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# 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

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## 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.



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**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<sup>[1-3]</sup>. It is considered immunologically “cold”<sup>[4]</sup>; 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<sup>[5]</sup>. 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<sup>[6-7]</sup>. Currently, first-line hormone therapy (HT) with AIs or fulvestrant is recommended in ER+/HER2- metastatic breast cancer patients<sup>[8]</sup>. 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<sup>[9]</sup>. Therefore, following successful investigational clinical trials, some cyclin D-dependent kinase (CDK) 4/6 inhibitors (CKIs), particularly ribociclib<sup>[10]</sup>, palbociclib<sup>[11]</sup>, and abemaciclib<sup>[12]</sup>, 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<sup>[13]</sup>. 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 G0-G1 cell 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<sup>[14]</sup>. 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<sup>[15]</sup>, 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<sup>[15]</sup>, 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/ $\mu$ L.
- Hemoglobin > 10.5 g/dL.
- Platelet count > 125,000/ $\mu$ L.
- 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	61.4 (36-89)	60.1 (34-82)	0.577
Follow-up (months), mean + sd, range	52.6 ± 24.7 (14-149)	86.2 ± 41.3 (31-221)	< 0.0001
DFI			0.008
> 24 months	60 (63.2%)	36 (85.7%)	
≤ 24 months	35 (36.8%)	6 (14.3%)	
Kind of response			0.051
CR	1 (1%)	4 (9.6%)	
PR	33 (34.8%)	13 (30.9%)	
SD	61 (64.2%)	25 (59.5%)	
Hormone receptor status			< 0.0001 <sup>1</sup>
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 <sup>[34]</sup>			< 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			0.003 <sup>2</sup>
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; <sup>1</sup>ER+ vs. ER-; <sup>2</sup>visceral 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<sup>[14-15]</sup>.

### Cases

All 42 recruited cases received first-line hormone immunotherapy (HIT) [Tables 1-2]. Cases were recruited according to the 2:1 ratio of the experimental design (2 controls for every case recruited)<sup>[15]</sup>.

### 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<sup>[14]</sup>. 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 [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 chemotherapy. For most of them, this was anthracyclines and/or taxanes and vinorelbine and/or 5-FU as first and second regimens, respectively.

**Table 2.** First-line salvage HT and additional treatments in the 137 endocrine-dependent metastatic breast cancer patients

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

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

### *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<sup>[16-17]</sup>. 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<sup>[18]</sup>. 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<sup>[19]</sup>. 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<sup>[15]</sup>, 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<sup>[20-22]</sup> was in CR for more than 12 years from the beginning of their first-line hormone therapy<sup>[23]</sup>. Different tissue immune patterns and tumor microenvironments<sup>[24-25]</sup> have been reported. It has also been reported that bone metastases are immune-preserved<sup>[25-26]</sup>. 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.

### HIT tolerability

Good HIT tolerability was confirmed<sup>[14]</sup>. 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].

