Final results of a 2:1 control-case observational study using interferon beta and interleukin-2, in addition to first-line hormone therapy, in estrogen receptor-positive, endocrine-responsive metastatic breast cancer patients

Andrea Nicolini¹, Giuseppe Rossi², Paola Ferrari¹, Riccardo Morganti³, Angelo Carpi⁴

¹Department of Oncology, Transplantations and New Technologies in Medicine, University of Pisa, Pisa 56100, Italy  
²National Research Council (CNR), Epidemiology and Biostatistics Unit, Institute of Clinical Physiology and G. Monasterio Foundation, Pisa 56100, Italy  
³Statistics Section, University Hospital of Pisa, Pisa 56100, Italy  
⁴Department of Clinical and Experimental Medicine, University of Pisa, Pisa 56100, Italy

Correspondence to: Prof. Andrea Nicolini, Department of Oncology, Transplantations and New Technologies in Medicine, University of Pisa, via Roma 57, 56127 Pisa, Italy. E-mail: andrea.nicolini@med.unipi.it

How to cite this article: Nicolini A, Rossi G, Ferrari P, Morganti R, Carpi A. Final results of a 2:1 control-case observational study using interferon beta and interleukin-2, in addition to first-line hormone therapy, in estrogen receptor-positive, endocrine-responsive metastatic breast cancer patients. J Cancer Metastasis Treat 2022;8:13. https://dx.doi.org/10.20517/2394-4722.2021.209

Received: 16 Dec 2021  First Decision: 28 Jan 2022  Revised: 6 Feb 2022  Accepted: 18 Mar 2022  Published: 7 Apr 2022

Academic Editor: William P. Schiemann, Lucio Miele  Copy Editor: Jia-Xin Zhang  Production Editor: Jia-Xin Zhang

Abstract

Aim: We conducted a pilot study that combines immunotherapy (cyclic interleukin-2 interferon-beta sequence) and hormone therapy (HT) to overcome endocrine resistance in metastatic breast cancer.

Methods: The final results of a 2:1 control-case retrospective observational study are here shown following 22 additional months of postoperative follow-up and 6 further controls. There were 95 controls and 42 cases in total. The 95 controls were ER+/HER2- metastatic breast cancer patients who underwent first-line HT with aromatase inhibitors (AIs) or fulvestrant. Twenty-eight of them (28.9%) also received biological drugs including cyclin kinase inhibitors (CKIs). The 42 cases were ER+ metastatic breast cancer patients who received interferon beta-interleukin-2 immunotherapy in addition to first-line HT. Selective estrogen receptor modulators/down-regulators (SERMs/SERDs) were used for HT in 39 (92.9%) of them and AIs in the remaining 3.
**Results:** Median progression-free survival (PFS) and overall survival (OS) were significantly longer in the 42 studied patients who received hormone immunotherapy (HIT) than in the 95 controls (median time 33 vs. 18 months, \( P = 0.002 \), and 81 vs. 62 months, \( P = 0.019 \)). In the analysis adjusted for disease-free interval (DFI), hormone receptor, HER2 status, visceral involvement, AIs, and biological therapy, the PFS and OS hazard ratio (HR) further increased in favor of the 42 cases (\( P = 0.004 \) and \( P = 0.044 \) respectively). In the same ER+/HER2-metastatic breast cancer patients treated with both AIs and CKIs, a median PFS ranging from 25.3 to 28.18 months and a median OS of 37.5 months were observed.

**Conclusions:** This study strongly suggests multi-center randomized clinical trials should be performed to enter our proposed immunotherapy into clinical practice.

**Keywords:** Breast cancer, metastasis, hormone-dependent, hormone resistance, immunotherapy

**INTRODUCTION**

ER+/luminal, including ER+/HER2-, breast cancer is the most common type of metastatic breast cancer\(^1\text{-}^3\). It is considered immunologically "cold"\(^4\); therefore, immunological therapy is not suitable for it. In this setting, therapies that interfere with E2 signaling, such as selective estrogen receptor modulators or down-regulators (SERMs or SERDs) and aromatase inhibitors (AIs), have been seminal in reducing breast cancer mortality over the past three decades\(^5\). Nevertheless, acquired resistance occurs in about 30-50% of ER+ breast cancer patients subjected to these hormonal therapies; thus, additional or substitutive therapy to maintain the clinical benefit is required\(^6\text{-}^7\). Currently, first-line hormone therapy (HT) with AIs or fulvestrant is recommended in ER+/HER2- metastatic breast cancer patients\(^8\). Moreover, the CCND1-CDK4/6-RB pathway, which is innately fundamental to cell cycle control and governs whether a cell moves on or is arrested at the G1-S phase, has recently been recognized as a helpful molecular target to prolong the clinical benefit of first-line hormonal therapy in ER+/HER2-luminal metastatic breast cancer patients\(^9\). Therefore, following successful investigational clinical trials, some cyclin D-dependent kinase (CDK) 4/6 inhibitors (CKIs), particularly ribociclib\(^10\), palbociclib\(^11\), and abemaciclib\(^12\), have received FDA approval and are recommended in combination with AIs or fulvestrant as first-line treatment in ER+/HER2-metastatic breast cancer patients. Contrary to the current thinking, in 1992, we hypothesized that, due to the anti-proliferative action of the anti-estrogens in ER+ metastatic breast cancer, the tumoral cell G0-G1 state promoted a contemporaneous downregulation of the mechanisms that favored immune evasion\(^13\). According to our hypothesis, in these patients, multiple ER-mediated mechanisms, including immunological ones, rather than a single or few pathways, accounted for the acquired resistance to conventional anti-estrogens. If so, during clinical benefit on anti-estrogens, in the metastatic tumor microenvironment (TME), the tumoral cell G0-G1 state promoted the counteraction/reversion of the multiple mechanisms that sustained tumor growth and immune inhibition. This could lead to the stimulation of an active immune response due to interferon beta and interleukin-2 immunotherapy. In a pilot open-label study, patients who received interferon beta and interleukin-2 in addition to conventional HT were compared with a small group of historical controls or with literature data where treated subjects underwent conventional HT alone. This pilot study showed a more than 100% increase in progression-free (PFS) and overall (OS) survivals without any relevant side effects in patients who also underwent immunotherapy\(^14\). Thereafter, due to the difficulties in launching a multi-center confirmatory randomized clinical trial, we resorted to a 2:1 control-case observational study where the studied patients with clinical benefit during first-line hormone therapy were compared with a relatively large group of comparable subjects who did not undergo additional immune therapy and were treated at the same oncological department. In the first report\(^15\), the OS was subjected to preliminary analysis, and the Kaplan-Meyer curve was interrupted after 80 months due to the relatively short follow-up of the control group. Here, after an
extension of the follow-up time and inclusion of some more controls, the final results are presented and discussed.

