The prognostic significance of serum interferon-gamma (IFN-γ) in hormonally dependent breast cancer

Running head: IFN-γ in prognosis of breast cancer

Nataša Todorović-Raković[1], Jelena Milovanović[1], John Greenman[2], Marko Radulovic[1]

[1]Department of Experimental Oncology, Institute of Oncology and Radiology of Serbia, Belgrade, Serbia

[2]Department of Biomedical Sciences, University of Hull, Hull, UK

Nataša Todorović-Raković: todorovicn@ncrc.ac.rs orcid.org/0000-0001-8975-3129
Jelena Milovanović: jelena.mil10@gmail.com orcid.org/0000-0002-5778-5393
John Greenman: J.Greenman@hull.ac.uk orcid.org/0000-0002-6003-6051
Marko Radulovic: marko@radulovic.net orcid.org/0000-0002-2314-7457

*Corresponding author:
Jelena Milovanović
Department of Experimental Oncology
Institute of Oncology and Radiology of Serbia
Pasterova 14, 11000 Belgrade, Serbia.
Tel: +381 11 20 67 290
E-mail: jelena.mil10@gmail.com

Abstract

Background: Interferon-γ (IFN-γ) is a pleiotropic immunomodulatory cytokine. Because of its contradictory and even dualistic roles in malignancies, its potential as a biomarker remains to be unraveled.

Aim: To evaluate the prognostic significance of serum IFN-γ in hormonally treated breast cancer patients.

Material and methods: The study included 72 premenopausal breast cancer patients with known clinicopathological characteristics. All patients received adjuvant hormonal therapy based on hormone receptor-positivity. The median follow-up period was 93 months. IFN-γ serum protein levels were determined by quantitative ELISA. Prognostic performance was evaluated by the receiver operating characteristic (ROC), Cox proportional hazards regression and Kaplan-Meier analyses. Classification of patients into IFN-γ^{low} and IFN-γ^{high} subgroups was performed by the use of the outcome-oriented cut-off point categorization approach.

Results: The best prognostic performance was achieved by IFN-γ (AUC=0.24 and p=0.01 for distant events, AUC=0.29 and p=0.01 for local and distant events combined). Age and IFN-γ were prognostically significant in instances of all types of outcomes and IFN-γ was the independent prognostic parameter (Cox regression). There was a significant difference between IFN-γ values of patients without any events and those with distant metastases (Mann-Whitney test, p=0.007). IFN-γ levels correlated significantly with nodal status and tumor stage (Spearman's rank order, r=-0.283 and r=-0.238, respectively). Distant recurrence incidence was 4% for the IFN-γ^{high} subgroup and 33% for the IFN-γ^{low} subgroup (Kaplan–Meier analysis).

Conclusions: Raised serum IFN-γ levels associate independently with favorable disease outcome in hormonally dependent breast cancer.

Keywords*: interferon gamma; breast cancer; hormonal therapy; tamoxifen; prognosis.*

1. Introduction

Interferon-γ (IFN-γ) is the sole member of the type II interferon family, a pleiotropic cytokine with primarily antiviral but also immunomodulatory functions, which plays an important role in coordinating both innate and adaptive immune responses [1]. Its expression is mainly from natural killer (NK) and natural killer T (NKT) cells in innate immunity while CD8+ and CD4+ T-cells are major paracrine sources of IFN-γ during the adaptive immune response [2]. These cells are stimulated by interleukins produced *in situ*, tumor- or pathogen- antigens, and by IFN-γ itself through a positive feedback loop [3]. Besides its autocrine effects on the IFN-γ-producing cells, IFN-γ also acts on stromal cells in an inflamed or tumor microenvironment (e.g. macrophages, dendritic cells, B cells, endothelial cells), as well as on tumor cells. The effect of IFN-γ is mediated through the induction of interferon signature genes (ISGs) that alter the function of the target cells [1, 4].

Based on the current knowledge, the effect of host-derived IFN-γ can be both anti-tumorigenic and pro-tumorigenic because of its complex effects in immunoediting [5]. Although the anti-tumoral effects of IFN-γ i.e. inhibition of the growth of several tumor cell lines including breast cancer cells, has been demonstrated in different studies, tumor cells can escape from the control of this cytokine in the early stages of tumor development, either due to a decreased expression of IFN-γ and/or through an alteration of either its receptors or transduction elements [6]. Furthermore, IFN-γ may actually reduce immune responses through enhanced activation of distinct immunosuppressive mechanisms that allow tumor progression and metastasis [1]. A recent study demonstrated that in the presence of IFN-γ producing cytotoxic T-cells, tumor cells developed genetic instability, which supported their genetic evolution and immune escape [7].

Approximately 70% of human breast cancers are hormone-dependent and express hormone receptors – estrogen receptor (ER) and/or progesterone receptor (PR) [8]. The introduction of adjuvant (postoperative) systemic therapy leads to a significant improvement in post-surgical survival and a reduction in disease relapse, especially in women with ER-positive (ER+) breast cancer, who may receive hormonal therapy alone or in combination with cytotoxic therapy [9]. Due to hormone receptor-positivity of breast cancer, these patients were treated with adjuvant endocrine therapy: tamoxifen alone or a combination of tamoxifen and luteinizing hormone-releasing hormone (LHRH) agonist goserelin [10]. Tamoxifen is known to have a dual mechanism of action: to compete with 17β-estradiol (E_2) at the receptor site and to block the

promotional role of E_2 in breast cancer, to bind DNA after metabolic activation and to initiate carcinogenesis [11]. LHRH agonists are recommended in younger breast cancer patients as they induce temporary ovarian suppression and thus preserve ovarian function from the toxic effects of chemotherapy [12]; these premenopausal women are at relatively high risk of relapse [13]. However, despite the effectiveness of hormonal therapy, ER+ breast cancers still show high recurrence rates, largely due to the phenomenon of resistance to hormonal therapy.

