 nm was detected by the Microplate ELISA reader El 311 (Behring, Marburg, Germany). Concentrations were calculated from the constructed linear standard curve (average coefficient of correlation $r=0.9997$ from four independent assays). Each data point represents the mean of duplicate wells, which differed up to 7% both within and between the assays. The sensitivity of the kit was 20 pg/ml TGF-$\beta_1$. Detection of PF4 was performed by an antibody-based PF4 Behring ELISA kit, according to the manufacturers' instructions.The absorbance at 492 nm was measured by a Beckman DU-8 spectrophotometer.

Table 1
Subjects' characteristics (race-Caucasian)

| BC patients | *n* | (%) Total | Healthy donors | *n* | (%) Total |
|---|---|---|---|---|---|
| Patients' characteristics | 44 | (100) | Donors' characteristics | 36 | (100) |
| Age at study entry (years) median (range) | 54 (37–74) | | Age at study entry (years) median (range) | 44 (24–61) | |
| Menopausal status | | | Menopausal status | | |
| premenopausal | 8 | (18) | premenopausal | 18 | (50) |
| peri/postmenopausal | 36 | (82) | peri/postmenopausal | 18 | (50) |
| Time from diagnosis of invasive BC (months) | | | | | |
| none (included at diagnosis) | 17 | (39) | | | |
| $<12$ months | 2 | (5) | | | |
| $\geqslant 12$ months | 25 | (57) | | | |
| Previous treatment of BC | | | | | |
| none (recent biopsy alone) | 20 | (45) | | | |
| surgery +/-RT +/-adjuvant therapy | 19 | (43) | | | |
| RT alone +/-systemic treatment | 3 | (7) | | | |
| Systemic treatment of MBC[a] | 2 | (5) | | | |
| Intended treatment of actual advanced disease | | | | | |
| neo-adjuvant chemotherapy | 9 | (20) | | | |
| chemotherapy of MBC ± RT ± ET | 16 | (36) | | | |
| ET (endocrine treatment) ± RT | 19 | (43) | | | |
| Tumour characteristics | | | | | |
| Clinical stage[b] (at study entry) | | | | | |
| IIIB | 9 | (20) | | | |
| IV-localised metastases | 12 | (27) | | | |
| IV-multiple metastases | 23 | (52) | | | |
| Histological type of invasive BC | | | | | |
| IDC[c] | 14 | (32) | | | |
| ILC[c] | 17 | (39) | | | |
| other or unknown IC | 13 | (30) | | | |
| Histopathological grade | | | | | |
| G1 | 1 | (2) | | | |
| G2 | 24 | (55) | | | |
| G3 | 5 | (11) | | | |
| GX[d] | 14 | (32) | | | |
| Steroid receptor status | | | | | |
| ER-positive | 16 | (36) | | | |
| ER-negative | 21 | (48) | | | |
| ER n.d.[e] | 7 | (16) | | | |
| PR-positive | 14 | (32) | | | |
| PR-negative | 24 | (55) | | | |
| PR n.d.[e] | 6 | (14) | | | |
| Site of actual tumour lesions | | | | | |
| Breast only +/− regional lymphatics | 9 | (20) | | | |
| Distant metastases (stage IV) | 35 | (80) | | | |
| bone involvement: BM +[f] | 18/35 | (51) | | | |
| BM− | 17/35 | (49) | | | |
| visceral involvement: liver + | 10/35 | (29) | | | |
| liver− | 25/35 | (71) | | | |

BC, breast cancer; RT, radiotherapy; IC, infiltrating carcinoma ER, Oestrogen Receptor; PR, Progesterone Receptor.
[a] MBC, metastatic breast cancer.
[b] Clinical staging was based on American Joint Committee on Cancer/International Union Against Cancer (AJCC/UICC) TNM classification.
[c] IDC, infiltrating ductal carcinoma; ILC, infiltrating lobular carcinoma.
[d] GX, Grade cannot be assessed.
[e] n.d., Not done.
[f] BM +/−, bone metastases present/absent.

All determinations were performed in duplicate, the discrepancy between duplicates being within 3%.