**METHODS**

**The study design and setting**

The study was a 2:1 ratio control-case observational study of metastatic breast cancer patients that showed clinical benefit during the first-line salvage HT. The enrolment interval was longer than usual because all patients were recruited at the same oncological department, with a relatively low recruitment rate. In addition, the enrolment intervals were at least in part different for cases (1992-2013) and controls (2006-2018). Controls were included at the Oncology Center, which is part of the same Oncology Department and was launched in 2006. The study started in 1992 as an open-label exploratory trial. Following the surprisingly promising and, since 2005, more often reported results, we encountered unexpected difficulties in launching a sponsored prospective confirmatory randomized clinical trial, which was likely because the experimental drugs had both a low cost and an expired license. On the other hand, bureaucracy was an insurmountable hurdle to launching a governmental trial. Therefore, we resorted to a more feasible 2:1 control-case retrospective observational study. All data were collected from the charts of included patients at the Oncology Department of Pisa University and processed from April to October 2020. Following our initial report,[15] we spent 22 additional months of postoperative follow-up and included 6 more controls (total controls, n = 95). All data were analyzed again and are briefly described here. The principal characteristics of cases and controls are shown in Table 1.

**Criteria for inclusion of cases and controls**

The following were the inclusion criteria:

- Age > 18 years.

- Distant metastases stable or responsive to first-line SERMs, SERDs, or AIs in patients who had undergone primary mastectomy for breast cancer.

- Eastern Cooperative Oncology Group performance status < 2.

- White blood cells > 3500/µL.

- Hemoglobin > 10.5 g/dL.

- Platelet count > 125,000/µL.

- Creatininemia < 1.5 mg/dL.

- Serum bilirubin < 1.5 times the upper limit of normal.

- Aspartate aminotransferase and alanine transferase < 3 times the upper limit of normal.

- No severe and uncontrolled heart disease.
Table 1. Principal characteristics of the 137 metastatic breast cancer patients who showed clinical benefit during hormone therapy

| Patient characteristic | Controls (1st-line HT) | Cases (1st-line HIT) | P-value |
|------------------------|------------------------|----------------------|---------|
| **N**                  | 95                     | 42                   |         |
| **Gender**             |                        |                      | 0.919   |
| Female                 | 93 (97.9%)             | 41 (97.6%)           |         |
| Male                   | 2 (2.1%)               | 1 (2.4%)             |         |
| **Menopausal status**  |                        |                      | 0.788   |
| Post-menopausal        | 75 (78.9%)             | 34 (80.9%)           |         |
| Pre-perimenopausal     | 20 (21.1%)             | 8 (19.1%)            |         |
| **Age (years), average, range** | 52.6 ± 24.7 (14-149)  | 86.2 ± 41.3 (31-221) | < 0.0001|
| **Follow-up (months), mean ± sd, range** | 60 (63.2%) | 36 (85.7%) | 0.008 |
| ≤ 24 months            | 35 (36.8%)             | 6 (14.3%)            |         |
| **Kind of response**   |                        |                      | 0.051   |
| CR                     | 0 (0.1%)               | 4 (9.6%)             |         |
| PR                     | 33 (34.8%)             | 13 (30.9%)           |         |
| SD                     | 61 (64.2%)             | 25 (59.5%)           |         |
| **Hormone receptor status** |                     |                      | < 0.0001|
| ER+/PR+                | 84 (88.4%)             | 21 (50%)             |         |
| ER+/PR-                | 11 (11.6%)             | 6 (14.3%)            |         |
| ER-/PR+                | 0                      | 1 (2.4%)             |         |
| ER-/PR-                | 0                      | 9 (21.4%)            |         |
| NA                     | 0                      | 5 (11.9%)            |         |
| **Ki67/Mib-1 cut-off [34]** |                     |                      | < 0.0001|
| 25%                    | 20 (21%)               | 13 (30.9%)           |         |
| ≤ 25%                  | 66 (69.5%)             | 7 (16.7%)            |         |
| NA                     | 9 (9.5%)               | 22 (52.4%)           |         |
| **HER2**               |                        |                      | < 0.0001|
| Positive               | 0                      | 10 (26.1%)           |         |
| Negative               | 91 (95.8%)             | 26 (59.5%)           |         |
| NA                     | 4 (4.2%)               | 6 (14.3%)            |         |
| **Site of metastases** |                        |                      | 0.003²  |
| Bone                   | 38 (40%)               | 20 (47.6%)           |         |
| Visceral               | 2 (2.1%)               | 7 (16.7%)            |         |
| Soft tissue            | 17 (17.8%)             | 1 (2.3%)             |         |
| Bone and visceral      | 2 (2.1%)               | 10 (23.8%)           |         |
| Bone and soft tissue   | 22 (23.2%)             | 2 (4.8%)             |         |
| Visceral and soft tissue | 7 (7.4%)             | 0                    |         |
| Bone, visceral and soft tissue | 7 (7.4%) | 2 (4.8%) |         |
| **Number of lesions**  |                        |                      | 0.271   |
| 3                      | 67 (70.5%)             | 27 (64.3%)           |         |
| < 3                    | 24 (25.3%)             | 15 (35.7%)           |         |
| NA                     | 4 (4.2%)               | 0                    |         |

HT: Hormone therapy; HIT: hormone immunotherapy; NA: not available; ¹ER+ vs. ER-; ²visceral vs. non visceral.
• Availability to regularly carry out clinical-instrumental monitoring.

