

# Final Results of a 2:1 Control Case Observational Study with Interferon Beta and Interleukin-2 in Addition to First-Line Hormone Therapy in ER+ Endocrine Responsive Metastatic Breast Cancer Patients.

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## Research Article

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# Abstract

Following 22 additional months of post-operative follow-up and 6 further controls (total controls, n= 95) here the final results of a 2:1 control-case retrospective observational study are shown. Controls were ER+HER-2- metastatic breast cancer patients who were given first-line hormone therapy (HT) with aromatase inhibitors (AIs) or fulvestrant. Moreover 28 of them (28.9%) also received biological drugs including cyclin kinase inhibitors (CKis). Cases were 42 ER+ metastatic breast cancer patients who received the addition of interferon beta-interleukin-2 immunotherapy to first-line HT with selective estrogen receptor modulators/down-regulators (SERMs/SERDs). Median PFS and OS maintained 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, HER-2 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 type of patients treated with AIs plus CKis a median PFS ranging from 25.3 to 28.18 months and a median OS of 37.5 months occurred. Overall, this strongly suggests multi-centre randomised clinical trials to enter our proposed immunotherapy into clinical practice.

## Key Messages

- In metastatic endocrine-dependent breast cancer patients acquired resistance is a common hurdle.
- The final results of a 2:1 control-case retrospective observational study are shown. ER+HER-2- controls received aromatase inhibitors (AIs) or fulvestrant as first-line hormone therapy (HT) (some also received biological drugs including cyclin kinase inhibitors (CKI)). Cases were ER+ patients treated with interferon beta-interleukin-2 immunotherapy plus selective estrogen receptor modulators/down-regulators.
- Median PFS and OS maintained significantly longer in the cases who received hormone immunotherapy than in the controls (median time 33 vs 18 months,  $p=0.002$  and 81 vs 62 months,  $p=0.019$  respectively). In the same type of patients treated with AIs or fulvestrant plus CKi a median PFS ranging from 25.3 to 28.18 months and a median OS of 37.5 months occurred. Moreover, unlike CKis, our proposed immunotherapy did not show any relevant adverse event and the cost is about 8 to 18 times lower.

## Introduction

ER+/luminal, including ER + HER2- breast cancer is the commonest type of metastatic breast cancer [1–3]. It is considered immunologically "cold" [4] therefore not suitable for immunological therapy. In this setting, the therapies interfering with E2 signaling, such as selective estrogen receptor modulators or downregulators (SERMs or SERDs) and aromatase inhibitors (AIs), have been seminal in reducing breast cancer mortality over the past three decades [5]. Despite this, acquired resistance occurs in about 30%-50% ER + breast cancer patients on these hormonal therapies so requiring additional or substitutive therapy to maintain the clinical benefit [6–7]. Currently, first-line hormone therapy (HT) with AIs or

fulvestrant is recommended in ER + HER-2- metastatic breast cancer patients [8]. Moreover, recently the CCND1-CDK4/6-RB pathway which is innately fundamental for the cell cycle control and governs whether a cell move on or arrests at the G1-S phase, has been recognized as a potential helpful molecular target to prolong the clinical benefit of ER + HER2- luminal metastatic breast cancer on first-line hormonal therapy [9]. Therefore, some cyclin D-dependent kinase (CDK) 4/6 inhibitors (CKIs), particularly ribociclib [10], palbociclib [11] and lastly abemaciclib [12] following successful investigational clinical trials, have received FDA approval and are recommended in combination with AIs or fulvestrant as first-line treatment of ER + HER2- metastatic breast cancer patients. Contrary to the ongoing view, in 1992, we had hypothesized that the tumoral cell G0-G1 state due to the anti-proliferative action of the anti-estrogens in ER + metastatic breast cancer promoted a contemporaneous down-regulation of the mechanisms favoring the immune evasion [13]. According to our hypothesis, in these patients multiple ERs mediated mechanisms, including the immunological ones, rather than a single or few pathways accounted for the arising of the acquired resistance to conventional anti-estrogens. If so, during a clinical benefit on antiestrogens in the metastatic tumor microenvironment (TME) the tumoral G0-G1 cell state promoted counteraction/reversion of the multiple mechanisms sustaining tumor growth and the immune inhibition. This could allow interferon beta and interleukin-2 immunotherapy to stimulate an active immune response. First, in a pilot open-label study, patients receiving 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 were given conventional HT alone. This pilot study showed a more than doubled increase in progression-free (PFS) and overall (OS) survivals without any relevant side effect in patients also receiving immunotherapy [14]. Thereafter, due to the difficulties to launch a multicenter confirmatory randomized clinical trial we resorted to a 2:1 control case observational study where the studied patients in clinical benefit during first-line hormone therapy were compared with a relatively large group of comparable subjects who were not given additional immune therapy and were recruited at the same oncological department. In the first report [15] OS underwent a preliminary analysis and the Kaplan-Meier curve was interrupted at 80 months due to the relatively short follow-up of the control group. Here, after more prolonged follow-up and some additional controls recruited, the final results are presented and discussed.