Nonetheless, *de novo* and acquired resistance remain the major problems in treatment of breast cancer patients and identification of resistance biomarkers remains unresolved. Although IFN-γ has been studied for a long time, because of its contradictory and even dualistic roles in malignancies, its potential as a biomarker remains to be unraveled. Therefore, this study aimed to evaluate the relationship between serum protein levels of IFN-γ and clinical outcome in hormonally-treated breast cancer patients.

2. Material and methods

2.1 Patients

This retrospective study included 72 premenopausal women with breast cancer. Clinicopathological characteristics of the patients at the time of primary diagnosis are presented in Table 1. Patients were diagnosed at different stages of the disease, but none of them had metastases at the time of diagnosis. All patients underwent surgical resection, their median age was 45 years. Histological specimens were examined and classified according to the criteria of the American Joint Committee on Cancer / Union International Contre le Cancer (AJCC/UICC) for TNM stage, histological type, tumor grade and receptor status. Patient data were received in an anonymised form without indirect identifiers that could enable re-identification (Safe-Harbour methodology of the 2012 Health Insurance Portability and Accountability Act).

This non-interventional, retrospective, study was approved by the Institutional Ethics committee of the Institute of Oncology and Radiology of Serbia and conforms to The Code of Ethics of the World Medical Association (Declaration of Helsinki), printed in the British Medical Journal (18^{th} July 1964) and its later amendments.

All patients received adjuvant (postoperative) hormonal therapy based on estrogen receptor (ER) and/or progesterone receptor (PR) proportion scoring according to Allred et al. [14]. Endocrine therapy consisted of tamoxifen alone or a combination of tamoxifen and LHRH

agonist goserelin (Zoladex®). According to the standard protocol at our hospital, postmenopausal breast cancer patients were switched to aromatase inhibitors (AIs) as part of adjuvant endocrine treatment. Therefore, in this study we included the premenopausal breast cancer patient group which was uniformly positive for hormone receptors that received tamoxifen alone or a combination of tamoxifen and goserelin. Such design has reduced the group heterogeneity which could mask or alter the prognostic role of IFN-γ. Taken together, the inclusion criteria were: premenopausal status, hormone receptor-positivity and hormone therapy. Fifty-two patients received tamoxifen alone over 5 years after operation while 20 patients (≤44 years of age) received a combination of tamoxifen and goserelin (LHRH agonist) for 3 years after the operation and then continued with tamoxifen alone for 2 years (up to 5 years).

To provide insight into the prognostic performance of serum IFN-γ in breast cancer, we evaluated whether IFN-γ serum levels were associated with the, retrospectively recorded, actual occurrence of distant and local events. Local recurrence refers to the development of locoregional changes (in the same breast or regional lymph nodes), while distant recurrence refers to distant metastasis such as the bone, lung, liver and brain [15]. The follow-up period was from 60 up to 172 months, with a median of 93 months.

2.2 Measurement of IFN-γ levels in serum

Five milliliters of peripheral blood were taken from all patients postoperatively. Blood samples were centrifuged at 950 g for 10 min and serums were stored at ≤ −70 °C.

IFN-γ levels were determined by ELISA according to the manufacturer's instructions (Human IFN-γ Quantikine HS ELISA, R&D Systems, Minneapolis, USA).

2.3 Measurement of hormone levels in serum

Follicle-stimulating hormone (FSH), luteinizing hormone (LH) and estradiol levels were measured by ELISA according to the manufacturer's instructions (Human Diagnostics GmbH, Wiesbaden, Germany).

2.4 Prognostic performance evaluation

The receiver operating characteristic (ROC) analysis by the area under the ROC curve (AUC) was employed as a quantitative measure of discrimination efficiency. Discrimination is the capability to stratify patients who experience the event and patients who do not experience

the event. AUC ranges from 0.5 (chance accuracy) to 1.0 (perfect accuracy), with the intermediate benchmarks of: 0.4-0.5 or 0.5-0.6 (poor), 0.3-0.4 or 0.6-0.7 (fair), 0.2-0.3 or 0.7-0.8 (moderate), 0.1-0.2 or 0.8-0.9 (good) and 0.0-0.1 or 0.9-1.0 (excellent) [16]. Kaplan–Meier analysis was done for the period from tumor extraction surgery until the occurrence of local and distant events (IBM SPSS Statistics for Windows version 24, IBM Corp. Chicago, IL, USA). ROC analysis was based on continuous feature values, while Cox proportional hazards regression used categorized feature values.

Categorization of the continuous values measured in serum was achieved by the outcome-oriented optimal cut-off point selection by use of the log-rank test and the X-tile 3.6.1 software from Yale University (New Haven, CT, USA) [17]. Univariate Cox proportional hazards regression test was performed for comparison of the prognosticated and actual, local and distant events. The HR designates the effect size by Cox regression, corresponding to recurrence rates in high- and low-risk groups of patients (IBM SPSS). Each feature satisfied the proportional hazards assumption based on the Schoenfeld residuals by phtest (Stata/MP 13 package, StataCorp, College Station, TX, USA). Multivariate stepwise Cox proportional hazards regression analysis was performed to test for the independence of each prognostic factor. Variables categorized by the outcome were added to a full model using the forward selection entry criterion of $p \leq 0.05$ in univariate analysis and removed using backward elimination by the selection stay criterion of $p < 0.05$ in IBM SPSS Statistics for Windows.

