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Mini review
Cytokines in breast cancer
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Abstract
In recent decades many advances have occurred in the understanding of the role of cytokines in breast cancer. New signalling pathways of interleukin (IL)-1 family, IL-6, IL-11, IL-18, interferons (IFNs) and interferon regulatory factors 1 (IRF-1) and 2 (IRF-2) have been found within tumour microenvironments and in metastatic sites. Some cytokines (IL-1, IL-6, IL-11, TGFβ) stimulate while others (IL-12, IL-18, IFNs) inhibit breast cancer proliferation and/or invasion. Similarly, high circulating levels of some cytokines seem to be favourable (soluble IL-2R) while others are unfavourable (IL-1β, IL-6, IL-8, IL-10, IL-18, gp130) prognostic indicators. So far IL-2, IFNα, IFNβ and occasionally IFNγ, IL-6, IL-12 have been the cytokines used for anti tumour treatment of advanced breast cancer either to induce or increase hormone sensitivity and/or to stimulate cellular immunity. Disappointing results occurred in most trials; however, two long-term pilot studies suggest that IL-2 and IFNβ, when used appropriately can have a positive effect on clinical benefit and overall survival of patients with minimal residual disease after chemotherapy or with disseminated disease controlled by conventional endocrine therapy.

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Keywords: Breast cancer; Cytokines; Prognosis; Treatment

1. Introduction
Cytokines are glycoproteins of low molecular weight, which are rapidly synthesized and usually secreted by different healthy and diseased cells (mainly mononuclear phagocytes and activated T lymphocytes) mainly after stimulation. They act on many different adjacent target cells (pleiotropism) often in an additive, synergistic, or antagonistic manner. In multicellular organisms, cytokines are intercellular mediators that regulate survival, growth, differentiation, and the effector functions of cells [1]. Therefore, it is not surprising that cytokines significantly affect the growth of tumors in vivo. On the other hand, they are also produced by cancer cells and represent a network with a large variety of molecularly and functionally different members that may act as tumor growth-promoting or inhibiting factors. As they affect the growth and function of immunocompetent cells, they can activate or modulate specific or non-specific antitumor responses. Furthermore, because cytokines are mediators of the effector response from innate and acquired cellular immunities [2], they are probably involved in the mechanism from tumour cell evasion of the immunosurveillance system.

This review summarizes principal recent findings on the relationship between cytokines and breast cancer and their role in effecting patient prognosis. Additionally, this review deeply explores the body of knowledge acquired in the last decade for therapeutic antitumoral purpose in advanced breast cancer.

2. Biological studies
2.1. Interleukins
2.1.1. Interactions with breast cancer cells
The interleukin (IL) -1 family of cytokines (IL-1α, IL-1β), the IL-1 receptor antagonist (IL-1Ra) and receptors (IL-1RI and IL-1RII) have been found to be frequently expressed
in breast cancer cell lines, in human breast cancer tissue, and within the tumour microenvironment [3–5]. This local expression of IL-1/IL-1R cytokine family can control, via autocrine and/or paracrine mechanisms, the tumour cell subpopulation expression of other protumorigenic cytokines, such as the expression of IL-8, and subsequently contribute to protumorigenic activities, i.e. angiogenesis, tumour proliferation, and local tumour invasion [4,5]. It has been reported that IL-1β signalling pathway may be different in ER positive MCF-7 versus ER negative MDA-MB231 breast carcinoma cells [6].

IL-6 has been found in high concentrations in human breast cancer cell lines and in breast tumour samples [7–9]. Fibroblasts, macrophages and lymphocytes (mainly Th2 cells) are thought to be an important source of IL-6. This cytokine exerts its effects through glycoprotein (gp) 130-mediated activation of signalling pathways (including the JAK/STAT and MAP kinase pathways) resulting in the transcriptional regulation of genes involved in cell proliferation, survival and differentiation. IL-6 promotes tumour growth by upregulating anti-apoptotic and angiogenic proteins in tumour cells [1,10–12]. Increased IL-6 production also increases estradiol-17β-HSD) type I which converts estrone (E1) to the biologically active estrogen, estradiol (E2) [13]. Therefore, it has been hypothesized that IL-6 and IL-1β stimulate proliferation of breast cancer cells through estrogen production by activating steroid-catalyzing enzymes in the tissue [7]. IL-6 may favour proliferation and metastasis of cancer cells, development of osteolysis and humoral hypercalcemia, and it has also been suggested to be a cachectic factor in cancer patients [8].

Bone metastases from breast cancer induce osteoclast formation by stimulating osteoblastic production of IL-11 with the subsequent release of prostaglandin E2 and inhibition of GM-CSF production by cells within the bone microenvironment [14]. This mechanism seems to importantly contribute to breast cancer cell induced osteoclast formation and their resorptive activity.

IL-12 is a heterodimeric molecule composed of an α chain (p 35 subunit) and a β chain (p 40 subunit) linked to form the biologically active 74 kDa heterodimer [15]. The principal sources of IL-12 are macrophages, dendritic cells, monocytes, neutrophils, and to a lesser extent, B cells. Concomitant gene expression for IL-12 and interferon γ was demonstrated by reverse transcriptase-polymerase chain reaction in all 10 cases of infiltrating ductal carcinoma [16].

IL-18 injected intraperitoneally, not only inhibited osteolytic growth at bone metastatic sites of human breast cancer cells, MDA-231 cells, in nude mice, but also suppressed an early stage of bone metastasis. However, no significant effect on proliferation of subcutaneously injected cancer cells was found [17].

