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Research Paper

Association of androgen receptor and tumour-infiltrating lymphocytes with bone recurrence in triple-negative breast cancer

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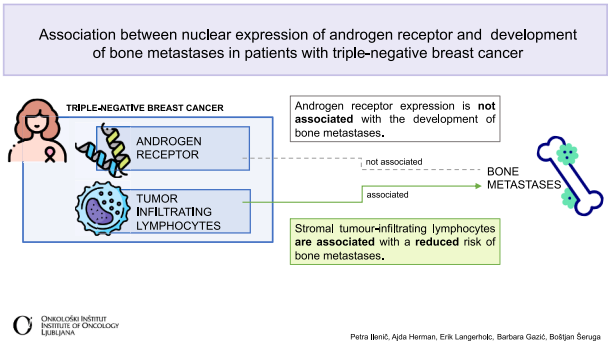
HIGHLIGHTS

- Bone recurrence in triple-negative breast cancer (TNBC) is unfavourable.
- Androgen receptor (AR) not associated with bone recurrence in TNBC.
- Stromal infiltration with lymphocytes decreases a risk for bone relapse in TNBC.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: As compared to endocrine responsive breast cancer bone is less frequent site of distant recurrence in triple-negative breast cancer (TNBC). A biomarker which predicts bone recurrence would allow a more personalized treatment approach with adjuvant bisphosphonates in TNBC. Here we hypothesised that tumour expression of androgen receptor (AR) is associated with bone recurrence in TNBC.

Materials and methods: Patients with operable TNBC who were treated at the Institute of Oncology Ljubljana between 2005 and 2015 and developed distant recurrence were included into our study. Nuclear expression of AR in the tissue of primary tumours was determined immunohistochemically by using the Androgen Receptor (SP107) Rabbit Monoclonal Antibody. We applied a logistic regression model to test the association between expression of AR and development of bone metastases. The model was adjusted for selected known prognostic factors and possible confounders in TNBC, including the level of the stromal tumour-infiltrating lymphocytes (sTILs).

Results: At recurrence 45 (45 %) out of 100 patients presented with bone metastases. Additionally, seven (7 %) developed bone metastases metachronously. AR was expressed in primary tumours of 35 (35 %) women and 19 (54.3 %) developed bone recurrence. In 25 (25 %) patients sTILs were absent. Neither the proportion of AR

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positive cancer cells (OR = 1.00; 95 % CI 0.96–1.03; p = 1.00) nor the intensity of AR positive reaction (OR = 0.71; 95 % CI 0.02–21.4; p = 1.00) were significantly associated with bone recurrence. However, women with at least mild level of the sTILs were at significantly lower risk for bone recurrence as compared to those without any sTILs (OR = 0.01; 95 % CI < 0.01–0.08; p = 0.01).
Conclusions: Expression of AR is not significantly associated with the development of bone metastases in TNBC. However, patients with absent sTILs in their primary tumours are highly susceptible for recurrence in the bone and might particularly benefit from adjuvant bisphosphonates.

1. Introduction

Bone is less frequent site of distant recurrence in triple-negative breast cancer (TNBC) as compared to the oestrogen receptor (ER)-positive breast cancer; for example, a study conducted by Dent et al. [1] showed that bone was a site of the first distant recurrence in 36.1 % and 58.6 % women with TNBC and other breast cancer subtypes, respectively. However, bone metastases are an unfavourable prognostic factor in advanced TNBC [2]. Apart from the prevention of treatment-induced bone loss, bisphosphonates may also decrease the risk of recurrence of breast cancer in the bone. Results of the large *meta*-analysis which included more than 18,000 patients with early breast cancer showed that the risk for the development of bone metastases and subsequently for breast cancer mortality can be reduced by adjuvant treatment with bisphosphonates in postmenopausal women with early breast cancer, including those with TNBC [3]. The American Society of clinical Oncology (ASCO) and the European Society of Medical Oncology (ESMO) currently recommend 2–5 years of treatment with adjuvant bisphosphonates (i.e., zoledronate or daily oral clodronate or ibandronate) in post-menopausal women or premenopausal women treated with gonadotropin-releasing hormone analogues with early breast cancer deemed at significant risk for recurrence [4,5]. However, for the majority of post-menopausal women with early TNBC treatment with adjuvant bisphosphonates may not be necessary as only about a third of those with recurrent disease present with bone metastases [1]. Furthermore, beside some well-known rare but serious adverse effects, such as hypocalcaemia, osteonecrosis of the jaw and subtrochanteric femoral fracture bisphosphonates can cause a wide range of other less serious but quite common adverse effects [6]. In contrast, patients with TNBC who are at risk for bone recurrence need effective systemic anticancer therapy. Therefore, a biomarker which predicts recurrence in the bone would allow a more personalized treatment approach with systemic treatment in women with early TNBC.