## Material And Methods

### *The study design and setting*

The study was a 2:1 ratio control-case observational study recruiting metastatic breast cancer patients in clinical benefit during first-line salvage HT. The enrolment interval was longer than usually because all patients were recruited with a relatively low recruitment rate at the same single oncological center. Also, it was at least in part different in cases (years 1992-2013) than in controls (years 2006-2018). The study started in 1992 as an open-label exploratory trial. Following the surprisingly promising and since 2005 more times reported results [14,16-20] we encountered unexpected difficulties in launching a sponsored prospective confirmatory randomised clinical trial likely because both the experimental drugs were at low cost with an expired license. On the other side bureaucracy revealed an insurmountable hurdle to launch

a governmental trial. Then we decided to resort to a more feasible 2:1 control-case retrospective observational study. All data were collected from the charts of each recruited patient at the Oncology Department of Pisa University and were processed from April to October 2020. Following our initial report [15], 22 additional months of post-operative follow-up spent and 6 further controls were consecutively enrolled (total controls, n = 95) then all data again have been analyzed and are briefly described here.

### *Inclusion and exclusion criteria*

For both cases and controls inclusion criteria were: distant metastases stable or responsive to first-line SERMs, SERDs or AIs in patients that had undergone primary mastectomy for breast cancer, an ECOG performance status  $\leq 2$ , white blood cells  $\geq 3500$ /microL, haemoglobin  $\geq 10.5$  g/dL, platelet count  $> 125000$ /microL, 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 and availability to regularly carry out clinical-instrumental monitoring were also required. Previous or concomitant malignancy without a definite cure was an exclusion criterion both for cases and controls. The need of corticosteroids was an additional exclusion criterion for cases [14-15].

### *Cases*

All 42 recruited cases received first-line hormone immunotherapy (HIT) (Table 2)

Cases were enrolled according to the 2:1 ratio of the experimental design (2 controls for every patient studied) [15].

### *Conventional first-line HT and following 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 the progression, in 39 of the 42 enrolled patients, SERMs were replaced with AIs. One of the 3 remaining progressing patients, who had been treated with AIs as first-line salvage HT, was given fulvestrant, a more recent SERD and 1 received conventional chemotherapy (CT) due to anti-estrogen resistance. The last patient is still responding to anastrozole. Patients progressing onto second-line salvage HT received the standard CT, for most of them, cyclophosphamide methotrexate fluorouracil (CMF) and/or anthracyclines was 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 a taxane alone or in combination.

### *Immunotherapy*

After two months at least during which the metastatic disease of the candidates to recruitment had not progressed during conventional first-line salvage HT (induction time), all 42 recruited patients in addition to HT were given 3.000.000 IU of interferon beta i.m. every other day (3 times a week) for 4 weeks,

followed by 3.000.000 IU of interleukin-2 s.c. every other day (3 times a week) for a further 4 weeks. For the successive 2 weeks, HT only was given to all the enrolled subjects then the same HIT schedule was started again. Thus, each cycle of HIT was 10 weeks long and HIT cycles were continued until progression. Four to 6 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 4 weeks was decreased to 2 weeks and SERM daily dose, that during interferon beta treatment was increased, no more was changed [14]. 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 consecutive metastatic breast cancer patients sharing the same eligibility criteria with the cases and treated from January 2006 to 10<sup>th</sup> December 2018 at the same Oncology Centre, Department of Oncology, Pisa University Hospital.