2.1.2. Prognostic role

In human breast cancer cells, IL-10 protein expression correlated with both poor differentiation and decreasing epithelial ERα expression [3,5]. Elevated serum levels of IL-1β correlated with a high rate of recurrence in patients with breast cancer [18].

An association of prognostically favourable factors with higher serum values of soluble IL-2 receptors has been reported [19].

In serum of breast cancer patients, IL-6 correlated directly, although not always [10], with clinical stage [20,21] and the rate of recurrence [18]. In a few studies on metastatic breast cancer, multivariate analysis identified high serum IL-6 levels as an independent adverse prognostic variable for progression free and overall survival [22–25]. Accordingly, the finding of low serum IL-6 levels prior to and the maintenance of relatively low IL-6 levels after 4 weeks of medroxyprogesterone acetate treatment, was beneficial in 65 patients with advanced or recurrent breast cancer [26].

Controversial findings have been reported with regard to the relationship between IL-6 expression [27,28] or the polymorphism at nucleotide-174 within the promoter region of IL-6 gene [29,30] and clinical outcome.

Serum IL-8 levels were found higher in 69 patients with either operable or advanced breast cancer as compared to healthy women and they correlated directly with clinical stage of breast cancer. Elevated serum IL-8 levels were also associated with the occult cytotkeratin-positive bone marrow cells [31], thus suggesting a correlation with poor prognosis [20,31]. Another study with 43 breast cancer patients suggested that tissue and plasma β chemokine RANTES (a member of the same family as IL-8) plays a role in carcinogenesis and that a RANTES assay in tissue surrounding a tumour or in the tumour area after excision, may help to predict unfavourable prognosis in breast cancer patients [32].

IL-10 levels in blood samples of breast cancer patients were [20] or were not [32] higher than controls and correlated directly with clinical stage of the disease [20].

Serum IL-18 levels were higher in breast cancer patients than in control subjects, higher in advanced than in early stages of the disease, and higher in metastatic than in non-metastatic patients [33,34].

2.1.3. Effects on cellular immunity

This section reports the effects on cellular immunity of the main interleukins used in clinical trials. IL-2 was purified from mitogen-stimulated lymphocyte cultures and, in vivo, is produced exclusively by activated T cells [35,36]. IL-2 induces the proliferation of activated T cells and the differentiation of cytotoxic T lymphocytes (CTL); it also has effects on other immune cells including natural killer (NK) cells, B cells, monocyte/macrophages, and neutrophils [35–37]. Three different IL-2 receptor (R) complexes exist; they include three receptor subunits located on T and NK cells. The isolated IL-2Rα binds IL-2 with low-affinity (Kd ~ 10^{-8} M) without transducing a signal; the heterodimeric IL-2Rβγ binds IL-2 with intermediate affinity (Kd ~ 10^{-9} M) and transduces intracellular signals while the heterotrimeric IL-2Rα β γ binds IL-2 with high affinity (Kd ~ 10^{-11} M)
and also signals. The IL-2Rγ also is referred to as the common γ chain (γc). The intermediate affinity receptor IL-2Rβγc is expressed in most (~90%) human NK cells that are CD56dim NK cells. CD56dim NK cells have low surface density expression of CD56 and high expression of CD16 and NK receptors (NKR)s. NKR,s recognise MHC class I ligands and regulate (inhibit or activate) the functional response to target cells. CD56dim NK cells are potent mediators of antibody-dependent cellular cytotoxicity (ADCC), lymphokine-activated killing (LAK) activity and natural cytotoxicity. They do not express L-selectin and produce a low amount of cytokines (IFNγ, TNFα, TNFβ3, IL-10) in response to monokine stimulation. CD56bright NK cells are ~10% of human NK cells and express the high affinity IL-2Rαβγc. CD56bright NK cells have high-density expression of CD56, low-density expression of CD16 and NKR,s. They are poorly cytotoxic but show potent LAK activity following activation with IL-2; they highly express the adhesion molecule L-selectin and through cytokine receptors produce elevated levels of cytokine in response to monokine stimulation [37]. In cancer patients, CD56bright, unlike CD56dim, NK cells proliferate in response to subcutaneously administered low or ultralow (~1 million IU/m², pM serum concentration) doses of IL-2 that selectively saturate and signal through IL2Rαβγc. Due to the constitutively high expression of L-selectin, the expansion of CD56bright NK cells may result in an increased number of NK cells capable of trafficking to secondary lymphoid organs where they could interact with other immune cells as T cells and antigen-presenting cells [37]. Higher doses (720,000 IU/kg/q, nM serum concentration) of IL-2, administered by continuous infusion or bolus, further stimulated NK cells, activated T cells, monocyte/macrophages, and activated B cells, which express the intermediate-affinity IL-2Rβγc. These high IL-2 doses increase the toxicity by CD56bright and CD56dim NK cells as well as their LAK activity and ADCC, so that severe side effects can occur (arterial hypotension, capillary leak syndrome). In an effort to provide less toxic regimens, a different schedule of IL-2 using an intermediate dose (72,000 IU/kg/q) was drawn. However, this different schedule still resulted in relatively high (nM) serum concentrations and was toxic [37].

Regarding IL-6 antitumour activity, induction of T cell and B cell differentiation, stimulation of cytotoxic T cells and help in producing lymphokine-activated killer cells have been reported. Also, through the increased synthesis of C-reactive protein (CRP) IL-6 indirectly influenced the binding of this protein to phospholipids on tumour cells, activating C1q of the complement system, which may lead to tumour cell lysis [11,12].