The corrected plasma TGF-$\beta_1$ value was calculated by the formula: TGF-$\beta_{1\ \text{corrected (plasma)}}$ = TGF-$\beta_{1\ \text{measured (plasma)}}$ $-R\times[\text{PF4}]_{\text{measured (plasma)}}$, using as $R=[\text{TGF-}\beta_1]_{\text{serum}}/[\text{PF4}]_{\text{serum}}$, as previously described in Ref. [22]. The $R$ values for both the HD (mean value: $0.0244\pm0.0096$, $n=8$) and BC patients (mean value: $0.0135\pm0098$, $n=6$), were used to correct for each individual plasma TGF-$\beta_1$ value of the HD and BC patients, respectively.

### 2.3.3. Statistical methods

The non-parametric Mann–Whitney U test was performed to examine the relationship between the subgroup distribution of the individual plasma TGF-$\beta_1$ concentrations. Subsequent analysis has revealed a symmetrical distribution of individual TGF-$\beta_1$ and PF4 values for both BC patients and HD. Therefore, in several instances (Fig. 1 and Table 2) the student's 2-tailed test was used for statistical analysis of multiple means.

The correlation of TGF-$\beta_1$ values versus age was performed by a correlation coefficient analysis and TGF-$\beta_1$ versus oestrogen receptor (ER) content by the Spearman's rank correlation test. The level of significance was set at $P<0.05$.

## 3. Results

### 3.1. Validation of plasma preparation protocol by the PF4 correction method

Fig. 1 and Table 2 indicate the results obtained by measurements of the platelet-specific marker, PF4, in blood specimens of 13 healthy donors and 6 BC patients. The corrected and measured plasma TGF-$\beta_1$ values, presented in Fig. 1, reveal negligible differences in the mean values of 2.8 and 5.2% for HD and BC patients, respectively. The data points are derived from the absolute PF4 values given in Table 2, as described in Methods. Taken together, these findings demonstrate an insignificant release of PF4 and TGF-$\beta_1$ from both platelet carryover and *in vitro* platelet activation in our plasma samples, and this is encouraging for our subsequent plasma TGF-$\beta_1$ analysis, as well as any future large-scale analyses.

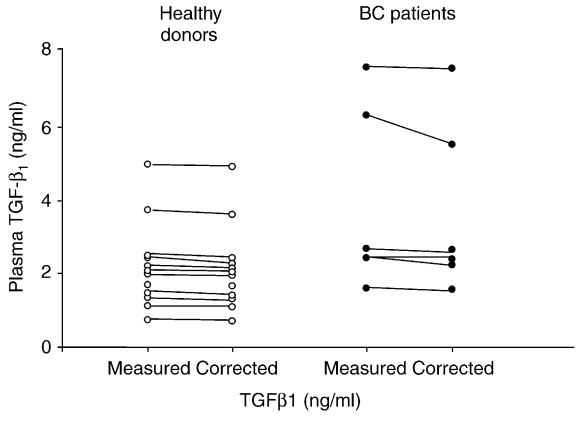


Fig. 1. Comparative concentrations of the "measured" and the "corrected" plasma TGF-$\beta_1$, obtained before and after PF4 correction for *in vitro* platelet activation. Lines connect matched measured and corrected values for each individual healthy donor (○) and breast cancer (BC) patient (●).

Table 2
Mean values[a] of circulating PF4 in plasma and serum of HD and BC patients

| Subjects | Plasma PF4 (ng/ml±S.E.M.) | Serum PF4 (ng/ml±S.E.M.) | % Platelet degranulation in plasma |
|---|---|---|---|
| HD | 2.5±0.4 | 1000±100 | 0.25% |
| BC patients | 17±9** | 3300±400*** | 0.51% |

HD, healthy donors; BC, breast cancer; S.E.M., standard error of mean.
**$P<0.02$.
***$P<0.001$.
[a] Mean values obtained from PF4 values for HD (plasma range: 1.3–6.4 ng/ml, serum range: 590–1800 ng/ml, $n=13$) and BC patients (plasma range: 2.03–60 ng/ml, serum range: 1660–4580 ng/ml, $n=6$); $n$, number of patients; $P$, level of significance.