2.5 Validation

The p-values and confidence intervals (95%CI) of the obtained HRs and AUCs were corrected for bias using the bootstrap internal validation in IBM SPSS Statistics version 24 for Windows. Bootstrap resample validation is a very powerful tool for testing model stability by constructing confidence intervals and calculating p-values [18]. The bootstrap variant of resampling with replacement produces new "surrogate" data sets with the same number of cases as the original data set. This is achieved by a random selection of observations from the original sample until the same number of observations is achieved, followed by calculation of prognostic estimates such as the 95%CI and p-value. The performed bootstrap is defined as “resampling with replacement” because the selected observations are not removed from the pool during resampling. Therefore, some measurements may be selected multiple times while certain

observations may not appear in a resample. By creating 1000 different resamples bootstrapping offers a more stable estimate of the prognostic performance.

3. Results

3.1. Sample size calculation

The sample size calculation was based on a pilot experiment with 35 patients. The calculation parameters obtained from the pilot experiment were: target power of 0.8, effect size by hazard ratio (HR) of 5, significance level of 0.05, variability in standard deviations (SD) of 0.65 and the event rate of 12%. We calculated the variability for each feature as a distance between average values of the patient subgroups with and without the actual recurrence, expressed in SD.

The required numbers were 60 patients with eight events. The actual patient number recruited was 72 with ten distant events and eight local events; and the average SD distance between the subgroups with and without recurrence was 0.68 for distant events and 0.61 for local events respectively. The event rate was 11% for local and 14% for distant events. The effect size for IFN-γ was 0.19 or 5.3 for local events and 0.12 or 8.3 for distant events. This resulted in the actual power of 0.82 for prognostication of the local events and 0.995 for distant events. Calculations were performed by the two-sided stpower cox test (Stata/MP 13 software, StataCorp, College Station, TX, USA).

3.2 Prognostic performance evaluation and validation

Table 1 presents clinicopathological characteristics of the premenopausal patients at the time of primary diagnosis. Table 2 presents the statistical evaluation of the association between the available variables and the, retrospectively recorded, actual local and/or distant events. During the follow-up time, 25% of patients developed local or distant recurrence. In our study the time frame for local recurrences was 26 to 89 months whilst for distant recurrences it was 20 to 56 months. The statistical association was calculated against three outcomes: only local, only distant or distant+local events.

Age and IFN-γ were prognostically significant in instances of all types of outcomes (Table 2). Local events could not be prognosticated by any of the tested parameters according to the criteria of ROC analysis, whilst distant events could be by age, PR, nodal status and IFN-γ

(Table 2). Local and distant events combined could be prognosticated by age and IFN-γ. By the measure of AUC, the best prognostic performance was achieved by IFN-γ (AUC=0.24 and p=0.01 for distant events, AUC=0.29 and p=0.01 for local and distant events combined). ROC analyses of the IFN-γ serum levels in the prognosis of distant and local events are presented in Figure 1 (A-C).

In univariate Cox proportional hazards regression analysis, the most pronounced HR was observed with age, FSH, tumor size and IFN-γ for local and distant events combined (Table 2). AUC values below 0.5 and HRs below 1.0 indicate a prognostic association with good disease outcome. For instance, HR of 0.04 obtained for IFN-γ indicated that patients with IFN-γ serum protein levels above the outcome-oriented threshold had a 25-fold lower risk of incurring an event in comparison to patients with IFN-γ levels below the threshold. When distant metastases and local relapses were separated as events, the most pronounced HR by the Cox regression was observed with age, estradiol, tumor size and IFN-γ for both distant and local events (Table 2).

The multivariate Cox proportional hazards regression analysis of the metastasis risk included age, estradiol, FSH, LH, PR, nodal status, tumor size, HER2 and IFN-γ because they all satisfied the forward entry criterion of $p \leq 0.05$ in the univariate analysis by distant events. Multivariate analysis was performed considering distant events because these are the most relevant for disease outcome. Furthermore, IFN-γ prognosticated distant metastases better than local recurrences (Table 2). This analysis highlighted IFN-γ and lymph node status as the independent prognostic parameters (Table 3). This result was also supported by a Mann-Whitney rank sum test, which showed a significant difference between IFN-γ values of patients without any events and those with distant metastases (p=0.007) but not between patients without any events and those with local relapses (p=0.14).

The average±standard deviation (SD) IFN-γ measured values were 35.7±8.3 pg/mL for patients without recurrences, 18.3±2.8 pg/mL for patients with distant metastases and 23.0±3.2 pg/mL for patients with local relapses. In our patient group distant metastases were seen in the bones (4), lungs (3), CNS (2) and liver (1). When events were divided based on distant metastatic sites, their numbers were too low for reliable statistical analysis according to the sample size calculation. By Spearman's rank order correlation test, IFN-γ levels correlated significantly with age, nodal status and tumor stage (Table 4). A positive correlation was found between age and IFN-γ levels (r=0.324) and a negative correlation between nodal status and

IFN-γ levels (r=-0.283) as well as between tumor stage and IFN-γ levels (r=-0.238). Taken together, higher serum protein levels of IFN-γ indicated lower recurrence risk.

Figure 1 (D-F) presents Kaplan-Meier plots for IFN-γ in prognostication of distant and local events. Classification of patients into IFN-γ^{low} and IFN-γ^{high} subgroups was performed by the use of the outcome-oriented cut-off point categorization approach. P-values were calculated by the Cox proportional hazards regression test. A wider separation between upper and lower curves indicates better prognostic performance. Considering distant and local events combined, recurrence incidence was 0% for the IFN-γ^{high} subgroup and 28% for the IFN-γ^{low} subgroup (Figure 1D). When distant metastases and local relapses were separated as events, distant recurrence incidence was 4% for the IFN-γ^{high} subgroup and 33% for the IFN-γ^{low} subgroup (Figure 1E), while local recurrence incidence was 6% for the IFN-γ^{high} subgroup and 24% for the IFN-γ^{low} subgroup (Figure 1F).