The following were the exclusion criteria:

• Previous or concomitant malignancy without a definite cure.

• The need for corticosteroids for cases only \(^{[14-15]}\).

**Cases**

All 42 recruited cases received first-line hormone immunotherapy (HIT) \([\text{Tables 1-2}]\). Cases were recruited according to the 2:1 ratio of the experimental design (2 controls for every case recruited) \(^{[15]}\).

**Conventional first-line HT and subsequent therapeutic regimens**

All 42 cases received SERMs as first-line salvage HT, i.e., tamoxifen (20 mg/day) (1992-1999 and 2003-2008) or toremifene (60 mg/day) (1998-2002), or AIs, i.e., anastrozole (1 mg/day) or letrozole (2.5 mg/day) (2008-2013). At progression, SERMs were replaced with AIs in 39 of the 42 enrolled patients. One of the three remaining progressing patients, who had been treated with AIs as first-line salvage HT, was given fulvestrant, a more recent SERD, and one received conventional chemotherapy (CT) due to anti-estrogen resistance. The last patient is still responding to anastrozole at the time of writing this report. Patients progressing to second-line salvage HT received the standard CT. For most of them, cyclophosphamide methotrexate fluorouracil and/or anthracyclines were the first regimen, followed by vinorelbine and/or 5-FU as a successive regimen. Only a minority of the cases received a further CT regimen with taxanes.

**Immunotherapy**

After two months, in which the metastatic disease of the candidates to be included had not progressed during conventional first-line salvage HT (induction time), all 42 recruited patients, in addition to undergoing HT, were given 3,000,000 IU of interferon beta i.m. every other day (three times a week) for four weeks, followed by 3,000,000 IU of interleukin-2 s.c. every other day (three times a week) for a further four weeks. For successive two weeks, HT only was given to all the included subjects, and then the same HIT schedule was started again. Thus, each cycle of HIT was ten weeks long, and HIT cycles were continued until progression. The treatment schema has been previously reported \(^{[14]}\). Four to six years after the beginning of the pilot study, the initial design of the study was adjusted. Interestingly, the rest interval between two successive cycles of immune therapy that lasted four weeks was decreased to two weeks; in addition, SERM daily dose, which in the initial design of the study was increased during interferon beta treatment, remained unchanged. All 42 patients gave written informed consent, and the study was approved by the Council of the Department of Internal Medicine of Pisa University.

**Controls**

Controls were the first 95 metastatic breast cancer patients who satisfied the same eligibility criteria as the cases and were treated from January 2006 to 10 December 2018 at the same Oncology Center, Department of Oncology, Pisa University Hospital.

**Conventional first-line HT and subsequent therapeutic regimens** \([\text{Table 1} ]\)

Most controls were ER-positive, HER2-negative patients who received AIs (letrozole, anastrozole, or exemestane) or SERM/SERD (tamoxifen/fulvestrant) as first-line salvage HT. In most controls, fulvestrant was the second-line salvage HT. Then, patients progressing to second-line HT underwent standard
Table 2. First-line salvage HT and additional treatments in the 137 endocrine-dependent metastatic breast cancer patients.

| Therapy                                      | Controls (HT) | Cases (HIT) |
|----------------------------------------------|---------------|-------------|
| **N = 95**                                  |               | **N = 42**  |
| **First line hormone-therapy**               |               |             |
| SERM/SERD, total 1                          | 17 (17.9%)    | 39 (92.9%)  |
| Tamoxifen                                   | 5             | 27          |
| Toremifene                                  | 0             | 12          |
| Fulvestrant                                 | 12            | 0           |
| AI, total                                   | 78 (82.1%)    | 3 (7.1%)    |
| Anastrozole                                 | 8             | 2           |
| Letrozole                                    | 53            | 1           |
| Exemestane                                  | 17            | 0           |
| **Additional treatments**                    |               |             |
| Molecular target therapies 2                 | 28 (28.9%)    | 0 (0%)      |
| AI plus mTOR inhibitors                      | 8             | 0           |
| AI plus bevacizumab                          | 6             | 0           |
| AI plus palbociclib                          | 6             | 0           |
| SERD plus bevacizumab                        | 1             | 0           |
| SERM plus bevacizumab                        | 3             | 0           |
| SERD plus palbociclib                        | 4             | 0           |
| Immunotherapy 3                              | 0             | 42          |

HT: Hormone therapy; HIT: hormone immunotherapy; SERM: selective estrogen receptor modulator; SERD: selective estrogen receptor down-regulator; AI: aromatase inhibitor; \(^1 P < 0.0001; ^2 P = 0.0002; ^3\) sequential low-dose beta-interferon-interleukin-2 cycles (see the Materials and Methods Section); among controls, 20 peri-/pre-menopausal patients received luteinizing hormone releasing hormone (LHRH) agonist for at least two years.

chemotherapy. For most of them, this was anthracyclines and/or taxanes and vinorelbine and/or 5-FU as first and second regimens, respectively.

Additional treatments to first-line salvage HT [Table 2]

Twenty-eight (28.9%) controls received biological therapy in addition to first-line salvage HT. According to the current guidelines, the main aim of biological therapy (everolimus, bevacizumab, and palbociclib) was to overcome or delay the occurrence of hormone resistance \(^{16-17}\). In 10 of these 28 controls who underwent biological therapy, palbociclib, a CKI, was administered in combination with AIs or SERMs/SERDs. Besides, 20 (21%) peri-/pre-menopausal controls were given luteinizing hormone-releasing hormone (LHRH) agonists for up to two years. In 15 (75%) of them, LHRH agonists were given in addition to SERM/SERD or AI, while LHRH agonists were administered with SERM/SERD or AI and bevacizumab or palbociclib in the remaining 5 (25%).