**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)**

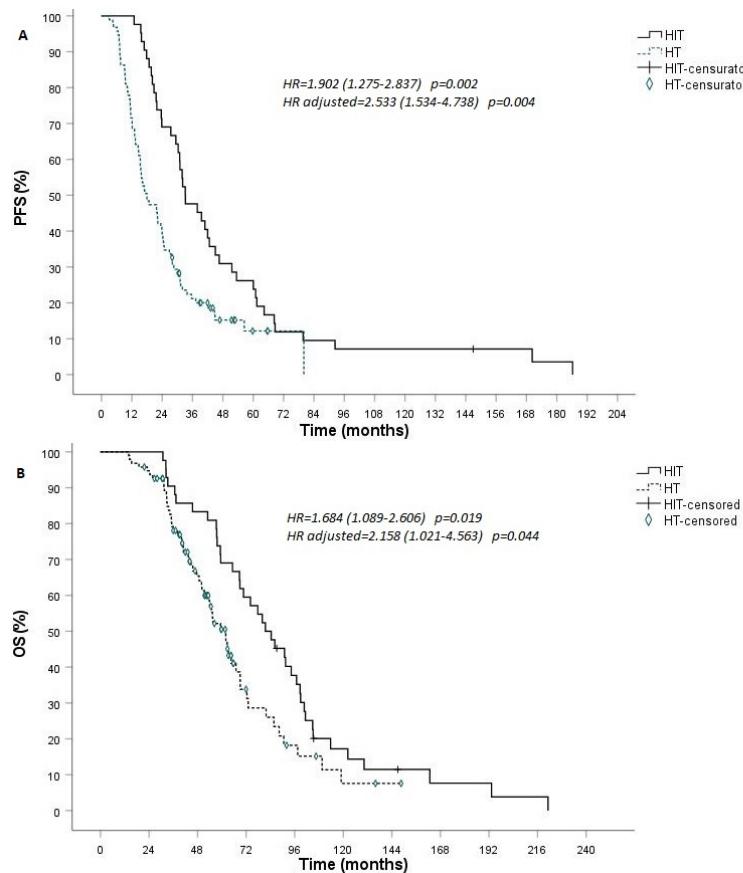
Endpoint	Unadjusted analysis		Adjusted analysis		
	HR (95%CI)	P-value	RC	HR (95%CI)	P-value
PFS	1.902 (1.275-2.837)	0.002	0.929	2.533 (1.534-4.738)	0.004
OS	1.684 (1.089-2.606)	0.019	0.769	2.158 (1.021-4.563)	0.044

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

**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	First-line HIT			
	Grade 0-1*		Grade 1-2*	
Predefined	N	%	N	%
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.



**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).

## 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<sup>[27]</sup> and the latter to triple-negative breast cancer (TNBC) patients<sup>[28]</sup>. ER-positive, including ER+/HER2-, luminal breast cancer represents 60-80% of all breast malignancies, with the incidence increasing with age<sup>[3,29]</sup>. ER-positive luminal breast cancer is considered immunologically “cold”<sup>[4]</sup>, 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<sup>[14]</sup>. Despite these surprisingly promising and, since 2005, more often reported results<sup>[13-15]</sup>, 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<sup>[15]</sup>.

### 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<sup>[30-31]</sup>. 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<sup>[16-17]</sup> 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<sup>[32-33]</sup>. 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<sup>[16-17]</sup>, 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 luminal molecular subtype, namely the subtype with a better prognosis<sup>[34-35]</sup>, while TNBC has the worst prognosis<sup>[34-35]</sup>. Accordingly, ER-negative/HER2-positive status, compared to ER-positive/HER2-negative status, is widely recognized as an unfavorable prognostic/predictive marker<sup>[36-37]</sup>. 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<sup>[32-33]</sup>. In addition, as just above mentioned, the cases, unlike some controls, could not benefit from 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<sup>[16-17]</sup>. 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<sup>[10]</sup> to 28.2 months when abemaciclib was used<sup>[12]</sup>. In ribociclib and abemaciclib trials, the median OS has not yet been reached. Mature data in palbociclib trial<sup>[11]</sup> 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<sup>[14]</sup>. 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<sup>[38]</sup>. 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<sup>[39]</sup>. 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<sup>[40]</sup> or cMYC amplifications/MAPK signaling defects<sup>[41]</sup> 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.

Conceived the initial experimental design, conducted the study and wrote the manuscript: Nicolini A

Conceived the retrospective control-case observational study and carried out statistical analysis of previously published reports: Rossi G

Conducted the study and revised the manuscript: Ferrari P

Carried out statistical analysis: Morganti R

Conducted the study and revised the manuscript: Carpi A

### 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 42 patients of the study group took part of an initial open pilot study started in 1992 where patients continued to be recruited up to 2013. In 1992 for this pilot study only needed the approval from the Council of the Department of Internal Medicine (Principal Investigator's Department) of Pisa University which was obtained; in addition any included patient gave a witnessed written informed consent; The 95 comparable controls (control group) of the retrospective observational study were treated in the Oncology Department of Pisa university (launched about 2003) from 2006 to 2017. All data of these 95 controls were collected from the charts of each included patient and were processed from April to December 2018. All original data are maintained in the archive of the Oncology Department of Pisa University and are available to be checked out at any time.

**Consent for publication**

Not applicable.

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