4. Discussion

In our previous study, based upon the long-term follow-up of 73 pN0M0 breast cancer patients after surgery & radiotherapy receiving no subsequent systemic therapy, we investigated the prognostic value of intratumoral IFN-γ mRNA and protein levels [19]. Over the entire period of 14 years of follow-up neither IFN-γ mRNA nor IFN-γ protein levels were significantly associated with breast cancer outcome by ROC analysis or Cox regression criteria, but intratumoral IFN-γ mRNA was associated significantly with favorable disease outcome over the first 7 years of follow-up. [19]. The current study demonstrates the prognostic value of measuring IFN-γ in the serum of patients with breast cancer; this allows easy sampling and repeated measurements. Furthermore, ELISA is a widely used and inexpensive method in routine clinical laboratory practice. The main finding of the study, in a patient group with a median follow-up of 7.5 years, is that raised serum IFN-γ protein levels associated significantly with favorable disease outcome.

A recent study examined the molecular subtype-specific prognostic significance of IFN-γ as a single gene as well as an IFN-γ signature covering the signalling pathway in breast cancer patients [20]. Heimes et al. found that the independent prognostic significance of IFN-γ as a single gene was limited to basal-like breast cancer but the IFN-γ-associated gene signature was the independent prognostic factor in the whole cohort [20]. Higher expression of the IFN-γ-

signature was associated with a better prognosis and that is in accordance with our results. Also in agreement with our results was another study that investigated the prognostic significance of intratumoral IFN-γ mRNA in invasive cervical cancer patients and found that the good-prognosis patient subgroup had higher IFN-γ mRNA expression [21]. Two other studies reported that the IFN-γ gene signature predicted significant improvement in both progression-free survival and overall survival in uterine and ovarian cancer for patients with higher intratumoral IFN-γ expression [22, 23]. In hepatocellular cancer patients, low serum IFN-γ levels were associated significantly with greater tumor size and advanced stage, and patients with lower baseline IFN-γ levels had a higher risk of recurrence [24]. Similarly, we found in this study low serum IFN-γ levels associated significantly with the unfavorable clinicopathological features of nodal spread and advanced stage. Finally, a recent study investigated the association between IFN-γ levels and clinical outcomes in non-small-cell lung cancer patients receiving immunotherapy [25]. In this study median progression-free survival was significantly longer in the subgroup of patients with higher IFN-γ levels. Furthermore, IFN-γ levels in the non-progressing disease group were significantly higher than in those patients who did show progression [25]. Our results in hormonally dependent breast cancer are in agreement with all of these previous studies on breast and other solid malignancies. Although several breast cancer studies have shown that IFN-γ levels are inversely correlated with intratumoral ER and PR expression [19, 26], in this study we found no correlation between IFN-γ and hormone levels, nor between IFN-γ and the ER and PR hormone receptors.

IFN-γ is a pleiotropic immunomodulatory cytokine that exerts contradictory and polarizing roles in malignancies, manifested by the opposing actions depending on the cellular, microenvironmental or molecular context [27]. Several studies have shown that IFN-γ induces an immunoevasive/survival gene expression signature, including upregulation of CTLA4, in skin melanocytes in the context of exposure to genotoxic ultraviolet radiation (UVR), which may play an important role in protecting melanocytes from eradication by the UVR-induced inflammatory response [28, 29]. Such protective functions of IFN-γ signalling could be exploited by cancer cells to evade immune-mediated destruction and to survive long-term until they accumulate enough mutations to get fully transformed [27].

Considering the effect of hormonal therapy on breast cancer, it exerts multiple effects on the expression of tumor biological variables. Lindner et al. showed that tamoxifen enhanced

interferon-regulated gene expression in breast cancer cells and this enhancement was an early event in the anti-tumoral activity [30]. Another study showed that IFN-γ increased the growth inhibitory effect of tamoxifen in breast metastatic carcinomas [6]. On the contrary, RNA sequencing of tamoxifen-resistant breast cancer cells indicated that expression of ISGs by tumor cells is involved in acquired treatment-induced resistance [31]. Furthermore, high ISGs expression levels were associated with worse outcome in breast cancer patients treated with adjuvant tamoxifen [31]. Ning et al. showed the ability of IFN-γ to induce apoptosis and restore the responsiveness of breast cancer cells to antiestrogen therapy [32]. Moreover, IFN-γ induced the expression of IFN regulatory factor 1 (IRF1), a tumor suppressor gene that can increase antiestrogen responsiveness, which implied that upregulating IRF1 might be a successful approach in the treatment of ER+ breast cancers that have acquired resistance to antiestrogen therapy [32]. From our results, we can speculate that hormonal therapy and endogenous IFN-γ might exert synergistic effect resulting in improvement of the patient's outcome, as the high IFN-γ expression might positively affect the efficiency of hormonal therapy.

Although we satisfied the sample size requirement and the patient group was highly homogenized, limitations of this study include the patient group size. Additional studies in external and larger patient groups are needed to further verify the clinical validity of the reported prognostic value of the serum IFN-γ levels.

5. Conclusions

In conclusion, raised serum IFN-γ levels associate independently with favorable disease outcome in hormonally dependent breast cancer. Moreover, low serum IFN-γ levels associate with the unfavorable clinicopathological features of nodal spread and advanced stage. Although there are findings suggesting that IFN-γ is a hormonally regulated factor, our results showed no correlation between IFN-γ and hormone levels, neither between IFN-γ and hormone receptors.