### 3.2. TGF-$\beta_1$ levels versus tumour factors in advanced BC

Fig. 2 shows plasma TGF-$\beta_1$ levels in HD (median value: 1.30 ng/ml, range: 0.41–4.93 ng/ml, $n=36$) relative to the advanced BC patients (stages IIIB/IV),

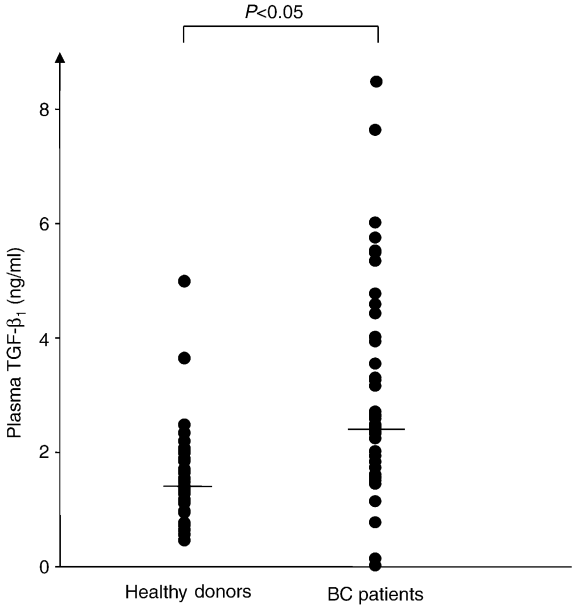


Fig. 2. Plasma TGF-$\beta_1$ concentrations of healthy donors relative to the advanced breast cancer(BC) patients (stages IIIB/IV). Horizontal bars represent median values; $P$, level of significance.

Table 3
Comparison of plasma TGF-$\beta_1$ levels between subgroups of BC patients with progressive disease (stages IIIB/IV) based on clinical tumour factors

| Clinical tumour factor | Subgroups of BC patients | *P value |
|---|---|---|
| Distant metastases | | |
| a. site: | bone-positive ($n=18$) vs. bone-negative ($n=17$) | 0.3 |
| | liver-positive ($n=10$) vs. liver-negative ($n=25$) | 0.4 |
| b. number: | single/localised ($n=12$) vs. multiple($n=23$) | 0.05 |
| Histological type | IDC** ($n=14$) vs. ILC** ($n=17$) | 0.08 |
| SR content*** | ER-positive ($n=16$) vs. ER-negative ($n=21$) | 0.3 |
| | PR-positive ($n=14$) vs. PR-negative ($n=24$) | 0.4 |

*$P$, level of significance; $n$, number of patients.
**IDC, infiltrating ductal carcinoma; ILC, infiltrating lobular carcinoma.
***Oestrogen receptor (ER) and progesterone receptor (PR) contents were determined by the [$^3$H] radio-receptor assay; SR, steroid receptor.

described in Table 1. The data indicate that circulating TGF-$\beta_1$ levels in BC patients (median value: 2.40 ng/ml, range: 0.13–8.48 ng/ml, $n=44$) were significantly elevated ($P<0.05$) when compared with HD of a similar age range (Fig. 2). This elevation was independent of the site and number of distant metastases, histopathological type and steroid receptor (SR) content of the breast tumour (Table 3). In contrast, there was an association with progressive stages of the disease, as observed by the statistically higher TGF-$\beta_1$ levels for the stage IIIB (median value: 2.11 ng/ml, range: 0.75–3.54 ng/ml, $n=9$, $P<0.05$) and the highly pronounced increase for stage IV patients (median value: 2.46 ng/ml, range: 0.13–8.48 ng/ml, $n=35$, $P<0.0001$) patients compared with HD (Fig. 3).

### 3.3. TGF-$\beta_1$ levels versus BC host factors

Fig. 4 shows the lack of correlation of TGF-$\beta_1$ levels with either premenopausal status (HD versus BC) or menopausal status within the HD and BC subject groups. However, the data in Fig. 4 reveal a highly significant plasma increase in TGF-$\beta_1$ levels in postmenopausal BC patients (median value: 2.52 ng/ml, range: 0.13–8.48 ng/ml, $n=36$, $P<0.001$) relative to postmenopausal HD (median value: 1.54 ng/ml, range: 0.51–4.93 ng/ml, $n=18$). In a further analysis according to the time intervals from first diagnosis ($\Delta t$), postmenopausal BC patients formed two subgroups: A. newly arrived patients ($\Delta t=0$), diagnosed for the first time at advanced stages/before any treatment ($n=14$) and B. previously diagnosed patients ($n=20$) i.e. analysed at the onset of metastatic disease after prolonged time intervals ($\Delta t \leqslant 156$ months) after an initial diagnosis/adjuvant treatment. Curiously, plasma TGF-$\beta_1$ levels of the former group (A) were highly pronounced (mean value: $3.78 \pm 0.63$), the value being gradually decreased down to 41.5% (data not shown) with an increasing $\Delta t$ in the latter group (B).