Conventional first-line HT and following therapeutic regimens (Table 2).

Most controls were ER-positive HER2-negative patients who were given 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 standard chemotherapy was given to patients progressing onto second-line HT, 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 in addition to first-line salvage HT received biological therapy. According to the ongoing guidelines the main aim of biological therapy (everolimus, bevacizumab, palbociclib) was to overcome or delay the occurrence of hormone resistance [21-22]. In 10 of these 28 controls receiving additional biological therapy, palbociclib, a CKi, was administered in combination with AIs or SERMs/SERDs. Besides, 20 (21%) peri/pre-menopausal controls were given LHRH agonists for up to 2 years, in 15 (75%) of them, LHRH agonists were given in addition to SERM/SERD or AI and in the remaining 5 (25%) LHRH agonists were administered with SERM/SERD or AI plus bevacizumab or palbociclib.

### *Follow-up*

Disease-free interval (DFI) was the time from primary surgery for the metastatic disease ascertained by imaging techniques. On entry, a complete work-up to document the presence and the extent of metastatic disease was carried out in all recruited subjects. Bone scans, abdominal ultrasonography and chest X-ray together with the so-called '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. At the need, invasive cyto-histology procedure was additionally performed. CR,

PR, SD and progressive disease (PD) were assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 [23]. Every 2-4 months, all patients underwent control visits. Consistent with ASCO guidelines, routine blood examinations as, well as serum CEA-CA15.3 tumour marker (TM) panel measurement were regularly carried out at any control visit [24]. The conventional and/or gold standard instrumental examinations were commonly performed every 6-12 months by any patient recruited to the study for accurate monitoring of the metastatic disease. At the need, again, the cyto-histology procedure was used to ascertain or to confirm new lesions and metastatic disease progression. PFS was the interval time from the beginning of first-line salvage HT to metastatic disease progression documented by CT and/or MRI and/or PET, and cyto-histology when necessary. OS was the interval time from the beginning of first-line HT to the last observation or death for any reason. The last observation in the 137 patients took place on 30<sup>st</sup> October 2020

### *Statistical analysis*

Absolute and relative frequency were used to describe categorical data while mean and standard deviation described 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 by chi-square test and t-test (two-tailed) respectively. PFS and OS curves were built by 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. The Ki67/Mib-1 rate has not been 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 technology. PFS was the primary endpoint, and the Kaplan–Meier curve has been described up to the last observation.

## **Results**

There was a statistically significant difference in the mean follow-up time ( $p < 0.0001$ ), HER2 positive ( $p < 0.0001$ ), visceral metastases ( $p = 0.003$ ) and the rate of patients with Ki67/Mib-1  $> 25\%$  ( $p < 0.0001$ ) in favour 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 also shows that a significantly higher proportion of the controls than the cases were given AIs and biological therapy. In this updating report compared with the previous one [15] PFS and OS maintained 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 1 A-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, it 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 [25-27] is in CR more than 12 years from the beginning of first-line hormone therapy [28]. Different tissue immune patterns and tumor microenvironments [29-30] have been reported as well as that bone metastases are immune preserved [30-31]. Despite this, during first line HIT, no significant discrepancy of metastatic disease evolution occurred in different tissues. In fact, in the 14 cases with an initial metastatic involvement of more organs (see Table 1), the same evolution (CR, PR, SD during clinical benefit and PD at the progression) was contemporaneously observed by the instrumental examinations in the 2 (12 cases) or 3 (2 cases) involved organs.

### *HIT tolerability*

Good HIT tolerability was confirmed [14]. No grade 3-4 adverse event was reported. Besides grade 0-1 or grade 1-2 flu-like syndrome and injection site reaction were the commonest side effects which occurred in about 80% and 90% of the cases respectively.