This study provides the first prognostic evaluation of IFN-γ in a patient group homogenous for hormone receptors. Clinical applicability of the study is based on the relevance for breast cancer immunotherapy research and the importance of prognosis for the identification of patients at high risk of recurrence who may benefit from more aggressive personalized treatments. Furthermore, this study is of high importance for advancing the prognosis of breast cancer because IFN-γ can easily be measured in serum, on repeated occasions, using ELISA, a

well-established, cost-effective analysis technology widely used in routine clinical laboratory practice.

Financial disclosure

This work was supported by the Ministry of Education, Science and Technological development of the Republic of Serbia (451-03-9/2021-14/200043).

Declaration of competing interest

The authors declare no conflict of interest.

Authors' contributions

All authors have made substantial contributions to: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) and final approval of the version to be submitted.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

6. References

[1] D. Jorgovanovic, M. Song, L. Wang, Y. Zhang, Roles of IFN-gamma in tumor progression and regression: a review, Biomark Res 8 (2020) 49.

[2] J.D. Burke, H.A. Young, IFN-gamma: A cytokine at the right time, is in the right place, Semin Immunol 43 (2019) 101280.

[3] E. Alspach, D.M. Lussier, R.D. Schreiber, Interferon gamma and Its Important Roles in Promoting and Inhibiting Spontaneous and Therapeutic Cancer Immunity, Cold Spring Harb Perspect Biol 11(3) (2019).

[4] B.S. Parker, J. Rautela, P.J. Hertzog, Antitumour actions of interferons: implications for cancer therapy, Nature reviews. Cancer 16(3) (2016) 131-44.

[5] N. Todorovic-Rakovic, The role of cytokines in the evolution of cancer: IFN-gamma paradigm, Cytokine (2021) 155442.

[6] I. Garcia-Tunon, M. Ricote, A.A. Ruiz, B. Fraile, R. Paniagua, M. Royuela, Influence of IFN-gamma and its receptors in human breast cancer, BMC Cancer 7 (2007) 158.

[7] K. Takeda, M. Nakayama, Y. Hayakawa, Y. Kojima, H. Ikeda, N. Imai, K. Ogasawara, K. Okumura, D.M. Thomas, M.J. Smyth, IFN-gamma is required for cytotoxic T cell-dependent cancer genome immunoediting, Nat Commun 8 (2017) 14607.

[8] E. Lim, O. Metzger-Filho, E.P. Winer, The natural history of hormone receptor-positive breast cancer, Oncology (Williston Park) 26(8) (2012) 688-94, 696.

[9] F. Lumachi, A. Brunello, M. Maruzzo, U. Basso, S.M. Basso, Treatment of estrogen receptor-positive breast cancer, Curr Med Chem 20(5) (2013) 596-604.

[10] M. Lambertini, L. Del Mastro, G. Viglietti, N.F. Ponde, C. Solinas, E. de Azambuja, Ovarian Function Suppression in Premenopausal Women with Early-Stage Breast Cancer, Current treatment options in oncology 18(1) (2017) 4.

[11] F. Yu, W. Bender, The mechanism of tamoxifen in breast cancer prevention, Breast Cancer Research 3(S1) (2001) A74,-bcr404.

[12] L. Del Mastro, G. Rossi, M. Lambertini, F. Poggio, P. Pronzato, New insights on the role of luteinizing hormone releasing hormone agonists in premenopausal early breast cancer patients, Cancer Treat Rev 42 (2016) 18-23.

[13] B. Conte, F. Poggio, L. Del Mastro, Luteininzing hormone releasing hormones analogs in combination with tamoxifen for the adjuvant treatment of premenopausal women with hormone receptor positive breast cancer, Expert opinion on pharmacotherapy 18(13) (2017) 1357-1362.

[14] D.C. Allred, M.A. Bustamante, C.O. Daniel, H.V. Gaskill, A.B. Cruz, Jr., Immunocytochemical analysis of estrogen receptors in human breast carcinomas. Evaluation of 130 cases and review of the literature regarding concordance with biochemical assay and clinical relevance, Arch Surg 125(1) (1990) 107-13.

[15] W. Chen, A.D. Hoffmann, H. Liu, X. Liu, Organotropism: new insights into molecular mechanisms of breast cancer metastasis, NPJ Precis Oncol 2(1) (2018) 4.

[16] J. Yang, X. Guo, H. Zhang, W. Zhang, J. Song, H. Xu, X. Ma, Differential diagnosis of pancreatic serous cystadenoma and mucinous cystadenoma: utility of textural features in combination with morphological characteristics, BMC cancer 19(1) (2019) 1223.

[17] R.L. Camp, M. Dolled-Filhart, D.L. Rimm, X-tile: a new bio-informatics tool for biomarker assessment and outcome-based cut-point optimization, Clin. Cancer Res. 10(21) (2004) 7252-9.

[18] B. Efron, Bootstrap Methods: Another Look at the Jackknife, The Annals of Statistics 7(1) (1979) 1-26.