The above mentioned study [16] indicated that breast cancer induces a local IL-12-dependent type I immune response likely directed towards tumour-associated antigens. The major actions of IL-12 are on T and NK cells that primarily express IL-12 receptors (IL-12Rβ1 and IL-12Rβ2) [38]. IL-12 is the main cytokine that regulates differentiation of CD4+ T cells to the Th1 phenotype that produces IFNγ and promotes cell-mediated immunity. Furthermore, IL-12, by inducing the Th1 response, also augments the production of antibody classes able to activate the complement cascade and to opsonise tumour cells exposing them to the cytotoxic activity of phagocytic and NK cells.

IL-12 induces proliferation and increases cytotoxic activity of CTLs and NK cells. Moreover, in these cells through IL-12Rβ1 and IL-12Rβ2, it activates the JAK/STAT pathway and induces high production of IFNγ which, in turn, stimulates further IL-12 production by immunocompetent cells. IFNγ, TNF, and a cascade of secondary and tertiary proinflammatory cytokines induced by IL-12 also stimulated the production of CXCR3 ligands, which are powerful inhibitors of tumour angiogenesis [15,38]. Synergistic actions of IL-12 with IL-2 have been shown. Low (pM) IL-2 doses can synergise with IL-12 for more efficient CD56bright NK cell IFNγ production [37] and IL-12/IL-2 combination synergistically enhanced Fas and FasL expression within tumors via an IFNγ-dependent mechanism in vivo. This combination also inhibited tumour neovascularisation and induced rapid destruction of tumour-associated endothelial cells and tumour regression [39].

2.2. Interferons

2.2.1. Interactions with breast cancer cells

α and β interferons are type I IFN proteins with antitumor activity [2]. They downregulate oncogene expression and induce tumour suppressor genes which result in antiproliferative activity. Antiproliferative and antiadhesive actions of IFNα have been shown in MCF-7 breast carcinoma cells [40]. The antiproliferative effect of IFNα2a and 2b on the growth of ZR-75-1 human breast cancer cells was synergistic with that of the antiestrogen, toremifene [41]. In human breast cancer, IFNα2a, combined with all-transretinoic acid (AT), did not potentiate the growth inhibition of AT [42]. With regard to hormone receptor modulation by IFNα, conflicting results have been reported [43–45].

Several genes associated with retinoid-IFN-induced mortality (GRIM) have been recently identified. GRIM-12 expression was induced by IFNβ/tamoxifen association at a post-transcriptional stage [46] and overexpression of GRIM-12 increased IFN/tamoxifen induced apoptosis. In two human breast cancer cell lines (the ER-positive MCF-7 and ER-negative MDA-MB-231 cells), the effects of huanglian extract (a widely used herb in traditional Chinese medicine with anticancer activities) on the expression of the common genes involved in carcinogenesis were examined. In MCF-7 cells, the huanglian extract provoked a dramatic increase in mRNA expression of IFNβ and TNFα [47]. Estrogen and progesterone receptor enhancement has been observed in vitro and in patients affected by advanced breast cancer treated with relatively low doses of IFNβ [43].

IFNγ, also called immune IFN or type II IFN, is mainly produced by CD4+ Th1, CD8+, and NK cells [2]. In
cancer xenografts, the antiproliferative action of IFNγ, probably due to enhanced cell death by up-regulation of some caspases [48–50] and an antiangiogenic activity, have been found [51].

In particular, many studies have been conducted on interferon regulatory factors 1 (IRF-1) and 2 (IRF-2) that are transcription factors in the interferon γ signal transduction pathway. IRF-1 acts as the effector arm of the interferon γ response with tumour suppressor activity. IRF-2 binds to the same DNA consensus sequence and opposes IRF-1 activity with oncogenic effects. High grade ductal carcinoma in situ (DCIS) or node-positive invasive ductal cancers were found less likely to express the tumour suppressor IRF-1 and much more likely the oncogenic IRF-2 protein than normal tissue [52]. In another study [48] on 187 specimens of clinically defined invasive breast carcinoma, a significant positive correlation between IRF-1 and IRF-2 expression and a negative correlation between IRF-1 expression and tumour grade were found. It has been hypothesised that IRF-1 acts as a tumour suppressor gene and induces apoptosis [48–50]. Loss of IRF-1 regulation also appears related to antiestrogen resistance. Antiestrogens induce both cytostasis (cell cycle arrest) and apoptosis. In some breast cancer cell lines, the lack of IRF-1 inhibited the ICI 182,780 (fulvestrant) induced arrest and strongly stimulated the cell cycle inhibitor p21 expression and promoter activity in both human estrogen responsive and estrogen independent breast cancer cell lines [54]. These studies showed that DIM can up regulate the expression and stimulate the secretion of IFN-γ in the human MCF-7 breast cancer cell line [54,55]. In a further report indole-3-carbinol (I3C), another naturally occurring compound of brassica vegetables with anticancer properties, has been investigated in MCF-7 human breast cancer cells [56]. I3C mediated its anticancer effects also by stimulating transcription of the IFNγRI gene and increasing the IFNγ response [56].

In human MCF-7 breast cancer cells, IFNγ and AT acted synergistically [57,58] to induce expression of FasR mRNA and FasR protein which promote tumour cellular apoptosis. IFNγ signalling through IFNγ receptor also activates signal transducer and activator of transcription-1 (STAT1) protein which belongs to the STAT family of transcription factors. STAT1 and STAT3 members modulate IL-6 signalling pathway and activate acute phase protein genes and a variety of other genes [1]. In another study [59] on MCF-7 human breast cancer cells, IFNγ induced the overexpression of retinoic acid inducible gene-1 (RIG-I) which, in turn, up-regulated the IFNγ stimulated gene 15, able to amplify the immunomodulatory effects of IFNγ. The genotype analysis of breast cancers showed that about 40% of the samples examined [60] expressed the “interferon-related” signature providing molecular support for a role for either inflammation or viral infection in the pathogenesis of breast cancer. In other reports [61,62], IFNγ polymorphism has been found in association with an increased risk of sporadic breast cancer development, probably related to a resultant compromised immune surveillance.