Results of a large *meta*-analysis showed that androgen receptor (AR) is expressed in 74.8 % and 31.8 % of primary tumours of patients with ER-positive and ER-negative breast cancer, respectively, which was prognostically favourable in the overall population of studied patients [7]. There is some limited evidence which suggests that AR might be implicated in the development of bone metastases in patients with breast cancer. Results of a study which sequenced RNA of the circulating tumour cells (CTCs) in women with ER-positive breast cancer showed that activation of the AR pathway is associated with the development of bone metastases [8]. However, a role of AR may be context-specific and distinct in different subtypes of breast cancer and its role in TNBC is not clear yet [9].

Here we hypothesised that nuclear expression of the AR in primary tumours of patients with TNBC is associated with the development of bone metastases.

2. Material and methods

2.1. Patients

This retrospective study was conducted at the Institute of Oncology Ljubljana (IOL) between October 2020 and April 2022. Patients with operable TNBC who started their treatment at the IOL between years 2005 and 2015 and later developed distant recurrence were included. The Cancer Registry of Slovenia was accessed to identify potentially eligible patients. All relevant demographic, clinical, and histopathological data of these patients were retrieved from patients' electronic health records. We excluded patients: (i) who did not undergo surgery for their primary breast tumour, (ii) who had concomitant second primary cancer, (iii) who developed ipsilateral loco-regional recurrence only, (iv) who developed recurrent disease within one month or more than five years after surgery of their primary tumour, (v) who were receiving bisphosphonate therapy for osteopenia/osteoporosis at the time of diagnosis of their early TNBC and (vi) in whom expression of AR in their primary tumour was not possible to determine due to the technical reasons (Fig. 1). This retrospective study was performed in compliance with relevant laws and institutional guidelines and approved by the National Medical Ethics Committee of the Republic of Slovenia on November 25th, 2020 (0120–430/2020/5).

The distant recurrence was defined as presence of metastases in distant organs and/or contralateral axillary lymph nodes without primary tumour in the contralateral breast. Patients were deemed to have bone recurrence when bone metastases were unequivocally shown by the bone scintigraphy, computer tomography (CT) and/or F-fluorodeoxyglucose positron emission tomography-computed tomography (FDG PET-CT). The site and date of the first distant recurrence were recorded for each patient. The sites of distant metastases were categorised into three groups: (i) bone, (ii) visceral organs (e.g., liver, lungs), and (iii) other tissues/organs.

The TNBC was defined by the absence of expression of ER (<1%), progesterone receptor (PR) (<1%) and overexpression of the Human epidermal growth factor receptor 2 (HER2) (immunohistochemically [IHC] ≤ 1 or Fluorescence in situ hybridization [FISH] ratio HER2/CEP17 < 2 in the case of IHC 2). The analyses of expression of the ER, PR and HER2 were performed by the specialised breast pathologist (BG).