## **Discussion**

### *ER positive luminal breast cancer is considered an immunologically "cold" breast cancer subtype*

In breast cancer, anti-HER-2 monoclonal antibodies and PD-L1 inhibitors, combined with conventional chemotherapy, are currently the only immunotherapy used in clinical practice. The former is given in HER-2 positive [32] and the latter in triple negative breast cancer patients (TNBC) [33]. However, ER positive, including ER+ HER-2-, luminal breast cancer represents 60% to 80% of all breast malignancies and the incidence increases with older age [3,34]. ER positive luminal breast cancer is considered immunologically "cold" [4] therefore not suitable for immunological therapy. Unlike this, 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 literature data [14]. Despite these surprisingly promising and since 2005 more times reported results [13-14,16-20], 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 oncologic centre [15].

### *Main different characteristics of cases compared with controls: impact on PFS and OS*

The different recruitment interval time was the reason for most discrepancies. By far it is known that ER and/or PgR positive breast cancer patients are expected to respond to anti-estrogen therapy. However, roughly 20% of false negative rate of hormone receptor status evaluation by IHC for different reasons had been reported [35-36]. So, mainly in the first half (years 1992-2003) of the interval time of cases recruitment, those in 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 triple-negative breast cancer (TNBC) patients. For the same reason, 10 other cases were HER-2 positive (Table 1). At the end of nineties, following molecular subtype classification, ER positive

HER-2 negative breast cancer patients were the selected population recommended by ongoing guidelines [21-22] to receive first-line anti-estrogen treatment. The interval time of controls' recruitment (years 2006-2018) followed that of cases (years 1992-2013). This accounts for more controls than cases being ER positive (100%) HER-2 negative (95.8%). The difference in accrual period again accounted for a higher proportion of controls treated with AIs (82% vs 7.1%) while SERMs/SERDs were given to 92.9% of the cases and 18% only of the control group ( $p < 0.0001$ ). Also, over the time SERMs (tamoxifen or toremifene) were replaced by AIs due to a clear superiority of AIs versus SERMs in adjuvant and metastatic settings [37-38]. Moreover 28 (28.9%) of the 95 controls were given molecular-targeted drugs (everolimus, palbociclib, bevacizumab) to overcome hormone resistance and prolong the clinical benefit during first line hormone therapy [21-22]. Instead, no case received any of these drugs which were not available or recommended at the time of their metastatic disease. ER-positive/HER2-negative patients take part in the luminal molecular subtype, namely the type with better prognosis [39-40] while TNBC is that with the worst prognosis [39-40]. Accordingly, ER negative/ HER-2 positive compared with ER positive/HER-2 negative are widely recognized unfavorable prognostic/predictive markers [41-42]. Moreover, as to the 10 HER-2 positive cases, 8 of them could not receive anti-HER-2 specific therapy due to the lack of availability of any of these types of drugs at the time of their metastatic disease. The two remaining patients among the different lines successive to hormone therapy were given lapatinib concomitant with capecitabine that was interrupted after 3-4 months following heavy side effects (diarrhea). Most cases (92.9%) received SERMs/SERDs, unlike most controls that received AIs (82%) as first-line HT. However, while the mean clinical benefit of first-line tamoxifen has been reported to be about 13 months, that of AIs was 3 months at least longer [37-38]. In addition, cases, unlike some controls could not take benefit from molecular target therapies which again were not available at the time of their metastatic disease. Also, visceral metastases more often occurred in cases than in controls. Overall, the principal prognostic/predictive characteristics (hormone receptor and HER 2 status, AIs as well as biological therapy and visceral involvement) except DFI of the patients recruited into the cases and controls were significantly in favour of controls (Table 1). Therefore, these discrepancies were expected to prolong median PFS and OS in the 95 controls compared with 42 cases. When all these factors were taken into consideration in the adjusted statistical analysis, both the end-points maintained a significant difference in favor of the 42 cases. Moreover, the HR for PFS and OS that was 1.902 and 1.684 in the unadjusted analysis increased to 2.533 and 2.158 respectively in the adjusted one in favour 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 [21-22]. This likely accounts for lower significance of OS ( $p = 0.019$ ) than PFS ( $p = 0.002$ ) in the cases compared with controls.

