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Original article

# Clinical and laboratory patterns during immune stimulation in hormone responsive metastatic breast cancer

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ABSTRACT

This study clarifies the relationship between clinical and laboratory patterns, in endocrine-responsive metastatic breast cancer patients treated with a cyclic beta-interferon and interleukin-2 sequence added to anti-estrogens. In 31 patients, a regular laboratory and immunological assessment was made. During clinical benefit, as opposed to progression, a significant increase in the total number of lymphocytes, CD4+, CD8+, NK cells, CRP and IL-12 was confirmed. Also, a significant CEA, TPA, CA15.3 decrease occurred 24–72 h after interleukin-2 administration. At the progression, both basally and after interleukin-2 stimulation, the mean values of CD4+ plus CD25+ cells were more than twice higher than during clinical benefit, with a decrease of CD4+ plus CD8+ (Teffector)/CD4+ CD25+ (Treg) ratio. Moreover, a significant increase for CEA and for all 3 markers (standardized values) was found 24–72 h after interleukin-2 administration. In patients who survived less than 5 years, the Treg cell increase occurred at a significantly shorter time interval than in those who survived longer than 5 years (20 vs 45.5 months, respectively; $P = 0.001$). These data show laboratory evidence of the effect of immunotherapy as well as that of hormone resistance occuring concomitantly with a laboratory pattern compatible with immune inhibition.



## 1. Introduction

Previously, we reported on the prolonged clinical benefit and survival in endocrine-responsive breast cancer patients with diffuse metastatic disease treated with anti-estrogens plus a cyclic administration of beta-interferon and interleukin-2 [1]. In these patients during the period of clinical benefit, a significant increase in the total lymphocytes, CD4+, CD8+ and CD16+ plus 56+ (NK) cells was seen. We hypothesized that during the period of clinical benefit, resting cancer cells do not inhibit the immune system, while at the onset of resistance, the cells recover their constitutive ability to inhibit it [2]. In a further report, CRP, IL-6, IL-10, IL-12, TNF-alpha, TGF-beta 1 and IFN-gamma were examined. It was suggested that the activation of cellular immunity and a specific cytokine cascade was related to the prolongation of clinical benefit due to the anti-estrogen therapy. It was observed that significant IL-10 increase and TGF-beta 1 decrease were concomitant with no significant interleukin-2 stimulation of cellular immunity and a very different pattern of the evaluated cytokines occurs during clinical benefit and at progression [3,4].

In the present study, after a prolonged follow-up, we have updated laboratory data including CD4+ plus CD25+ T cells and tumor markers (CEA, TPA, CA15.3). The principal aim is to clarify the relationship between laboratory data and disease time course.

## 2. Materials and methods

### 2.1. Patients

One patient from the initial study group of 32 patients was excluded because her metastatic disease involving a single bony segment was not confirmed by the regular radiological follow-up [3]. Six other recruited patients were also excluded from this analysis because their follow-up was shorter than 5 years.

The 31 remaining consecutive metastatic breast cancer (MBC) patients, with either stable disease or a response to first line anti-estrogens, were aged 61 ± 12 years (mean ± sd; range 34–82). The last postoperative observation was October 31, 2010, and the mean follow-up was 149 ± 46 (range 73–243) months.

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All the 31 patients provided witnessed written informed consent before participation and the study was approved by the Council of the Department of Internal Medicine of Pisa University.

### 2.2. Therapy

All the 31 patients received tamoxifen 20 mg/day or toremifene 60 mg/day as first line endocrine-therapy. Two to seven months after the beginning the anti-estrogen (this was an induction time to ascertain stable disease or response to first line anti-estrogen), cyclic administration of beta-interferon and interleukin-2 was added [3].

During the induction time and successively, tamoxifen (1992–1999, 20 mg/day) or toremifene (1998–2002, 60 mg/day) was given continuously. After the induction time, beta-interferon 3,000,000 IU im/day was added three days per week for 1 month (weeks 1–4). In these 4 weeks, the daily dose of tamoxifen and toremifene was increased to 30 mg and 90 mg, respectively, because in vitro and in vivo studies [1] suggested an upregulation of estrogen receptors by tamoxifen in human breast cancer. Successively, recombinant interleukin-2 3,000,000 IU sc per day (three days a week) for 4 weeks was added (weeks 5–8). The patient continued anti-estrogen therapy only during weeks 9–12. The cycle lasted 12 weeks (3 months), and was then repeated, the 13th week being the first week of the successive cycle. At each cycle, five days before the recombinant interleukin-2 and during its administration, melatonin 40 mg/day bm, at 8.30–9 pm, was given because it enhanced clinical efficacy of recombinant interleukin-2, stimulated estrogen receptor expression on breast cancer cells and reduced cachexia in advanced cancer patients [1].

When progression occurred, tamoxifen or toremifene were replaced with letrozole 2.5 mg per day. Patients receiving letrozole after the progression of metastatic disease were assessed again for two or more months (induction time). With the exception of 3 patients, the same schedule of immune therapy was adopted in the patients either responsive to therapy or who had stable disease. In 1 of the 3 remaining patients, letrozole was not yet available when resistance to tamoxifen developed, one patient refused any further oral treatment, one patient at the last observation was still responsive to first line salvage treatment with tamoxifen. Immunotherapy during the first and second line hormone therapy was self-administered in an outpatient setting. Patients progressing on letrozole received the standard chemotherapy, of antracyclines, taxanes, vinorelbine and/or 5-FU as the first, second and third line regimen, respectively [1,3].