TGF-$\beta_1$ levels were independent of the subject's age for both the HD and BC groups as a whole (data not shown). In addition, within the postmenopausal BC patients, a

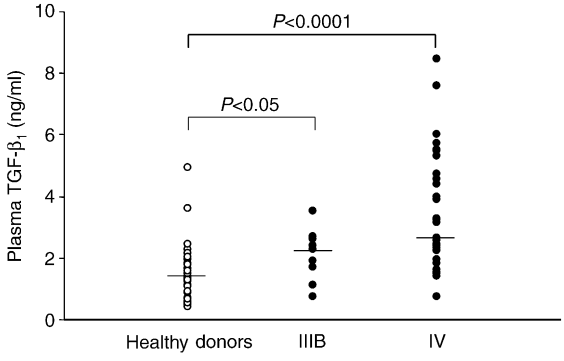


Fig. 3. Relationship between plasma TGF-$\beta_1$ levels of healthy donors (○) and the tumour stages (●): stage IIIB versus stage IV of breast cancer(BC) patients. Horizontal bars represent median values; $P$, level of significance.

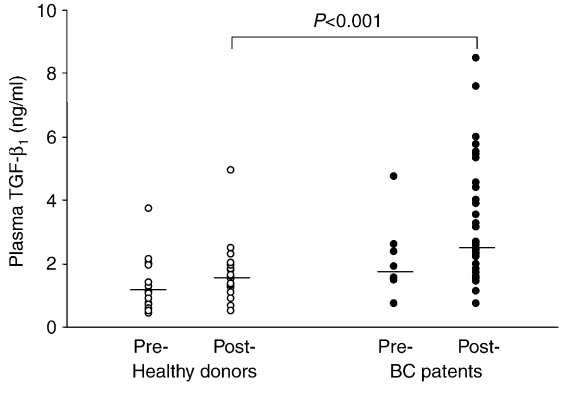


Fig. 4. Effect of menopausal status on plasma TGF-$\beta_1$ levels in pre-, premenopausal [pre-] and postmenopausal [post-] healthy donors (○) compared with the respective menopausal status of the advanced breast cancer (BC) patients (●) Horizontal bars represent median values; $P$, level of significance.

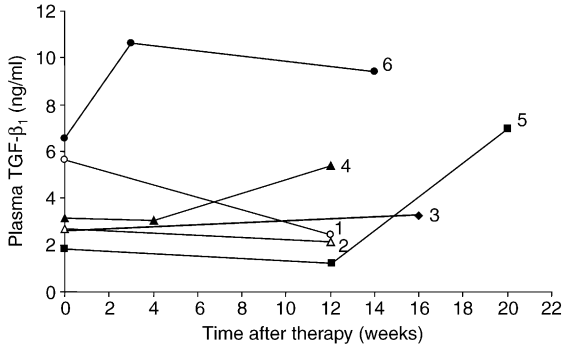


Fig. 5. Time curves of plasma TGF-$\beta_1$ values observed during the follow-up of six BC patients with advanced disease undergoing treatment, numbered 1–6. The treatment included either a single or combination therapy for metastatic disease, as described in Table 4.

lack of correlation of TGF-$\beta_1$ levels with respect to distant metastases (site/number), histopathological type and SR content was observed (data not shown).

However, our results illustrate a wide overlapping in TGF-$\beta_1$ values between normal donors and the patients in these analyses presented in Figs. 2–4.

### 3.4. Individualised TGF-$\beta_1$ monitoring of BC therapy

Fig. 5 illustrates time-dependent changes in plasma TGF-$\beta_1$ levels of six patients undergoing therapy for metastatic disease. The patients' therapeutic response was monitored and, together with their respective plasma TGF-$\beta_1$ changes, are presented in Table 4. The results reveal a decrease in TGF-$\beta_1$ value by 57, 21 and 33%, for patients who responded to treatment (numbered 1, 2 and 5, respectively), and an increase in TGF-$\beta_1$ values by 34, 69 and 62% for BC patients (numbered 3, 4 and 6, respectively), who failed to respond to systemic treatment (Fig. 5, Table 4). However, an exception was observed in one patient, numbered 5, who had post-therapeutic complications unrelated to the disease or the treatment (Fig. 5, Table 4).