#### *CKIs in addition to AIs or fulvestrant*

In metastatic ER+ HER2 negative breast cancer patients, CKIs in addition to AIs or fulvestrant, are currently recommended. These drugs which mainly are addressed to inhibit the G1-S checkpoint have been recently investigated. Early randomized clinical trials conducted with these drugs, in particular, ribociclib, palbociclib and more recently with abemaciclib have shown significant prolongation of PFS when added to AIs or fulvestrant. Therefore, these drugs received prompt FDA approval to enter into

clinical practice. In clinical trials carried out with CKIs in addition to AIs or fulvestrant, median PFS ranged from 25.3 with ribociclib [10] to 28.2 months with abemaciclib [12]. In ribociclib and abemaciclib trials, median OS has not yet been reached, however mature data in palbociclib trial [11], did not show any significant difference in treated patients vs controls (34.5 vs 37.5 months). Grade 3-4 AEs from any cause have been reported in > 10%, >15% and in 58% of patients in the arm additionally treated with ribociclib, palbociclib and abemaciclib respectively. Particularly grade 3 neutropenia occurred in >50% of patients additionally receiving ribociclib or palbociclib and in 22% with abemaciclib. In our observational 2:1 control-case study, PFS in the 42 cases treated with SERMs/SERDs plus immunotherapy was longer (31 months) than in clinical trials using CKIs in addition to AIs or fulvestrant (from 25.3 to 28.2 months). However, in our 42 cases no 3-4 grade AEs occurred and grade 1-2 flu-like syndrome (50%) and injection site reaction (61%) were the only more serious commonly observed AEs [14]. Furthermore, our proposed immune-therapy is 8 to 18 times cheaper than the CKIs.

### *Potential mechanistic rationale of HIT*

The association of our immunotherapy to 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 an immune stimulation of the effector T cells by interferon beta interleukin-2 sequence. Recently the potential of anti-estrogens to revert the immunosuppressive TME has been highlighted [43]. The G0-G1 state induced by anti-estrogens likely favour the stimulation of the effector immune cells. In our initial open-label exploratory clinical study also immunologic laboratory data supported this effect [17, 44]. The 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 check point 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. Despite the limitation of our study, which is retrospective and observational, the persistence of the promising results over a long time confirms our rationale and suggests that an active immune stimulation in metastatic patients in clinical benefit during first-line salvage hormone therapy is the main road to investigate. Interestingly our proposed immunotherapy may be added to AIs CKIs combination which implements an anti-proliferative action.

## **Conclusions**

Overall, these findings strongly suggest multi-centre randomised confirmatory clinical trials to eventually enter our proposed immunotherapy into clinical practice.

## **Declarations**

### *Funding*

No funding.

### *Conflict of interest*

No conflict of interest.

### *Ethics approval and consent*

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.

### *Availability of data and material*

All data used are available in the archive of the Department of Oncology, Oncologic Centre of Pisa University Hospital.

### *Author contributions*

All authors had full access to all data of the submitted study. A. N. conceived the initial experimental design, conducted the study and wrote the manuscript, G. R. conceived the retrospective control-case observational study and carried out statistical analysis of previously published reports, P. F. conducted the study and revised the manuscript, R. M. carried out statistical analysis, A. C. conducted the study and revised the manuscript.