No influence of hormone receptor status on the antiproliferative effects of IFNγ in breast carcinoma cells in vitro occurred [63] A synergistic inhibitory effect of IFNγ and α was obtained in human breast cancer xenografts; moreover, lesser responses occurred if they were given systemically rather than directly into the tumour [64,65]. Finally, in human tumour models, IFNs increased the apoptotic effect of 5-fluorouracil [66,67], and the cyclophosphamide, paclitaxel and doxorubicin cytotoxicity [67,68].

2.2.2. Prognostic role

In two studies on breast cancer patients, plasma IFNγ [32] and IFNγ genotype [28] were not correlated with disease course. In another study on 87 metastatic breast cancer patients, decreased survival was significantly associated with IFNγ gene polymorphism (C-A repeats within the first intron) and a higher IFNγ transcription [69].

2.2.3. Effects on cellular immunity

Alpha and β IFNs increase the expression of MHC class I molecules in tumour cells which can enhance immune recognition [66].

More recently, it has been shown that type I IFNs have further important immunological effects. They promote human Th1 type of immune response, stimulate proliferation and prolong survival of human cytotoxic lymphocytes, induce maturation, and improve function of dendritic cells [66]. Moreover, in clinical trials on melanoma and renal cell carcinoma, IFNs have been shown to be able to increase NK activity and T helper lymphocytes, as well as in vitro T-cell responses and tumour infiltrating lymphocytes [66]. Furthermore, IFNs have shown, in vitro and in vivo, anti-angiogenic activity [51,70]. Other studies in breast cancer patients reported that also low or intermediate doses of IFNβ provoked an enhancement of NK cell cytotoxicity [71,72]. The capability to type I IFNs to promote the rapid differentiation of human monocytes into mature dendritic cells has become of particular interest after the identification of “natural IFN-producing cells” as CD4+CD11c+ type II dendritic cells precursors also defined as plasmacytoid dendritic cells [66].

In breast cancer patients, the inhibitory action of adjuvant CMF chemotherapy on NK cells was antagonised by the addition of low IFNβ dose [71].

Further actions of IFNγ are the following: (a) increased expression of MHC class I and class II molecules; (b) activation of monocytes and macrophages able to induce a cytokine cascade (IL-1β, IL-6, TNFα and IL-8) and a successive complete activation of various subsets of T or B cells; (c) differentiation of CD4+ to Th1 cells and inhibition of Th2 proliferation [2,73,74].
2.3. Other cytokines and receptors (TGFβ, TNFα, gp130)

Mechanisms by which TGFβ arrests cell cycle in G1 phase in the nontransformed epithelial cells and loses this inhibiting property in human breast cancer have been identified and reviewed [75]. Fibroblasts provide structural and biochemical support for breast cancer (i.e. desmoplastic reaction); this effect did not occur following administration of antibodies against TNFα (or IL-11) [76].

Gene expression of TGFβ1 and TNFα was found in nine, and in a single instance, of 10 cases of infiltrating ductal carcinoma, respectively [16]. Additionally, no differences were found in TNFα-alleles and genotype frequencies between breast cancer patients and control subjects [62].

Constitutive activation of the STAT3 gene in human breast cancer cells inhibited tumour secretion of pro-inflammatory cytokines and produced soluble factors accounting for inhibited innate and acquired cellular immunity [77]. Interestingly, inhibition of cytokine receptor gp130 signalling in breast cancer blocked constitutive activation of STAT3 and inhibited, in vivo, malignancy growth. Besides, dominant negative gp130 protein MDA-231 breast cancer cells had markedly decreased engraftment, size, and number of metastases compared with control cells [78].

In a study [28], TGFβ1 low production genotypes (TGFβ1-10 CC) were associated with an increased risk of disease relapse. gp130 is one of two functional receptor subunits of IL-6. Similar to IL-6, expression of gp130 correlated with good prognosis for patients with breast cancer [27]. Conversely, serum gp130 levels were found to increase significantly at each progressive tumour stage without or after chemo/radiotherapy [10].

Table 1 summarizes the principal positive or negative biological actions on tumour growth and useful prognostic information of cytokines in breast cancer. Studies with controversial results of the same cytokines are also reported.

3. Clinical trials in advanced breast cancer patients

Many cytokines have shown a potent therapeutic antitumour effect in preclinical models. However, translation to clinical practice is limited, and at present, IFNα and IL-2 are the only cytokines approved for oncologic indications [79].

Table 2 shows the rationale and the therapeutic schedule of the cytokines that have been used for antitumour treatment of advanced breast cancer.

3.1. IL-2 given as single agent or with other cytokines and/or other drugs

The main characteristics of the clinical trials with IL-2 given alone or with other cytokines and/or other drugs in the treatment of advanced breast cancer are shown in Table 3a. Five [80–83,86] out of the seven trials with IL-2 alone [80–84] or IL-2 plus melatonin [85,86] were pilot studies. Three [81,83,86] of the five pilot studies, one phase I and one phase II trials [84,85] enrolled different cancer histotypes, including few patients (from four to eight) with often heavily pretreated breast cancer. In these five studies, the small sample size did not permit any reliable consideration. In the two remaining pilot studies [80,82] enrolling a slightly larger number of advanced breast cancer patients, IL-2 treatment was considered ineffective.