[19] N. Todorovic-Rakovic, M. Radulovic, T. Vujasinovic, J. Milovanovic, D. Nikolic-Vukosavljevic, The prognostic time dependence of intra-tumoural IFNgamma mRNA and protein in patients with breast

cancer followed for 14 years after surgery and radiotherapy, without subsequent systemic therapy, Eur Cytokine Netw 28(4) (2017) 151-156.
[20] A.S. Heimes, F. Hartner, K. Almstedt, S. Krajnak, A. Lebrecht, M.J. Battista, K. Edlund, W. Brenner, A. Hasenburg, U. Sahin, M. Gehrmann, J.G. Hengstler, M. Schmidt, Prognostic Significance of Interferon-gamma and Its Signaling Pathway in Early Breast Cancer Depends on the Molecular Subtypes, Int J Mol Sci 21(19) (2020).
[21] E. Tartour, A. Gey, X. Sastre-Garau, I. Lombard Surin, V. Mosseri, W.H. Fridman, Prognostic value of intratumoral interferon gamma messenger RNA expression in invasive cervical carcinomas, J Natl Cancer Inst 90(4) (1998) 287-94.
[22] C. Marth, H. Fiegl, A.G. Zeimet, E. Muller-Holzner, M. Deibl, W. Doppler, G. Daxenbichler, Interferon-gamma expression is an independent prognostic factor in ovarian cancer, Am J Obstet Gynecol 191(5) (2004) 1598-605.
[23] H. Mahdi, P.G. Rose, F.W. Abdul-Karim, B.J. Monk, Y. Ni, The role of proinflammatory immune (IFN-gamma) gene signature in predicting prognosis and response to chemotherapy in uterine carcinoma, J. Clin. Oncol. 37(15_suppl) (2019) e14209-e14209.
[24] I.C. Lee, Y.H. Huang, G.Y. Chau, T.I. Huo, C.W. Su, J.C. Wu, H.C. Lin, Serum interferon gamma level predicts recurrence in hepatocellular carcinoma patients after curative treatments, Int J Cancer 133(12) (2013) 2895-902.
[25] T. Kanai, H. Suzuki, H. Yoshida, A. Matsushita, H. Kawasumi, Y. Samejima, Y. Noda, S. Nasu, A. Tanaka, N. Morishita, S. Hashimoto, K. Kawahara, Y. Tamura, N. Okamoto, T. Tanaka, T. Hirashima, Significance of Quantitative Interferon-gamma Levels in Non-small-cell Lung Cancer Patients' Response to Immune Checkpoint Inhibitors, Anticancer Res 40(5) (2020) 2787-2793.
[26] C. Chavey, F. Bibeau, S. Gourgou-Bourgade, S. Burlinchon, F. Boissiere, D. Laune, S. Roques, G. Lazennec, Oestrogen receptor negative breast cancers exhibit high cytokine content, Breast Cancer Res 9(1) (2007) R15.
[27] M.R. Zaidi, The Interferon-Gamma Paradox in Cancer, J Interferon Cytokine Res 39(1) (2019) 30-38.
[28] M.R. Zaidi, S. Davis, F.P. Noonan, C. Graff-Cherry, T.S. Hawley, R.L. Walker, L. Feigenbaum, E. Fuchs, L. Lyakh, H.A. Young, T.J. Hornyak, H. Arnheiter, G. Trinchieri, P.S. Meltzer, E.C. De Fabo, G. Merlino, Interferon-gamma links ultraviolet radiation to melanomagenesis in mice, Nature 469(7331) (2011) 548-53.
[29] M.R. Zaidi, E.C. De Fabo, F.P. Noonan, G. Merlino, Shedding light on melanocyte pathobiology in vivo, Cancer Res 72(7) (2012) 1591-5.
[30] D.J. Lindner, V. Kolla, D.V. Kalvakolanu, E.C. Borden, Tamoxifen enhances interferon-regulated gene expression in breast cancer cells, Mol Cell Biochem 167(1-2) (1997) 169-77.
[31] A.E.M. Post, M. Smid, A. Nagelkerke, J.W.M. Martens, J. Bussink, F. Sweep, P.N. Span, Interferon-Stimulated Genes Are Involved in Cross-resistance to Radiotherapy in Tamoxifen-Resistant Breast Cancer, Clin Cancer Res 24(14) (2018) 3397-3408.
[32] Y. Ning, R.B. Riggins, J.E. Mulla, H. Chung, A. Zwart, R. Clarke, IFNgamma restores breast cancer sensitivity to fulvestrant by regulating STAT1, IFN regulatory factor 1, NF-kappaB, BCL2 family members, and signaling to caspase-dependent apoptosis, Mol Cancer Ther 9(5) (2010) 1274-85.

7. Tables

Table 1. Clinicopathological characteristics at the time of primary diagnosis

Parameter	*Number of patients*	*%*
Age (years)		
≤ 45 (median)	32	44
> 45	40	56
Recurrence		
distant	10	14
local	8	11
(distant+local)	(18)	(25)
no recurrence	54	75
Tumour size (cm)		
≤ 2	32	45
2 – 5	26	36
> 5	14	19
Nodal status		
N0	35	49
N+	37	51
Histological type		
Invasive ductal	31	43
Invasive lobular	24	33
other types	17	24
Stage		
1	21	29
2	30	42
3	21	29
Histological grade		
G1	12	17
G2	53	73
G3	2	3
data not available	5	7
Hormone therapy		
Tamoxifen	52	72
Tamoxifen+Goserelin	20	28
Estrogen receptor status		
ER^{low}	8	11
ER^{high}	64	89
Progesterone receptor status		
PR^{low}	6	8
PR^{high}	59	82
data not available	7	10
***HER2* status**		
HER2−	62	86
HER2+	9	13
data not available	1	1

Abbreviations: ER, estrogen receptor; ER^{low}, ER Allred proportion score < 3; ER^{high}, ER Allred proportion score ≥ 3; PR, progesterone receptor; PR^{low}, PR Allred proportion score < 3; PR^{high}, PR Allred proportion score ≥ 3; *HER2*, human epidermal growth factor receptor 2; *HER2–*, HER2 gene not amplified; *HER2+*, HER2 gene amplification.