### 2.3. Clinical benefit and disease progression

Performance status was assessed by the ECOG scale. An initial treatment assessment was carried out at the end of the induction time, i.e. after 2 to 7 months from the first line hormone salvage therapy with anti-estrogens. Evaluation of complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD) was performed using modified World Health Organization criteria [4].

Before starting immunotherapy, all patients underwent a complete work-up to document the extent of disease [3].

### 2.4. Laboratory and immunologic assessment

At each control visit, routine laboratory examinations and serum measurement of a CEA-TPA-CA15.3 tumor marker (TM) panel was carried out, and an immunological assessment was made. The evaluated immunologic parameters included: the total number of lymphocytes, CD4+, CD8+, CD16+ plus 56+ (NK) cells and C-reactive protein (CRP). Additionally, since January 2000, CD4+ plus 25+ cells and the following cytokines were measured: interleukin 6 (IL-6), interleukin 10 (IL-10), interleukin 12 (IL-12), tumour necrosis factor α (TNFα), transforming factor β1 (TGFβ1) and interferon γ (IFNγ). During any four-weeks cycle, all of these parameters were measured in the peripheral blood before the first interleukin-2 administration and 24 to 72 h after the last interleukin-2 administration. CRP was evaluated by immunephelometry while all the cytokines were assayed by a quantitative immunosorbent assay. Cut-off values were: $< 0.5$ mg/dL, $< 8.5$ pg/mL, $< 8.9$ pg/mL, $< 14$ pg/mL, $< 44.3$ ng/mL, $< 2.8$ U/mL for CRP, IL-6, IL-10, TNFα, TGFβ1 and IFNγ, respectively; within and between assay coefficient of variation was 2.8%, 4.2%, 4.8%, 6.5%, 4.7%, 5.5% and 3.4%, 8.2%, 10.6%, 12.6%, 10.8% and 11.6%, respectively. For IL-12, from January 2000 to November 18, 2002, the cut-off value was $< 80$ pg/mL. After November 18, 2002, the cut-off value was $< 590$ pg/mL. The within and between assay coefficient of variation was 5.2% and 11% when the cut-off was $< 80$ pg/mL, compared to 5.5% and 11.5% when the cut-off was $< 590$ pg/mL. The following commercial kits were used: CardioPhase hsCRP (Dade Behring, Marburg) for CRP, Technogenetics s.r.l. (Wien) for IL-6, IL-10, TNFα and IFNγ and Biosurce Europe s.a. (Belgium) for TGFβ1 and IL-12 when the cut-off value was $< 80$ pg/mL. When the IL-12 cut-off value was $< 590$ pg/mL, the commercial kit Technogenetics s.r.l. (Wien), was used. For IL-12, only the coupled values obtained with the same assay were considered. Immunological assessment was performed continually during the study.

In current scientific literature Treg cells are defined as CD4+ plus CD25+ cells. Very recently, an improved definition by additional marker (CD127) of T regulatory cells has been reported. Yet, in this study, most data on Treg cells preceded this advance, that in our country, it is available in lab from 2009–2010. Nevertheless, recent studies have shown that in human tissues, transcription factor, Foxp3, serves as a lineage specification factor of Treg cells [5]. Moreover, "the majority of Foxp3+ cells were found within CD4+ T cell subsets and they expressed high amount of CD25" [6]. So, a good correlation has been shown between CD4+ plus CD25+ and Foxp3+ T cells.

From the beginning of 2001, serum CEA, TPA and CA15.3 values were measured soon before (basal value), 24–72 h after the last interleukin-2 administration and 4 weeks after the interruption of interleukin-2.

Serum CEA, TPA and CA15.3 concentrations were measured in fasting patients by commercial immuno-enzymatic assays: Abbott, Rome (Italy) for CEA and CA15.3; DRG, Marburg (Germany) for TPA. The within and between assay coefficients of variation for CEA, CA15.3 and TPA were all less than 5% and 6%, respectively. Serum levels $> 7$ ng/mL, $> 95$ U/L and $> 32$ U/mL were considered to be elevated for CEA, TPA and CA15.3, respectively.

### 2.5. Intensive follow-up

All patients were monitored by serial control visits every 3 months. At each control visit, all the patients underwent an accurate anamnesis, physical and routine laboratory examinations and a serum measurement of the CEA-TPA-CA15.3 tumor marker (TM) panel. When a patient was suspected of progression by clinical signs, TM panel and/or conventional instrumental exams (bone scintigraphy, liver echography and chest X-ray), more accurate, i.e. "gold standard" instrumental examinations [computed tomography (CT), magnetic resonance imaging (MRI) and whole PET] and, when necessary, cyto-histology was immediately performed [3,7]. Otherwise, for all patients, all conventional and "gold standard" instrumental exams were carried out at the time of study entry and successively approximately every 12 months. Among the more accurate and expensive instrumental examinations, CT aimed at the distant