Follow-up

The disease-free interval (DFI) was the time from primary surgery to the occurrence of the metastatic disease ascertained by imaging techniques. On entry, a complete work-up to document the presence and extent of metastatic disease was carried out in all recruited subjects. Bone scans, abdominal ultrasonography, and chest X-ray, together with the gold standard examinations [computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET)], were the instrumental tools used during the initial work-up. If necessary, an invasive cyto-histology procedure was additionally performed. Complete response (CR), partial response (PR), stable disease (SD), and progressive
disease (PD) were assessed according to the Response Evaluation Criteria in Solid Tumors 1.1\cite{18}. All patients underwent control visits every 2-4 months. Consistent with the American Society of Clinical Oncology guidelines, routine blood examinations, as well as serum CEA-CA15.3 tumor marker panel measurement, were regularly carried out during any control visit\cite{19}. The conventional and/or gold standard instrumental examinations were regularly performed every 6-9 months on any patient recruited for the study for accurate monitoring of the metastatic disease. If necessary, the cyto-histology procedure was again used to ascertain or confirm new lesions and metastatic disease progression. PFS was the interval from the beginning of first-line salvage HT to metastatic disease progression documented by CT and/or MRI. PET and/or cyto-histology were also carried out when necessary. OS was the interval from the beginning of first-line HT to the last observation or death for any reason. The last observation of the 137 patients took place on 30 October 2020.

**Statistical analysis**

Absolute and relative frequency were used to describe categorical data, while mean and standard deviation were used for continuous data. Qualitative (gender, menopausal status, DFI, kind of response, hormone receptor status, Ki67/Mib-1 cut-off, HER2, site of metastases, number of lesions, and AIs as well as biological therapy) and quantitative (age and follow-up) variables, according to therapy (HT or HIT), were compared using chi-square test and t-test (two-tailed), respectively. PFS and OS curves were built using the Kaplan-Meier method, and the log-rank test was applied to evaluate differences between curves. Cox regression models, unadjusted and adjusted for DFI, hormone receptor and HER2 status, visceral involvement, AIs, and biological therapy, were also used for PFS and OS evaluation. The Ki67/Mib-1 rate was not included in the adjusted analysis due to the high number of missing data. The results of the Cox regression were expressed using both the hazard ratio (HR), with its related 95% confidence interval (95% CI), and regression coefficients (RC). Differences were considered significant at $P < 0.05$. All analyses were carried out using SPSS v.27 software. PFS was the primary endpoint, and the Kaplan-Meier curve was described up to the last observation.

**RESULTS**

There was a statistically significant difference in the mean follow-up time ($P < 0.0001$), HER2 positive status ($P < 0.0001$), visceral metastases ($P = 0.003$), and the rate of patients with Ki67/Mib-1 $> 25\%$ ($P < 0.0001$), in favor of the cases. Conversely, the percentage of patients with a DFI $\leq 24$ months ($P = 0.008$) and ER-positive patients ($P < 0.0001$) was significantly higher in the controls [Table 1]. Table 2 shows that a significantly higher proportion of controls than cases received AIs and biological therapy. In this updated study compared with the previous one\cite{15}, PFS and OS were significantly longer in the 42 studied patients than in the 95 controls ($P = 0.002$ and $P = 0.019$, respectively, in the unadjusted analysis) [Figure 1A and B]. Particularly, the median time was 33 vs. 18 months for PFS and 81 vs. 62 months for OS in the 42 studied patients compared to the 95 controls. In the unadjusted analysis, the PFS HR was 1.902 and that of OS was 1.684 in favor of the 42 studied patients. In the adjusted analysis, the PFS HR further increased to 2.533 and that of OS to 2.158 in favor of the 42 studied patients [Table 3]. Cumulative survival at 10 years was 15% in the 42 studied patients and 7% in the 95 comparable controls. One of the 42 studied patients with oligometastatic bony disease\cite{20-22} was in CR for more than 12 years from the beginning of their first-line hormone therapy\cite{23}. Different tissue immune patterns and tumor microenvironments\cite{24-25} have been reported. It has also been reported that bone metastases are immune-preserved\cite{25-26}. Despite this, no significant discrepancy in metastatic disease evolution occurred in the different tissues during first-line HIT. In fact, in the 14 cases with initial metastatic involvement of more organs [see Table 1], the same evolution (CR, PR, and SD during clinical benefit and PD at progression) was contemporaneously observed by instrumental examination in the two (12 cases) or three (2 cases) involved organs.
Table 3. PFS and OS unadjusted and adjusted for disease-free interval (DFI), hormone receptor and HER2 status, visceral involvement, and use of AIs and biological therapy in patients treated with hormone-immunotherapy (HIT: 0) compared to hormone therapy alone (HT: 1)

| Endpoint | Unadjusted analysis | Adjusted analysis |
|----------|---------------------|-------------------|
|          | HR (95%CI) | P-value | RC | HR (95%CI) | P-value |
| PFS      | 1.902 (1.275-2.837) | 0.002 | 0.929 | 2.533 (1.534-4.738) | 0.004 |
| OS       | 1.684 (1.089-2.606) | 0.019 | 0.769 | 2.158 (1.021-4.563) | 0.044 |

PFS: Progression-free survival; OS: overall survival; HR: hazard ratio; AI: aromatase inhibitors.

HIT tolerability
Good HIT tolerability was confirmed \[^{14}\]. No grade 3-4 adverse event was reported. Grade 0-1 or 1-2 events, flu-like syndrome, and injection site reaction were the most common side effects, which occurred in 60-93% and in 93% of the cases, respectively \[Table 4\].