Subcutaneous IL-2 and IFNα were consecutively [87] or simultaneously [88] given at a low dose in another pilot study and in one phase II non-randomised trial including a higher number of advanced breast cancer patients after
failure of previous conventional treatment. In the pilot study, high-risk breast cancer patients were successfully treated with high-dose chemotherapy and a IL-2-IFNα combination after transplantation of autologous hematopoietic stem cells activated by IL-2. The main aim was attained in 43% of the evaluable patients who developed autologous graft-versus-host disease (GVHD) in the hope of a graft-versus-tumour (GVT) effect contributing to a lower relapse rate. The results demonstrated the feasibility and the moderate toxicity of this regimen. The mean follow-up was short (13 months) and the

Table 2
Cytokines used for antitumoral treatment of advanced breast cancer patients: rationale and therapeutic schedule

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Rationale</th>
<th>Common schedules of therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>Stimulation of cellular immunity</td>
<td>3–5 days i.v. bolus 720,000 IU/kg (high dose; nM s.c.) ~1 million IU/m² per day for ~8 weeks subcutaneously (low/ultralow dose; PM s.c.)</td>
</tr>
<tr>
<td>Type I IFN (α, β)</td>
<td>Tumour growth inhibition; stimulation of cellular immunity; modulation of hormone receptors; enhanced cellular immune response to cytotoxic agents</td>
<td>1–10 MIU/m² every day or three-times a week for 2–4 weeks subcutaneously or i.m. or i.l. administration</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Tumour growth inhibition; stimulation of cellular immunity</td>
<td>1 × 10⁶ IU i.l. injections or 25 mg subcutaneously, days 1–5 of 4 weeks cycles</td>
</tr>
<tr>
<td>IL-6</td>
<td>Stimulation of cellular immunity</td>
<td>1–10 (25–30 MTD) µg/kg/day subcutaneously; 10–100 µg/kg/day c.i.</td>
</tr>
<tr>
<td>IL-12</td>
<td>Stimulation of cellular immunity</td>
<td>250–500 ng/kg/day i.v. bolus twice weekly or for 5 days</td>
</tr>
</tbody>
</table>

For details see text; abbreviations: s.c. = serum concentration; i.l. = intralocular.

Table 3a
Main clinical trials with interleukin-2 (IL-2) given as single agent or with other cytokines and/or other drugs as antitumoral treatment of advanced breast cancer

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Ref.</th>
<th>Patients enrolled (n)</th>
<th>Trial Type</th>
<th>Follow-up (months)</th>
<th>Disease stage</th>
<th>Immunological effects</th>
<th>Clinical outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>[80]</td>
<td>10 Pilot n.r.</td>
<td>Advanced</td>
<td>Yes</td>
<td>Ineffective (MR 2, SD 4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>[81]</td>
<td>4 Pilot n.r.</td>
<td>Advanced</td>
<td>Yes</td>
<td>Ineffective (PD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>[82]</td>
<td>21 Pilot 71 (Median)</td>
<td>Advanced n.r.</td>
<td>Yes</td>
<td>Ineffective (2 years PFS and OS 19% and 33%, respectively)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>[83]</td>
<td>2 Pilot 8+ (Median)</td>
<td>Metastatic</td>
<td>Yes</td>
<td>SD 1 (14 months)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>[84]</td>
<td>8 Phase I n.r.</td>
<td>Metastatic</td>
<td>Yes</td>
<td>Ineffective (two incomplete local tumour regressions)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2 vs. IL-2 and MLT</td>
<td>[85]</td>
<td>7 Phase II randomised 18 (Median)</td>
<td>Advanced</td>
<td>Yes</td>
<td>ORR 0% vs. 33%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2 and MLT</td>
<td>[86]</td>
<td>6 Pilot n.r.</td>
<td>Advanced</td>
<td>Yes</td>
<td>ORR 17%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2 and IFNα</td>
<td>[87]</td>
<td>34 Pilot 13 (Mean)</td>
<td>Locally advanced</td>
<td>Yes</td>
<td>2 year DFS and OS 68% and 75%, respectively</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>[88]</td>
<td>40 Phase II non-randomised n.r.</td>
<td>Advanced n.r.</td>
<td>Yes</td>
<td>Ineffective (ORR 3%; median OS 8.9 months; 8% C.B. ≥24 months)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2 and trastuzumab</td>
<td>[89]</td>
<td>53 Phase I n.r.</td>
<td>Metastatic</td>
<td>Yes</td>
<td>ORR 17%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2 and G-CSF</td>
<td>[90]</td>
<td>43 Phase I n.r.</td>
<td>Advanced</td>
<td>Yes</td>
<td>n.r.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2 and epirubicin vs. epirubicin</td>
<td>[91]</td>
<td>100 Phase III randomised n.r.</td>
<td>Metastatic n.r.</td>
<td>Yes</td>
<td>ORR 64% vs. 58% and TTP ≥12 months 33% vs. 12%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2, IFNβ, MLT and TAM or tamoxifen</td>
<td>[92]</td>
<td>29 Pilot 59 (Mean)</td>
<td>Metastatic</td>
<td>Yes</td>
<td>Median C.B. 38 months; median OS 103 months; 85% C.B. ≥24 months</td>
<td></td>
<td></td>
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</tbody>
</table>