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## Tables

**Table 1.** Principal characteristics of the 137 metastatic breast cancer patients in clinical benefit during hormone therapy.

| <b>Patients characteristic</b>                 | <b>Controls<br/>(1st line HT)</b> | <b>Cases<br/>(1st line HIT)</b> | <b>p-value</b>       |
|--|-----------------------------------|---------------------------------|----------------------|
| <b><i>N</i></b>                                | 95                                | 42                              |                      |
| <b><i>Gender</i></b>                           |                                   |                                 | 0.919                |
| Female   | 93 (97.9%)                        | 41 (97.6%)                      |                      |
| Male   | 2 (2.1%)                          | 1 (2.4%)                        |                      |
| <b><i>Menopausal status</i></b>                |                                   |                                 | 0.788                |
| <i>Post-menopausal</i>                         | 75 (78.9%)                        | 34 (80.9%)                      |                      |
| <i>Pre-perimenopausal</i>                      | 20 (21.1%)                        | 8 (19.1%)                       |                      |
| <b><i>Age (yrs), average, range</i></b>        | 61.4 (36-89)                      | 60.1 (34-82)                    | 0.577                |
| <b><i>Follow-up (mo), mean ± sd, range</i></b> | 52.6 ± 24.7 (14-149)              | 86.2± 41.3 (31-221)             | <0.0001              |
| <b><i>DFI</i></b>                              |                                   |                                 | 0.008                |
| > 24 mo  | 60 (63.2%)                        | 36 (85.7%)                      |                      |
| ≤ 24 mo  | 35 (36.8%)                        | 6 (14.3%)                       |                      |
| <b><i>Kind of response</i></b>                 |                                   |                                 | 0.051                |
| CR   | 1 (1%)                            | 4 (9.6%)                        |                      |
| PR   | 33 (34.8%)                        | 13 (30.9%)                      |                      |
| SD   | 61 (64.2%)                        | 25 (59.5%)                      |                      |
| <b><i>Hormone receptor status</i></b>          |                                   |                                 | <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%)                       |                      |
| <b><i>Ki67/Mib-1 cut-off [34]</i></b>          |                                   |                                 | <0.0001              |
| >25%   | 20 (21%)                          | 13 (30.9%)                      |                      |
| ≤25%   | 66 (69,5%)                        | 7 (16.7%)                       |                      |
| NA   | 9 (9.5%)                          | 22 (52.4%)                      |                      |
| <b><i>HER2</i></b>                             |                                   |                                 | <0.0001              |

|                                |            |            |                    |
|--------------------------------|------------|------------|--------------------|
| Positive                       | 0          | 10 (26.1%) |                    |
| Negative                       | 91 (95.8%) | 26 (59.5%) |                    |
| NA                             | 4 (4.2%)   | 6 (14.3%)  |                    |
| <b>Site of metastases</b>      |            |            | 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%)   |                    |
| <b>Number of lesions</b>       |            |            | 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

**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                    |
| <b><i>First line hormone-therapy</i></b> |                         |                         |
| SERM/SERD, 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                       |
| <b><i>Additional treatments</i></b>      |                         |                         |
| 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                       |
| SERM plus bevacizumab                    | 3                       | 0                       |
| SERD plus palbociclib                    | 4                       | 0                       |
| Immunotherapy <sup>3</sup>               | 0                       | 42                      |