DISCUSSION
ER-positive luminal breast cancer is considered an immunologically “cold” breast cancer subtype
In breast cancer, anti-HER2 monoclonal antibodies and PD-L1 inhibitors, combined with conventional chemotherapy, are currently the only immunotherapy drugs used in clinical practice. The former is given to HER-2 positive patients \[^{27}\] and the latter to triple-negative breast cancer (TNBC) patients \[^{28}\]. ER-positive, including ER+/HER2-, luminal breast cancer represents 60-80% of all breast malignancies, with the incidence increasing with age \[^{3-29}\]. ER-positive luminal breast cancer is considered immunologically “cold” \[^{4}\], and, therefore, immunological therapy is not suitable for it. Nevertheless, the addition of interferon beta-interleukin-2 immunotherapy to first-line salvage HT prolonged PFS and OS in an initial open-label exploratory clinical trial compared to 30 historical controls and the literature data \[^{14}\]. Despite these surprisingly promising and, since 2005, more often reported results \[^{13-15}\], we failed in launching a sponsored randomized confirmatory trial. Therefore, we resorted to a more feasible 2:1 control-case retrospective observational study conducted in a single oncology center \[^{15}\].

Main characteristics of cases compared with controls: impact on PFS and OS
ER- and/or PgR-positive breast cancer patients are expected to respond to anti-estrogen therapy. However, a roughly 20% false-negative rate of hormone receptor status evaluation by IHC has been reported for different reasons \[^{30-31}\]. Thus, mainly in the first half (1992-2003) of the interval time of case recruitment, those with clinical benefit during first-line anti-estrogen salvage therapy (induction time) were enrolled even if they were ER-negative. Therefore, 10 (23.8%) of the 42 cases were ER-negative \( (P < 0.0001) \) including three TNBC patients. For the same reason, 10 other cases were HER2-positive \[Table 1\]. At the end of the 1990s, following molecular subtype classification, ER-positive, HER2-negative breast cancer patients were then recommended by the guidelines \[^{16-17}\] to receive first-line anti-estrogen treatment. The interval for the inclusion of controls (2006-2018) followed that of cases (1992-2013). This accounted for more controls than cases being ER-positive (100%) and HER2-negative (95.8%). The difference in accrual periods accounted for a higher proportion of controls treated with AIs (82% vs. 7.1%), while SERMs/SERDs were given to 92.9% of cases and only 18% of controls \( (P < 0.0001) \). In addition, over time, SERMs (tamoxifen or toremifene) were replaced by AIs due to a clear superiority of AIs compared to SERMs in adjuvant and metastatic settings \[^{32-33}\]. Moreover, 28 (28.9%) of the 95 controls were given molecular-targeted drugs (everolimus, palbociclib, and bevacizumab) to overcome hormone resistance and prolong the clinical benefit during first-line hormone therapy \[^{16-17}\], but no case received any of these drugs since they were not available or recommended at the time of their metastatic disease. ER-positive/HER2-negative patients belong to the
Table 4. Predefined and other side effects during first-line HIT with cyclic beta-interferon-interleukin-2 in addition to antiestrogens in the 42 cases

| Side effects          | Predefined N % | First-line HIT N % |
|-----------------------|----------------|--------------------|
|                       | Grade 0-1*     | Grade 1-2*         |
| Palpitations          | 2 5            | 0 0                |
| Cardiac ischemia      | 1 2            | 1 2                |
| Coughing              | 5 12           | 0 0                |
| Anorexia              | 9 20           | 4 9.5              |
| Dyspepsia             | 4 9.5          | 2 5                |
| Nausea                | 11 26          | 2 5                |
| Vomiting              | 10 24          | 0 0                |
| Constipation          | 2 5            | 0 0                |
| Dizziness             | 2 5            | 0 0                |
| Headache              | 2 5            | 2 5                |
| Insomnia              | 2 5            | 0 0                |
| Hot flashes           | 2 5            | 0 0                |
| Vaginal bleeding      | 5 12           | 0 0                |
| Laboratory exams      |                |                    |
| Hypercreatininemia    | 7 17           | 2 5                |
| Elevated AST          | 8 19           | 0 0                |
| Elevated ALT          | 7 17           | 2 5                |
| Elevated GGT          | 23 55          | 8 19               |
| Anemia                | 28 67          | 2 5                |
| Thrombocytopenia      | 5 12           | 0 0                |
| Leucopenia            | 8 19           | 0 0                |
| Injection site reaction | 13 31      | 26 62              |
| Flu-like syndrome     |                |                    |
| Fever                 | 12 29          | 21 50              |
| Asthenia              | 31 74          | 8 19               |
| Myalgia               | 23 55          | 2 5                |
| Arthralgia            | 23 55          | 2 5                |
| Others                |                |                    |
| Asthmatic syndrome    | 0 0            | 3 7                |
| Hypoalbuminemia       | 0 0            | 3 7                |
| Flatulence            | 2 5            | 2 5                |

Toxicity graded by the United States’ National Cancer Institute (NCI) common toxicity criteria (version 2.0). *Two values of grading were given because different grades occurred during different cycles in the same patient. HIT: Hormone-immunotherapy.

luminal molecular subtype, namely the subtype with a better prognosis[34-35], while TNBC has the worst prognosis[34-35]. Accordingly, ER-negative/HER2-positive status, compared to ER-positive/HER2-negative status, is widely recognized as an unfavorable prognostic/predictive marker[36-37]. Moreover, of the 10 HER2-positive cases, eight could not receive anti-HER2-specific therapy due to the unavailability of any such drugs at the time of their metastatic disease. The two remaining patients, among the different lines successive to hormone therapy, were given lapatinib concomitantly with capecitabine, with both drugs interrupted after 3-4 months following heavy side effects (diarrhea). Most cases (92.9%) received SERMs/SERDs as first-line HT, whereas most controls received AIs (82%). While the mean clinical benefit of first-line tamoxifen administration has been reported to be about 13 months, that of AI administration is at least three months longer[32-33]. In addition, as just above mentioned, the cases, unlike some controls, could not benefit from
Figure 1. Progression-free survival correlated with hormonal therapy (survival median time of HIT 33 months (95%CI: 24-42); survival median time of HT 18 months (95%CI: 12-23)) (A). Overall survival correlated with hormonal therapy [survival median time of HIT 81 months (95%CI: 64-99); survival median time of HT 62 months (95%CI: 54-70)] (B).