For details see text. Ref. = reference; n.r. = not reported; MLT = melatonin; IFN = interferon; G-CSF = granulocyte colony stimulating factor; TAM = tamoxifen; MR = minor response; SD = stable disease; PD = progressive disease; C.B. = clinical benefit = CR + PR + SD; ORR = overall response rate; TTP = time to progression; DFS = disease free survival; PFS = progression free survival; OS = overall survival; **with HER-2 (2 pts); HER-2** (6 pts) or HER-2*** (25 pts) tumours; RP = retinyl palmiatate; ** IFNβ 2 MIU (group A) vs. 6 MIU (group B) i.m. three-times a week for 2 weeks; ***arm a: MEGACE 160 mg daily; arm b: IFNβ 0.25 MIU + IFNY25 mg s.c. + MEGACE (as in arm a); arm c: IFNβ 1 MIU/m² + RP 100,000 IU/day b.m. plus TAM 30 mg/day b.m.; **** with HER-2** (7) or HER-2*** (5) tumours.
observed overall and disease-free survival rates were comparable to those reported in literature [87]. In the phase II non-randomised trial, 40 advanced breast cancer patients were recruited between May 1991 and March 1995 by 25 institutions throughout the States and the results were published in 2004. Immunological effects were not reported and the IL-2 IFNα combination was defined as ineffective [88].

IL-2 plus trastuzumab (Herceptin), a humanised anti-HER-2 mAb, was evaluated in a phase I trial that enrolled patients with different cancer histotypes. Most of them (33 out of 45) had metastatic breast cancer that overexpressed HER-2. All enrolled patients were not suitable for or previously failed effective standard therapy. Clinical benefit (2 CR, 2 PR and 9 SD) occurred in 56% of the evaluated patients; no correlation was found between immunological effects and clinical response; response duration reported for the only two complete responders was 4 and 33 months, respectively. Therefore, IL-2 did not increase the activity of trastuzumab [89]. In 43 advanced breast cancer patients, IL-2 and granulocyte-colony stimulating factor (G-CSF) were subcutaneously administered in combination to develop a GVHD and GVT effect [90]; no clinical antitumor outcome was reported. In a randomised study, 100 hormone refractory patients received IL-2 and epirubicin or epirubicin alone [91]. Encouraging results were obtained in favour of the IL-2-epirubicin arm; however, no immunological effect was described and the short follow-up, with no data on survival, rendered the findings inconclusive.

Table 3b
Main clinical trials with IFNα, IFNβ, IL-6, IL-12 given as single agent or with other cytokines (except IL-2) and/or other drugs as antitumoral treatment of advanced breast cancer

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Ref.</th>
<th>Patients enrolled (n)</th>
<th>Trial Type</th>
<th>Disease stage</th>
<th>Follow-up (months)</th>
<th>Immunological effects</th>
<th>Clinical outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNα</td>
<td>[44]</td>
<td>20 Pilot n.r.</td>
<td>Advanced n.r.</td>
<td>ORR 10%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNα and TAM</td>
<td>[94]</td>
<td>13 Pilot n.r.</td>
<td>Advanced n.r.</td>
<td>ORR 15.4%; median PFS 4 months (0–26 range)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>[95]</td>
<td>7 Pilot n.r.</td>
<td>Advanced n.r.</td>
<td>ORR 57% (duration of response 1–8 months)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNα, IFNγ alone</td>
<td>[97]</td>
<td>11 Pilot n.r.</td>
<td>Metastatic n.r.</td>
<td>(non-effective in overcoming TAM resistance) ORR 50%</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>or combined</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNβ</td>
<td>[98]</td>
<td>45 Pilot n.r.</td>
<td>Metastatic n.r.</td>
<td><strong>CR in 53% of cutaneous recurrences</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNβ and TAM</td>
<td>[99]</td>
<td>43 Pilot n.r.</td>
<td>Advanced n.r.</td>
<td>ORR 26% (median duration 6 months)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>[100]</td>
<td>30 Pilot n.r.</td>
<td>Advanced n.r.</td>
<td>ORR 13% (median duration 8 months)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>[101]</td>
<td>33 Pilot n.r.</td>
<td>Metastatic n.r.</td>
<td>ORR 27% (median duration 7 months)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNβ, RP and TAM</td>
<td>[43]</td>
<td>36 Phase II non-randomised</td>
<td>Metastatic n.r.</td>
<td>Median OS 32 months</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>or MAP</td>
<td>[102]</td>
<td>85 Phase II non-randomised</td>
<td>(Median)</td>
<td>ORR 59%; median OS 28 months (from trial entry) and 38 months (from first metastasis)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNβ, IFNγ and TAM</td>
<td>[103]</td>
<td>19 Phase II randomised</td>
<td>Metastatic Yes</td>
<td>Median OS 36 months; ORR 39% (TTP 18 months, median OS 39 months)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNβ, IFNγ, RP</td>
<td>[104]</td>
<td>60 Phase I randomised</td>
<td>Metastatic Yes</td>
<td>***Arm a: ORR 22%; arm b: ORR 30%; arm c: see Ref. [103]</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>and TAM or MAP</td>
<td></td>
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<tr>
<td>IL-6</td>
<td>[105]</td>
<td>1 Phase I n.r.</td>
<td>Advanced Yes</td>
<td>No antitumor response</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>IL-12 and</td>
<td>[106]</td>
<td>****12 Phase I n.r.</td>
<td>Metastatic Yes</td>
<td>Ineffective; ORR 8%</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>trastuzumab</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For details see text. Ref. = reference; n.r. = not reported; MLT = melatonin; IFN = interferon; G-CSF = granulocyte colony stimulating factor; TAM = tamoxifen; MR = minor response; SD = stable disease; PD = progressive disease; C.B. = clinical benefit = CR + PR + SD; ORR = overall response rate; TTP = time to progression; DFS = disease free survival; PFS = progression free survival; OS = overall survival; “with HER-2” (2 pts), HER-2” (6 pts) or HER-2*** (25 pts) tumours; RP = retinyl palmitate; ** IFNβ 2 MIU (group A) vs. 6 MIU (group B) i.m. three-times a week for 2 weeks; ***arm a: MEGACE 160 mg daily; arm b: IFNβ 0.25 MIU + IFNy25 mg s.c. + MEGACE (as in arm a); arm c: IFNβ 1 MIU/m² + RP 100.000 IU/day b.m. plus TAM 30 mg/day b.m.; ****with HER-2* (7) or HER-2*** (5) tumours.
In one study [92,93], metastatic patients, with responsive or stable disease during antiestrogen first line salvage therapy, were recruited for immunotherapy with IL-2, IFNβ and melatonin and subjected to prolonged follow-up. From 1992 to 2003, accrual was relatively slow. Nevertheless, the median duration of clinical benefit and median overall survival from the beginning of antiestrogen first line salvage therapy in 29 evaluated patients was 38 and 103 months, respectively. Patients with three-times more than in historical controls treated only with antiestrogens [93].