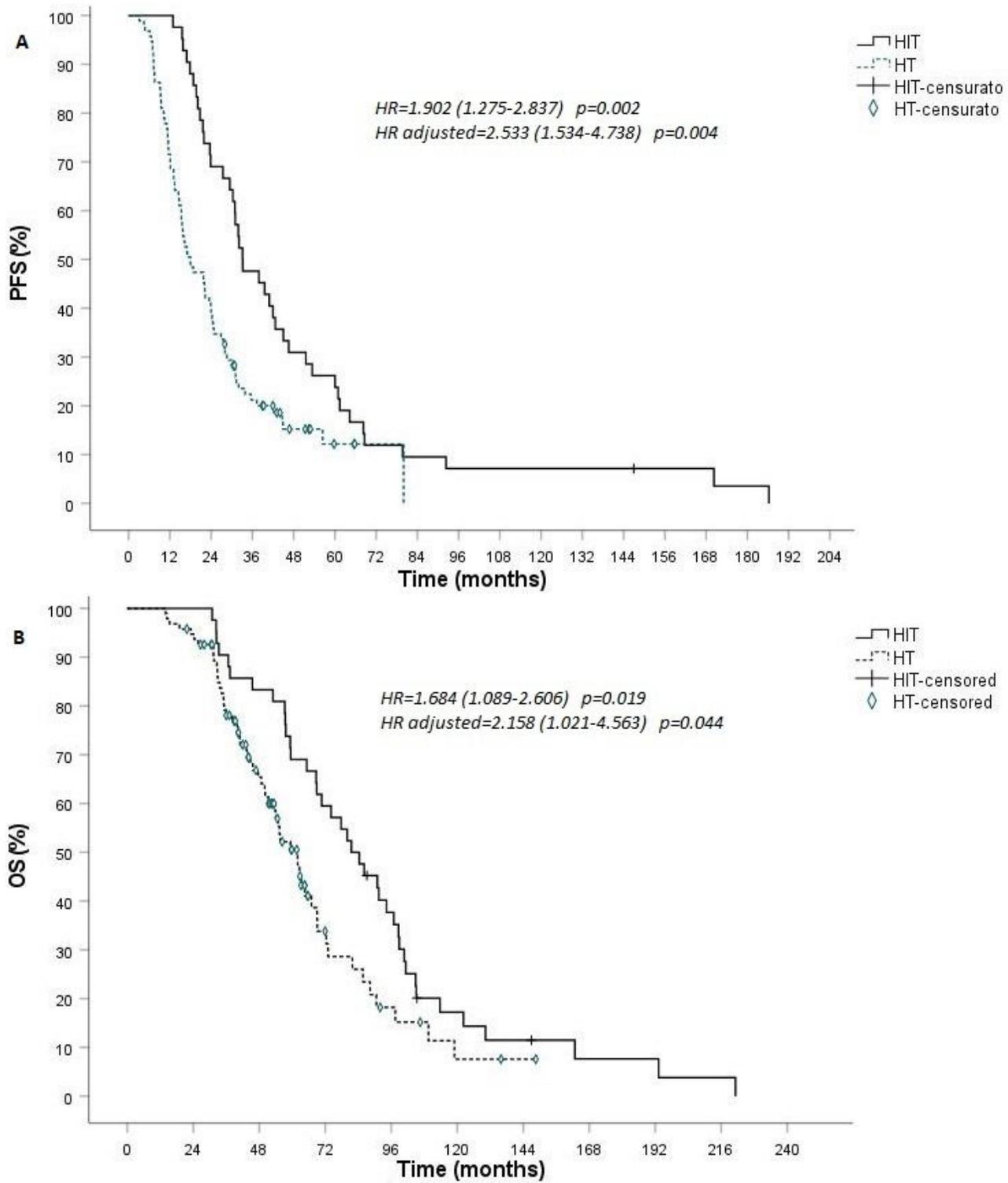
HT: hormone therapy, HIT: hormone immunotherapy, SERM: selective estrogen receptor modulator, SERD: selective estrogen receptor down-regulator, AI: aromatase inhibitor <sup>1</sup>p<0.0001, <sup>2</sup>p=0.0002, <sup>3</sup>sequential low-dose beta-interferon-interleukin-2 cycles (see materials and methods),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

**Table 3.** PFS and OS unadjusted and adjusted for disease-free interval (DFI), hormone receptor and HER2 status, visceral involvement, 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 |
| <b>PFS</b> | 1.902<br>(1.275-2.837) | 0.002   | 0.929             | 2.533<br>(1.534-4.738) | 0.004   |
| <b>OS</b>  | 1.684<br>(1.089-2.606) | 0.019   | 0.769             | 2.158<br>(1.021-4.563) | 0.044   |

PFS: progression free survival, OS: overall survival, RC: regression coefficient. Als: aromatase inhibitors

## Figures



**Figure 1**

Progression-free survival correlated to hormonal therapy [survival median time of HIT 33 (95% CI 24-42), survival median time of HT 18 (95% CI 12-23)] (A), Overall survival correlated to hormonal therapy [survival median time of HIT 81 (95% CI 64-99), survival median time of HT 62 (54-70)] (B)