Table 2. Prognostic performance of clinicopathological parameters and IFN-γ

	Distant and local events		Distant metastasis		Local recurrence	
Variable	AUC[a] 95% CI[c] P-value[c]	HR[b] 95% CI[c] P-value[c]	AUC[a] 95% CI[c] P-value[c]	HR[b] 95% CI[c] P-value[c]	AUC[a] 95% CI[c] P-value[c]	HR[b] 95% CI[c] P-value[c]
Age	0.32 0.17 – 0.46 **0.03***	0.21 0.05 – 0.54 **0.002***	0.28 0.13 – 0.42 **0.02***	0.12 0.02 – 0.42 **0.002***	0.35 0.15 – 0.55 0.18	0.19 0.002 – 0.83 **0.003***
Estradiol	0.52 0.37 – 0.67 0.81	0.34 0.03 – 0.92 0.09	0.54 0.36 – 0.73 0.66	26.7 22.9 – 33.8 **0.001***	0.47 0.29 – 0.65 0.79	0.03 0.02 – 0.04 **0.001***
LH	0.45 0.29 – 0.61 0.56	0.41 0.04 – 1.80 0.24	0.50 0.33 – 0.68 0.99	0.04 0.03 – 0.05 **0.001***	0.38 0.17 – 0.59 0.27	0.89 0.04 – 5.05 0.92
FSH	0.43 0.29 – 0.61 0.56	0.16 0.04 – 0.70 **0.001***	0.45 0.27 – 0.63 0.62	0.04 0.03 – 0.04 **0.001***	0.46 0.22 – 0.71 0.73	1.79 0.04 – 8.24 0.45
ER	0.45 0.28 – 0.61 0.51	0.97 0.73 – 1.29 0.76	0.43 0.23 – 0.63 0.46	0.92 0.66 – 1.32 0.54	0.49 0.27 – 0.71 0.92	1.01 0.74 – 1.95 0.94
PR	0.36 0.19 − 0.52 0.10	0.47 0.1 − 23.8 0.28	0.26 0.09 − 0.43 **0.02***	0.15 0.004 − 0.63 **0.01***	0.60 0.40 − 0.80 0.39	9.6 1.75 – 242.2 **0.03***
Histological grade	0.59 0.42 – 0.75 0.30	7.6 0.73 – 507.8 0.21	0.63 0.43 – 0.83 0.20	23.3 0.65 – 1248.9 0.08	0.51 0.27 – 0.75 0.95	1.68 0.17 – 1299 0.79
Nodal status	0.59 0.42 – 0.75 0.30	1.35 0.71 – 2.33 0.24	0.69 0.54 – 0.90 **0.03***	2.1 1.16 – 4.44 **0.004***	0.31 0.15 – 0.47 0.08	0.34 0.04 – 0.83 **0.04***
Tumor size	0.63 0.48 – 0.79 0.10	46.6 33.8 – 69.4 **0.001***	0.67 0.49 – 0.85 0.09	11.5 2.9 – 601.8 **0.002***	0.71 0.50 – 0.92 0.06	5.4 1.1 – 298.9 **0.005***
HER2	0.55 0.38 – 0.71 0.57	1.45 0.34 – 4.1 0.49	0.65 0.45 – 0.85 0.13	3.24 0.83 – 14.2 **0.05***	0.45 0.29 – 0.60 0.50	0.46 0.03 – 2.61 0.33
Stage	0.33 0.16 – 0.49 0.07	1.50 0.78 – 3.2 0.19	0.46 0.31 – 0.60 0.58	2.03 0.88 – 6.62 0.09	0.48 0.34 – 0.63 0.82	0.78 0.32 – 1.95 0.57
IFN-γ	0.29 0.14 – 0.42 **0.01***	0.04 0.03 – 0.04 **0.001***	0.24 0.08 – 0.40 **0.01***	0.12 0.004 – 0.58 **0.003***	0.37 0.18 – 0.56 0.23	0.19 0.003 – 0.92 **0.005***

[a] ROC analysis prognostic test, based on continuous parameter values prior to their categorisation.

[b] Univariate Cox proportional hazards regression test, based on categorized parameter data.

[c] bootstrap corrected

* $P \leq 0.05$

Abbreviations: LH, luteinizing hormone; FSH, follicle-stimulating hormone; ER, estrogen receptor; PR, progesterone receptor; *HER2*, human epidermal growth factor receptor 2; IFN-γ, interferon gamma.

Table 3. Multivariate Cox proportional hazards regression analysis of the prognostic features [a,b,c]

Parameter	P-value	HR 95% CI
IFN-γ	**0.01***	0.17 0.04 – 0.68
Nodal status	**0.05***	1.88 1.00 – 3.49

[a] Cox multivariate stepwise regression was performed by the forward entry criterion of $p \leq 0.05$ and the backward elimination criterion of $p < 0.05$. Only the remaining features are thus presented in this Table.

[b] Analysis was performed on the basis of distant metastases as events.

[c] Performed by use of categorized data.

* $P \leq 0.05$ indicated statistical significance.

Abbreviations: IFN-γ, interferon gamma.