In summary, these studies showed that in locally advanced or metastatic breast cancer patients IL-2 given at low dose, alone or in association with other cytokines and/or other drugs in an outpatient setting, is a well-tolerated drug [81–93]. The response rate in three studies including more than 10 breast cancer patients was 3% [88], 17% [89], or 64% [91] and its duration was reported only in a few patients. Median overall survival was reported in two studies [88,92,93]. In one study [88] it was 8.9 months, as expected without cytokine treatment, while in the other study it was much longer (103 months) [92,93]. Therefore, in all trials, except the last one, no relevant improvement of clinical outcome in the treated patients was obtained although occasional immunological effects were recorded.

3.2. IFNα, IFNβ, IFNγ, IL-6, IL-12 given as single agent or with other cytokines (except IL-2) and/or other drugs

The main characteristics of the clinical trials with IFNα, IFNβ, IFNγ, IL-6, and IL-12, given alone or with other cytokines and/or other drugs in the treatment of advanced breast cancer, are shown in Table 3b. In the first four trials [44,94–96], three pilot studies and one phase II non-randomised trial, although with different schedules, IFNα was given alone [44] or with tamoxifen [94–96]. In all four studies, immunological effects were not recorded. However, in all [44,94,96] but one of them [95], IFNα did not overcome tamoxifen resistance nor ameliorate clinical outcomes. In this study [95], limited favourable results were reported; metastatic skin or soft tissue lesions showed increased ER expression and in 57% of patients, a response lasting 1–8 months occurred. Similarly, in a pilot study IFNα and IFNγ, given locally, alone or combined, was effective with 53% of CR in the treatment of cutaneous recurrences associated with a well documented enhancement of intrallesional cell-mediated immunological response [97]. However, in these last two studies [95,97] the accrual was very low (7 and 11 patients, respectively). In four other pilot studies [98–101] IFNβ was given before and/or concomitantly with tamoxifen. Most patients had positive or unknown receptor status. In the first study [98] on patients with multiple soft tissue biopsyable metastatic breast cancer, a significant increase in tissue hormone receptors after IFNβ treatment occurred. However, the clinical response rate and duration of response were in the range of those described in similar patients treated only with tamoxifen. In two further pilot studies [99,100] advanced breast cancer patients treated with and non-responsive, initially or after clinical benefit, to tamoxifen were recruited to an IFNβ-tamoxifen combination trial. In the two studies, the overall response rate was 26 and 13%, and median duration of response was 6 and 8 months; stabilisation of disease occurred in 44 and 37% of the patients, respectively. However, these studies have not shown any significant prolongation of the expected overall survival. In the last pilot study [101], IFNβ did not improve the efficacy of tamoxifen in a population of unselected metastatic patients who had received no prior palliative hormone therapy, no adjuvant tamoxifen, or had terminated it at least 12 months previously. In all the studies with IFNβ, immunological effects were not recorded. In two phase II non-randomised trials, IFNβ and retinyl palmitate were administered concomitant with tamoxifen until progression [43,102]. In the first study, most patients had metastatic disease and this therapy was given as maintenance only to the responders to previous conventional chemotherapy. In the entire study population, median overall survival was 32 months, as expected without maintenance. However, in the 11 complete responders to prior chemotherapy, median overall survival reached the relatively long duration of 66 months [43]. In the other trial, the same schedule of therapy was administered to advanced breast cancer patients, pretreated with hormones or with chemotherapy, and with evidence of progressive disease. In the entire population, the overall response rate was 59% and median overall survival was 38 months [102].

In a phase II randomised trial conducted in metastatic patients, estrogen positive patients, previously treated with tamoxifen, received medroxyprogesterone only (arm a) or medroxyprogesterone plus IFNβ and IFNγ (arm b) while patients with negative estrogen receptors were treated with IFNβ, IFNγ and tamoxifen (arm c). In a first report [103], the results obtained in arm c displaying the best clinical outcome were analysed separately. In arm c, an objective overall response rate of 39% and 18-month median duration of response were achieved. Another 28% of patients demonstrated stable disease with a median duration of 10 months. Median overall survival in the entire group was 36 months. These results are comparable to those commonly observed in estrogen receptor positive patients. In the subsequent report [104], changes in cytokine production in the three groups of patients (arms a, b and c) were examined and a correlation between serum IFNγ increase and a relatively favourable clinical outcome was found.