## 4. Discussion

In the present study, an attempt has been made to assess the true value of plasma TGF-$\beta_1$ levels in the peripheral blood of advanced breast cancer patients. Special pre-analytical conditions have been set which proved to be effective in preventing the release of PF4 and TGF-$\beta_1$ from platelets. A similar approach to avoid spurious release of a marker by *in vitro* platelet activation has recently been performed for vascular endothelial growth factor (VEGF) by Dittadi and colleagues [7]. Our results revealed an insignificant contribution of *in vitro* platelet activation to the measured plasma TGF-$\beta_1$ level in both BC patients and HD, suggesting that the observed TGF-$\beta_1$ increase is associated with the presence of the tumour.

Breast cancer patients with progressive disease (stages IIIB/IV) were analysed and, despite the limited number of cases, the results revealed significantly elevated plasma TGF-$\beta_1$ values when compared with HD. Our data are consistent with previous reports demonstrating an augmented TGF-$\beta_1$ production in tumour tissue [25—27] and in the circulation [18,19,23,28] of

Table 4
The individualised effect of selected therapy on trend/change in plasma TGF-$\beta_1$ concentrations and relationship to the consequent therapeutic response for six BC patients with advanced disease described in Fig. 5

| No. | Therapy | | Plasma TGF-$\beta_1$ | | | Therapeutic response |
|---|---|---|---|---|---|---|
| | Type | Time interval (weeks) | Initial (ng/ml) | Final (ng/ml) | Trend/% change | |
| 1. | TAM + RT[a] | 12 | 5.65 | 2.45 | ↓57% | Partial remission |
| 2. | CT[a] | 12 | 2.69 | 2.13 | ↓21%[b] | Minor clinical benefit |
| 3. | TAM + CT | 16 | 2.43 | 3.25 | ↑34%[b] | Progressive disease |
| 4. | letrozole | 4 | 3.17 | 3.03 | ↓ 4% | Minor clinical benefit; |
| | | 12 | 3.17 | 5.37 | ↑69% | progressive disease |
| 5. | TAM + bisphosp[c] | 12 | 1.83 | 1.23 | ↓33% | Minor clinical benefit; |
| | | 20 | 1.83 | 6.97 | ↑281% | complete remission + complications[d] |
| 6. | CT | 3 | 6.55 | 10.63 | ↑62% | Progressive disease; |
| | | 14 | 6.55 | 9.41 | ↑44% | terminal illness |

[a] TAM, tamoxifen; RT, radiotherapy; CT, chemotherapy.
[b] ↑, increase; ↓, decrease.
[c] bisphosp, bisphosphonates.
[d] Gastrointestinal symptoms unrelated to the disease or treatment.

metastatic BC patients. They differ, on the other hand, from the investigations of two research groups who have reported unchanged plasma TGF-$\beta_1$ levels in advanced BC patients both before [22] and after [29] therapy. This discrepancy could be explained, as reviewed by Grainger and colleagues [24], by differing assay specificities and sensitivities, differing plasma preparation protocols [7,22], and/or by differing patient populations [29].

Our pre-clinical subgroup analysis indicates that the plasma TGF-$\beta_1$ increase is associated with both parameters of BC aggression and with newly diagnosed patients with aggressive untreated tumours. In addition, our follow-up of 6 individual patients has indicated a relationship between the increased plasma TGF-$\beta_1$ and patient's response to therapy, as previously suggested in Refs. [19,26,30]. In the present study, a significant increase in plasma TGF-$\beta_1$ in postmenopausal BC patients versus HD was observed, this is inconsistent with previous observations [22,24]. However, this elevation might have been attributed to the high number of newly diagnosed metastatic patients in the postmenopausal subgroup. In contrast, there was no statistically significant difference in TGF-$\beta_1$ levels between patients with single/localised lesions and those with multiple metastatic spread, suggesting that plasma TGF-$\beta_1$ could not be a marker of tumour mass (defined, for an individual patient, as the sum of all malignant lesions regardless of the location). Likewise, an insignificant relationship for TGF-$\beta_1$ levels and metastatic location, histological type, SR content, age and premenopausal status (BC versus HD), was observed. Nevertheless, due to the small numbers involved in some of the above described analyses, further large scale TGF-$\beta_1$ studies are necessary.

In conclusion, our findings provide preliminary evidence for a significantly elevated plasma TGF-$\beta_1$ level in breast cancer patients with a poor prognosis. Although the complete mechanism of action and the role of TGF-$\beta_1$ in breast cancer still remains to be elucidated, our present results suggest that this biomarker should be further assessed as a promising plasma marker for breast cancer patients with a poor prognosis.

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