molecular target therapies. Moreover, visceral metastases more often occurred in cases than in controls. Overall, the principal prognostic/predictive characteristics (hormone receptor and HER2 status, AI use as well as biological therapy, and visceral involvement) were significantly in favor of controls, except for DFI [Table 1]. Therefore, these discrepancies were expected to prolong the median PFS and OS in the 95 controls compared to the 42 cases. When all these factors were taken into consideration in the adjusted statistical analysis, both PFS and OS maintained a significant difference in favor of the 42 cases. Moreover, the HR for PFS and OS, which were 1.902 and 1.684, respectively, in the unadjusted analysis, increased to 2.533 and 2.158, respectively, in the adjusted one in favor of cases [Table 3]. Lastly, most of the 42 cases, unlike the 95 controls, could not benefit from the introduction of taxanes into current clinical practice\(^{[16-17]}\).
This likely accounts for the lower significance of OS ($P = 0.019$) than PFS ($P = 0.002$) in the cases compared to the controls.

**CKIs in addition to AIs or fulvestrant**

CKIs, in addition to AIs or fulvestrant, are currently recommended for the treatment of metastatic ER-positive/HER2-negative breast cancer patients. Ribociclib, palbociclib, and more recently abemaciclib have shown significant prolongation of PFS when added to AIs or fulvestrant; therefore, these drugs have received prompt FDA approval to be used in clinical practice. In clinical trials carried out with CKIs used in addition to AIs or fulvestrant, the median PFS ranged from 25.3 months when ribociclib was used $^{10}$ to 28.2 months when abemaciclib was used $^{12}$. In ribociclib and abemaciclib trials, the median OS has not yet been reached. Mature data in palbociclib trial $^{13}$ show no significant difference in treated patients vs. controls (34.5 vs. 37.5 months). Grade 3-4 AEs from any cause were reported in > 10%, > 15%, and 58% of patients in the arms additionally treated with ribociclib, palbociclib, and abemaciclib, respectively. Particularly, grade 3 neutropenia occurred in > 50% of patients who additionally received ribociclib or palbociclib and in 22% who additionally received abemaciclib. In our observational 2:1 control-case study, the PFS in the 42 cases treated with SERMs/SERDs plus immunotherapy drugs was longer (31 months) than that in clinical trials in which CKIs were used in addition to AIs or fulvestrant (25.3-28.2 months). No grade 3-4 AEs occurred in our 42 cases, and grade 1-2 flu-like syndrome (50%) and injection site reaction (61%) were the only serious commonly observed AEs $^{14}$. Furthermore, our proposed immunotherapy is 8-18 times cheaper than CKIs.

**Potential mechanistic rationale of HIT**

The association of our immunotherapy with anti-estrogens in ER+ metastatic breast cancer patients was based on the hypothesis that anti-estrogens reversed the inhibition of the immune system in the TME, thus allowing immune stimulation of the effector T cells by the interferon beta-interleukin-2 sequence. Recently, the potential of anti-estrogens to revert the immunosuppressive TME has been highlighted $^{38}$. The G0-G1 state induced by anti-estrogens likely favors the stimulation of the effector immune cells. In our initial open-label exploratory clinical study, immunologic laboratory data also support this effect $^{39}$. Immune stimulation by beta interferon and interleukin-2 uses a physiological pathway. This may explain why no important AEs occurred. Differently, the inhibition of the G1-S checkpoint by the CKIs involving tumoral and non-tumoral cells may account for the occurrence of some relevant AEs reported in the above-mentioned clinical trials. The persistence of the promising results over a long time confirms our rationale and suggests that active immune stimulation in metastatic patients that show clinical benefit during first-line salvage hormone therapy is the main process to investigate. Interestingly, our proposed immunotherapy may be included in an AI-CKI combination to obtain an anti-proliferative action.

**Limitations of the current study**

Following the difficulties we encountered to launch a prospective multi-center randomized clinical trial, we decided to perform a retrospective study, although we were aware that this kind of study represents a principal limitation. Possible differences in the distribution of key resistance mechanisms between cases and controls are another limitation, which pertains to most clinical studies in the same population. Regarding this last issue, well-designed investigational studies are necessary to possibly clarify if and which relationships occur among the thus far documented mechanisms of endocrine resistance as ESR1 mutations $^{40}$ or cMYC amplifications/MAPK signaling defects $^{41}$ and the above-described potential mechanistic rationale of HIT in the same type of breast cancer patients.

**Conclusion**

Overall, our findings strongly suggest that multi-center randomized confirmatory clinical trials should be performed to eventually enter our proposed immunotherapy into clinical practice.
DECLARATIONS

Authors' contributions
All authors had full access to all data of the submitted study. Nicolini A conceived the initial experimental design, conducted the study and wrote the manuscript. Rossi G conceived the retrospective control-case observational study and carried out statistical analysis of previously published reports. Ferrari P conducted the study and revised the manuscript. Morganti M. carried out statistical analysis. Carpi A conducted the study and revised the manuscript.

Availability of data and materials
All data used are available in the archives of the Department of Oncology, Oncologic Centre of Pisa University Hospital.

Financial support and sponsorship
None.

Conflicts of interest
All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate
All patients gave witnessed written informed consent, and the study was approved by the Council of the Department of Internal Medicine of Pisa University. The study was performed in accordance with the Declaration of Helsinki.