IL-6 did not produce any antitumor response in 11 patients with different histotypes of refractory advanced malignancies including one inflammatory breast cancer [105]. Eventually, in the last study highlighted in Table 3b on the effects of IL-12 and trastuzumab, increased circulating levels of IFNγ, TNFα, and antiangiogenic factors were found in patients with clinical benefit. Nevertheless, in this
phase I trial, IL-12 did not enhance clinical efficacy of trastuzumab [106]. In conclusion, IFNs given in an outpatient setting, are responsible for relatively limited side effects. The overall response rate (from 8 to 59%) is not so different from that usually seen in estrogen resistant and estrogen sensitive metastatic patients, respectively. Median overall survival was reported only in three different studies and ranged from 32 to 38 months. Median overall survival of 32 and 38 months shown in two [43,102] of these three studies is compatible with the studied population, also including patients with locally advanced disease [107,108], while median overall survival of 36 months in the other trial [103] is slightly higher than that usually expected in a similar population of patients with distant metastases [109–111]. A relatively prolonged survival was reported only in the subgroup of 11 patients on maintenance therapy with IFNβ, retinyl palmitate, and tamoxifen following complete remission due to chemotherapy.

With regard to IL-6 and IL-12, in spite of the reported immunological effects, they did not show any independent or synergistic clinical effect.

4. Discussion and conclusions

In recent decades, many experimental in vitro and in vivo studies have advanced the comprehension of the role of cytokines in oncology. Some cytokines (IL-1, IL-6, IL-11, TGFβ) stimulate while others (IL-12, IL-18, IFNs) inhibit breast cancer proliferation and/or invasion. Similarly, high circulating levels of some cytokines seem to be favourable (soluble IL-2R) while others are unfavourable (IL-1β, IL-6, IL-8, IL-10, IL-18, gp130) prognostic indicators. However, IL-2 is a potent stimulator of cellular immunity and, for this property, is the most selected interleukin for clinical trials. Interferons inhibit breast cancer proliferation and/or invasion and they also stimulate cellular immunity. Moreover, IFNβ, in vitro and in vivo, has been reported to enhance estrogen and progesterone receptors. Many clinical trials evaluated the pharmacokinetic characteristics and antitumor action of cytokines. So far, significant results have been reported with IL-2 and IFNα in advanced malignant melanoma and renal cancer [112–114]. In advanced breast cancer treatment, IL-2, IFNα, IFNβ and occasionally IFNγ, IL-6, and IL-12 have been used. While stimulation of the immunological cellular response was the principal aim of IL-2, IFNγ, IL-6 or IL-12 treatment, IFNα and IFNβ were given to induce or increase hormone dependency and overcome tamoxifen resistance (Tables 3a and 3b).

Median overall survival was observed in few trials and it was not better, except for one study [93], than that found in similar populations treated only with conventional therapy (Tables 3a and 3b). Therefore, the number of therapeutic studies with cytokines has decreased notably in recent years. Results were different in the non-randomised trial [43] on patients responsive to previous conventional chemotherapy and receiving a maintenance treatment with low dose IFNβ, retinyl palmitate and tamoxifen until relapse (Table 3b). In this series, the 11 patients with minimal residual disease, due to complete response to chemotherapy, showed median and 9-year survival rates of 66 months and 34%, respectively [43]. In a different series of 263 metastatic breast cancer patients in complete remission following combination chemotherapy, but not followed by any maintenance treatment, 42-month median survival and about 15% 9-year survival were observed [115]. However, the small sample size and some locally advanced breast cancer patients, may have contributed to the better outcome of the 11 complete responders with maintenance therapy [43]. Furthermore, in this trial [43], no immunological assessment was made. In a different long-term study [93], 29 consecutive breast cancer patients with distant metastases underwent immunotherapy with IFNβ and IL-2. Mean follow-up was 59 ± 37 months (mean ± S.D.). All patients were selected following clinical benefit on first line salvage therapy with antiestrogen. Unlike the previous study, where the encouraging result was observed in patients with minimal residual disease, all 29 patients at the beginning of immunotherapy had disseminated metastases. Therapy with cytokines significantly prolonged clinical benefit and overall survival in comparison to only antiestrogen therapy of historical control and literature data [92–93]. In fact, 38 months median clinical benefit, 103 months median overall survival and about 30% 9-year survival from the diagnosis of relapse were found, that is about three-times more than expected. During clinical benefit, stimulation of cellular immunity occurred after IL-2, consistent with previous reports [83,86,116], while it did not occur during disease progression [93]. Further recent immunological assessment confirmed findings on cellular immunity and showed a different definite cytokine pattern during clinical benefit and at the progression of disease [Nicolini et al., data not shown]. It was hypothesised that an immunological cellular response stimulated by IL-2 administration and by a provoked inflammatory cytokine cascade was responsible for the significant prolongation of the initial clinical benefit due to endocrine therapy [93] [Nicolini et al., data not shown]. Although with prolonged follow-up, this study suffers from being a non-randomised trial.

In conclusion, these limited favourable findings suggest that maintenance therapy with appropriate cytokines could significantly improve clinical outcomes of advanced breast cancer patients with minimal residual disease after chemotherapy or with disseminated disease controlled by conventional antiestrogens. Therefore, in these patients, IL-2 and IFNs should be used with suitable schedules in prospective randomised trials to confirm the prolongation of the clinical benefit and overall survival due to conventional chemo- or endocrine therapy.
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sion following combination chemotherapy for metastatic breast

therapy with recombinant interleukin-2 and interferon-alpha 2b in

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