Table 4. Correlations between serum levels of IFN-γ and the major clinicopathological parameters [a]

	IFN-γ	FSH	LH	E2	Grade	Nodal status	Tumor size	ER	PR	*HER2*	Age
IFN-γ	1.000	-	-	-	-	-	-	-	-	-	-
FSH	0.033	1.000	-	-	-	-	-	-	-	-	-
LH	-0.014	0.658*****	1.000	-	-	-	-	-		-	-
E2	0.185	-0.240*****	-0.070	1.000	-	-	-	-	-	-	-
Grade	-0.089	-0.124	-0.069	0.028	1.000	-	-	-	-	-	-
Nodal status	-0.283*****	-0.130	-0.098	-0.087	0.240*****	1.000	-	-	-	-	-
Tumor size	-0.164	-0.173	0.126	0.125	0.132	0.391*****	1.000	-	-	-	-
ER	0.182	-0.114	-0.035	0.141	0.109	-0.256*****	-0.185	1.000	-	-	-
PR	0.210	-0.029	-0.050	0.116	0.010	-0.187	-0.189	0.266*****	1.000	-	-
HER2	-0.158	0.024	-0.047	-0.117	-0.058	0.214	0.209	-0.284*****	-0.325*****	1.000	-
Age	0.324*****	0.158	0.186	0.011	-0.157	-0.384*****	-0.356*****	0.295*****	0.160	-0.368*****	1.000
Stage	-0.238*****	-0.201	0.014	0.042	0.218	0.738*****	0.780*****	-0.243*****	-0.121	0.160	-0.279*****

[a] Continuous numerical values were used for calculation of Spearman's coefficients except for nodal status, disease grade and stage, which are inherently categorical.

***** Spearman's correlation coefficients with $P \leq 0.05$

Abbreviations: IFN-γ, interferon gamma; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E2, estradiol; ER, estrogen receptor; PR, progesterone receptor; *HER2*, human epidermal growth factor receptor 2.

8. Figures

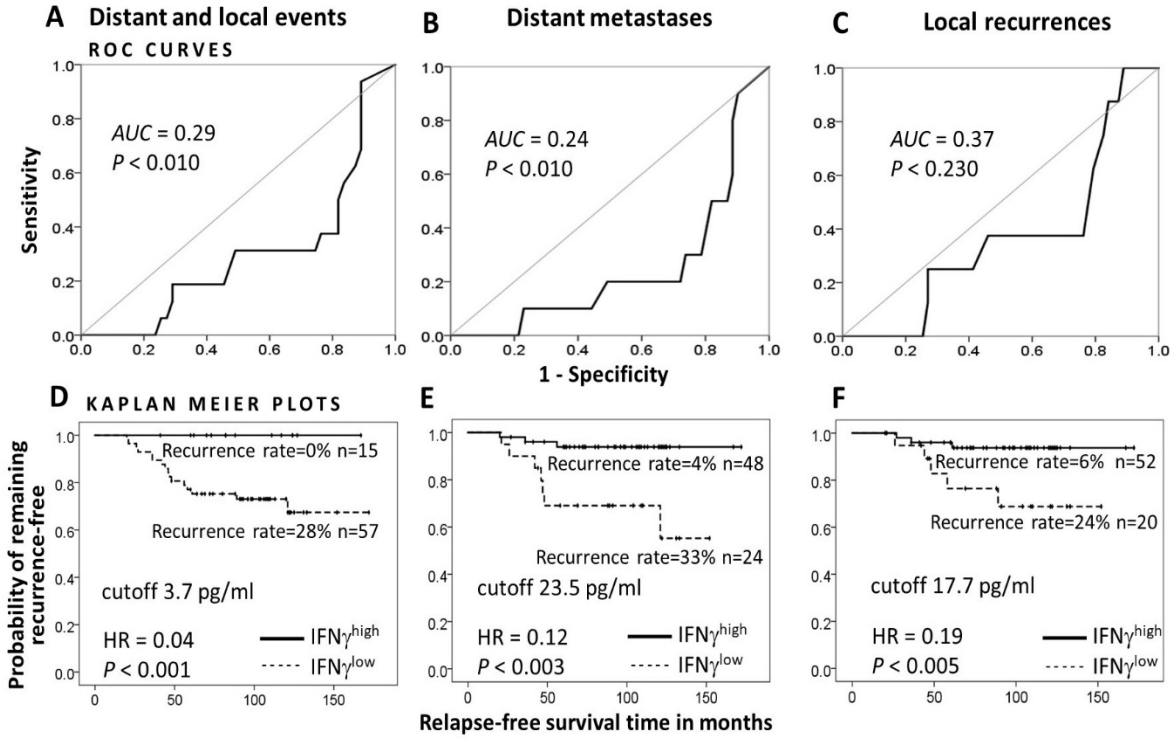

Figure 1. ROC and Kaplan-Meier analysis of the IFN-γ serum levels in prognosis of distant and local events as endpoints. (A) Prognostic performance of IFN-γ serum levels in prediction of both distant and local events. (B) Prognostic performance of IFN-γ serum levels in prediction of distant metastases. (C) Prognostic performance of IFN-γ serum levels in prediction of local recurrences. ROC analysis was based on continuous (non-categorized) feature values. (D) Kaplan-Meier prognostic analysis of IFN-γ serum levels with distant and local events as the endpoint. (E) Kaplan-Meier prognostic analysis of IFN-γ serum levels with distant metastasis as the endpoint. (F) Kaplan-Meier prognostic analysis of IFN-γ serum levels with local recurrence as the endpoint. Classification of patients into IFN-γ^{low} and IFN-γ^{high} subgroups was performed by the use of the outcome-oriented cut-off point categorization approach. The upper solid line in Kaplan-Meier plots represents the IFN-γ^{high} patient subgroup, while the lower dotted line indicates the IFN-γ^{low} subgroup. P-values were calculated by the Cox proportional hazards regression test.

Abbreviations: ROC: Receiver operating characteristic; AUC: Area under the ROC curve; IFN-γ, interferon gamma.