Consent for publication
Not applicable.

Copyright
© The Author(s) 2022.

REFERENCES
1. Howlader N, Altekruse SF, Li CI, et al. US incidence of breast cancer subtypes defined by joint hormone receptor and HER2 status. J Natl Cancer Inst 2014;106:dju055. DOI PubMed PMC
2. Parise CA, Bauer KR, Brown MM, Caggiano V. Breast cancer subtypes as defined by the estrogen receptor (ER), progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2) among women with invasive breast cancer in California, 1999-2004. Breast J 2009;15:593-602. DOI PubMed
3. Senkus E, Cardoso F, Pagani O. Time for more optimism in metastatic breast cancer? Cancer Treat Rev 2014;40:220-8. DOI PubMed
4. Vonderheide RH, Domchek SM, Clark AS. Immunotherapy for breast cancer: what are we missing? Clin Cancer Res 2017;23:2640-6. DOI PubMed PMC
5. Caswell-Jin JL, Plevritis SK, Tian L, et al. Change in survival in metastatic breast cancer with treatment advances: meta-analysis and systematic review. JNCI Cancer Spectr 2018;2:pky062. DOI PubMed PMC
6. Nayar U, Cohen O, Kapstad C, et al. Acquired HER2 mutations in ER+ metastatic breast cancer confer resistance to estrogen receptor-directed therapies. Nat Genet 2019;51:207-16. DOI PubMed
7. AlFakeeh A, Brezden-Masley C. Overcoming endocrine resistance in hormone receptor-positive breast cancer. Curr Oncol 2018;25:S18-27. DOI PubMed PMC
8. Najim O, Seghers S, Sergoyyne L, et al. The association between type of endocrine therapy and development of estrogen receptor-1 mutation(s) in patients with hormone-sensitive advanced breast cancer: a systematic review and meta-analysis of randomized and non-randomized trials. Biochim Biophys Acta Rev Cancer 2019;1872:188315. DOI PubMed
9. Piezzo M, Cocco S, Caputo R, et al. Targeting cell cycle in breast cancer: CDK4/6 inhibitors. Int J Mol Sci 2020;21:6479. DOI PubMed PMC
10. Hortobagyi GN, Stemmer SM, Burris HA, et al. Updated results from MONALEESA-2, a phase III trial of first-line ribociclib plus
letrozole versus placebo plus letrozole in hormone receptor-positive, HER2-negative advanced breast cancer. *Ann Oncol* 2018;29:1541-7. [DOI](https://doi.org/10.1093/annonc/mdy381) PubMed

11. Finn RS, Boer K, Bondarenko I, et al. Overall survival results from the randomized phase 2 study of palbociclib in combination with letrozole versus letrozole alone for first-line treatment of ER+/HER2- advanced breast cancer (PALOMA-1, TRIO-18). *Breast Cancer Res Treat* 2020;183:419-28. [DOI](https://doi.org/10.1007/s10549-020-05954-5) PubMed PMC

12. Johnston S, Martin M, Di Leo A, et al. Monarch 3 final PFS: a randomized study of abemaciclib as initial therapy for advanced breast cancer. *NPJ Breast Cancer* 2019;5:5. [DOI](https://doi.org/10.1038/s41523-019-0084-z) PubMed

13. Nicolini A, Carpi A, Ferrari P, Biava PM, Rossi G. Immunotherapy and hormone-therapy in metastatic breast cancer: a review and an update. *Curr Drug Targets* 2016;17:1127-39. [DOI](https://doi.org/10.2174/138945011766616033090442) PubMed

14. Nicolini A, Carpi A. Beta-interferon and interleukin-2 prolong more than three times the survival of 26 consecutive endocrine dependent breast cancer patients with distant metastases: an exploratory trial. *Biomed Pharmacother* 2005;59:253-63. [DOI](https://doi.org/10.1016/j.biopha.2004.12.002) PubMed

15. Nicolini A, Rossi G, Ferrari P, Morganti R, Carpi A. A new immunotherapy schedule in addition to first-line hormone therapy for metastatic breast cancer patients in a state of clinical benefit during hormone therapy. *J Mol Med (Berl)* 2020;98:375-82. [DOI](https://doi.org/10.1007/s00109-019-01791-6) PubMed

16. Available from: [https://www.nccn.org/professionals/physician_gls/pdf/breast.pdf](https://www.nccn.org/professionals/physician_gls/pdf/breast.pdf) [Last accessed on 29 Mar 2022].

17. Available from: [https://www.esmo.org/Guidelines/Breast-Cancer](https://www.esmo.org/Guidelines/Breast-Cancer) [Last accessed on 29 Mar 2022].

18. Eisenhauer EA, Therasse P, Bogaerts J, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). * Eur J Cancer* 2009;45:228-47. [DOI](https://doi.org/10.1016/j.ejca.2008.11.024) PubMed

19. Bast RC Jr, Ravdin P, Hayes DF, et al; American Society of Clinical Oncology Tumor Markers Expert Panel. 2000 update of recommendations for the use of tumor markers in breast and colorectal cancer: clinical practice guidelines of the American Society of Clinical Oncology. *J Clin Oncol* 2001;19:1865-78. [DOI](https://doi.org/10.1200/JCO.2001.19.15.1865) PubMed

20. David S, Tan J, Savas P, et al. Stereotactic ablative body radiotherapy (SABR) for bone only oligometastatic breast cancer: a prospective clinical trial. *Breast* 2020;49:55-62. [DOI](https://doi.org/10.1016/j.breast.2019.09.012) PubMed PMC

21. Coleman RE, Smith P, Rubens RD. Clinical course and prognostic factors following bone recurrence from breast cancer. *Br J Cancer* 1998;77:336-40. [DOI](https://doi.org/10.1054/bjcan.1998.0717) PubMed PMC

22. Pagani O, Senkus E, Wood W, et al; ES0-MBC Task Force. International guidelines for management of metastatic breast cancer: can metastatic breast cancer be cured? *J Natl Cancer Inst* 2010;102:456-63. [DOI](https://doi.org/10.1093/jnci/djp206) PubMed PMC

23. Nicolini A, Ferrari P, Morganti R, Carpi A. Treatment of metastatic or high-risk solid cancer patients by targeting the immune system and/or tumor burden: six cases reports. *Int J Mol Sci* 2019;20:5986. [DOI](https://doi.org/10.3390/ijms20235986) PubMed PMC

24. Oliver AJ, Lau PKH, Unsworth AS, et al. Tissue-dependent tumor microenvironments and their impact on immunotherapy responses. *Front Immunol* 2018;9:70. [DOI](https://doi.org/10.3389/fimmu.2018.00070) PubMed

25. Lee H, Na KJ, Choi H. Differences in tumor immune microenvironment in metastatic sites of breast cancer. *Front Oncol* 2021;11:649004. [DOI](https://doi.org/10.3389/fonc.2021.649004) PubMed PMC

26. Roato I, Ferracini R. Cancer stem cells, bone and tumor microenvironment: key players in bone metastases. *Cancers (Basel)* 2018;10:56. [DOI](https://doi.org/10.3390/cancers1005056) PubMed PMC

27. Escrivá-de-Romaní S, Arumí M, Bellet M, Saura C. HER2-positive breast cancer: Current and new therapeutic strategies. *Breast* 2018;39:80-9. [DOI](https://doi.org/10.1016/j.breast.2018.04.001) DOI

28. Kwa MJ, Adams S. Checkpoint inhibitors in triple-negative breast cancer (TNBC): Where to go from here. *Cancer* 2018;124:2086-103. [DOI](https://doi.org/10.1158/0008-5472.CAN-18-1318) PubMed

29. Howlader N, Altekruse SF, Li CI, et al. US incidence of breast cancer subtypes defined by joint hormone receptor and HER2 status. *J Natl Cancer Inst* 2014;106(5):djw055. [DOI](https://doi.org/10.1093/jnci/djw055) PubMed PMC

30. Alberts SR, Ingle JN, Roche PR, et al. Comparison of estrogen receptor determinations by a biochemical ligand-binding assay and immunohistochemical staining with monoclonal antibody ER1D5 in females with lymph node positive breast carcinoma entered on two prospective clinical trials. *Cancer* 1996;1978(4): 764-772. [DOI](https://doi.org/10.1002/(SICI)1097-0142(19960815)78:4) DOI

31. Allred DC, Bustamante MA, Daniel CO, Gaskill HV, Cruz AB 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* 1990;125:107-13. [DOI](https://doi.org/10.1001/archsurg.1990.01730010077018) PubMed

32. Mouridsen H, Gershovich M, Sun Y, et al. Phase III study of letrozole versus tamoxifen as first-line therapy of advanced breast cancer in postmenopausal women: analysis of survival and update of efficacy from the International Letrozole Breast Cancer Group. *J Clin Oncol* 2003;21:2109-42. [DOI](https://doi.org/10.1200/JCO.2003.04.076) PubMed

33. Thiürllmann B, Hess D, Köberle D, et al. Anastrozole (‘Arimidex’) versus tamoxifen as first-line therapy in postmenopausal women with advanced breast cancer: Results of the double-blind cross-over SAKK trial 21/95 - a sub-study of the TARGET (Tamoxifen or ‘Arimidex’ Randomized Group Efficacy and Tolerability) trial. *Breast Cancer Res Treat* 2004;85:247-54. [DOI](https://doi.org/10.1007/s10549-004-6202-6) PubMed

34. Kim YJ, Kim JS, Kim IA. Molecular subtype predicts incidence and prognosis of brain metastasis from breast cancer in SEER database. *J Cancer Res Clin Oncol* 2018;144:1803-16. [DOI](https://doi.org/10.1007/s00432-018-2880-1) PubMed

35. Kalimutho M, Parsons K, Mittal D, López JA, Srirahi S, Khanna KK. Targeted therapies for triple-negative breast cancer: combating a stubborn disease. *Trends Pharmacol Sci* 2015;36:822-46. [DOI](https://doi.org/10.1016/j.tips.2015.08.010) PubMed

36. Busina S. Correlation of serum levels of urokinase activation plasminogen (uPA) and its inhibitor (PAI-1) with hormonal and HER-2 Status in the Early invasive breast cancer. *Med Arch* 2018;72:335-40. [DOI](https://doi.org/10.1024/0300-8153.a003806) PubMed PMC
37. Nicolini A, Ferrari P, Duffy MJ. Prognostic and predictive biomarkers in breast cancer: Past, present and future. *Semin Cancer Biol* 2018;52:56-73. [DOI](https://dx.doi.org/10.1016/j.semcancer.2018.11.002) [PubMed]

38. Welte T, Zhang XH, Rosen JM. Repurposing antiestrogens for tumor immunotherapy. *Cancer Discov* 2017;7:17-9. [DOI](https://dx.doi.org/10.1158/2159-8290.CD-16-1097) [PubMed] [PMC](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5492707/)

39. Nicolini A, Rossi G, Ferrari P, Carpi A. Clinical and laboratory patterns during immune stimulation in hormone responsive metastatic breast cancer. *Biomed Pharmacother* 2014;68:171-8. [DOI](https://dx.doi.org/10.1016/j.biopha.2013.07.012) [PubMed]

40. Lei JT, Gou X, Seker S, Ellis MJ. [DOI](https://dx.doi.org/10.1016/j.ccr.2018.05.020) [PubMed] [PMC](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5926434/)

41. Razavi P, Chang MT, Xu G, et al. The genomic landscape of endocrine-resistant advanced breast cancers. *Cancer Cell* 2018;34:427-438.e6. [DOI](https://dx.doi.org/10.1016/j.ccell.2018.04.020) [PubMed] [PMC](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5926434/)