2.2. Assessment of AR and stromal tumour-infiltrating lymphocytes (sTILs)

The AR status was determined IHC on the 2–4 μm thick tumour tissue sections which were prepared from the formalin-fixed paraffin-embedded blocks of samples from primary tumours. The IHC staining was performed using the immunostainers (Benchmark ultra or Benchmark XT, Ventana medical systems, Tucson, AZ) with the following reagents: Cell Conditioning Solution buffer (Ventana medical systems, Tucson, AZ, Catalogue number: 950–124), Androgen Receptor (SP107) Rabbit Monoclonal Antibody (1:100 dilution, CellMarque, Rockli, CA, Catalogue number: 200R) and OptiView INH Detection Kit (Ventana medical systems, Tucson, AZ, Catalogue number: 760–700). Percentage of nuclei with AR expression was calculated as a proportion of all enumerated nuclei in ten high-power fields which stained positively for AR. Intensity of reaction in AR-positive samples was assessed semi-quantitatively as: (i) weak, (ii) moderate or (iii) strong. Again, the expression of AR was evaluated by the experienced breast pathologist (BG).

Histopathologic analysis of the sTILs was performed routinely on a single full-face hematoxylin-eosin stained tumour section by experienced BG at the IOL between years 2005 and 2015. The level of sTILs was defined as density of tumour stroma containing infiltrating

lymphocytes and reported semi-quantitatively as: (i) none, (ii) mild, (iii) moderate or (iv) strong infiltration. In the neoadjuvant setting assessment of sTILs was performed before any systemic treatment with chemotherapy.

2.3. Statistical methods

Two software programs were utilised for statistical analysis: *jamovi* and *Microsoft Excel*. Descriptive statistics were employed to summarise the characteristics of included patients and their tumours. A logistic regression model to assess the independent predictive value of AR expression and intensity of positive reaction in primary tumours for the development of bone metastases was applied. The binary dependent variable was defined as: (i) bone recurrence (with or without other distant metastases) and (ii) recurrence to other distant sites. The following well-established prognostic factors and possible confounders as covariates were included: pathological tumour (T) stage, pathological node (N) stage and the level of the sTILs [10].

A logistic regression model was applied to two cohorts of patients: (i) a cohort of all included patients as described above and (ii) a cohort of patients from which patients with metachronous bone metastases were excluded. The odds ratios (ORs) and their 95 % confidence intervals (CIs) were computed to examine the relationship between AR expression and the likelihood of developing bone metastases in TNBC patients. The fit of the logistic regression model was evaluated using the McFadden pseudo R2. We considered p-value < 0.05 as statistically significant. Additionally, to account for multiple comparisons, the p-values were adjusted using the Holm's method.

3. Results

3.1. Characteristics of patients

Out of 698 women initially identified in the Cancer Registry of Slovenia, 100 patients with metastatic TNBC were eligible for our study (Fig. 1). The mean age at the diagnosis of women with operable TNBC was 57.7 $\pm$ 13.6 years. The most common subtype of breast cancer was invasive ductal carcinoma in 88 (88 %) patients. Other clinical and histopathologic characteristics are presented in Table 1. While 61 (61 %) of patients underwent adjuvant chemotherapy, 23 (23 %) received neoadjuvant chemotherapy. Within the neoadjuvant setting, 12 (52.2 %) patients were administered anthracycline-based chemotherapy, and 11 (47.8 %) received a combination of anthracyclines and taxanes. In the adjuvant setting, anthracyclines, taxanes, both anthracyclines and taxanes and other chemotherapeutic regimens were administered in 19 (31.1 %), 6 (9.8 %), 22 (36.1 %) and 14 (23.0 %) patients, respectively. Sixty-six (66 %) patients also underwent treatment with adjuvant radiotherapy.

A median time from the initial diagnosis of early TNBC and the first distant recurrence was 18 months (range 1 – 60 months). Overall, bone metastases were detected in 52 (52 %) patients, of these 7 (13.5 %) patients developed bone metastases metachronously.

3.2. Nuclear expression of AR and assessment of sTILs

Expression of AR in $\geq$ 1 % of the cellular nuclei was present in primary tumours of 35 (35 %) patients. A distribution of the nuclear expression of AR according to the proportion of nuclei stained positive for AR are presented in Fig. 2. Overall, 17 (17 %) of tumour samples showed a high level of AR expression, with $\geq$ 75 % of nuclei stained positive for AR. A distribution of nuclear expression of AR in patients with and without bone metastases was comparable, as shown in Fig. 3.

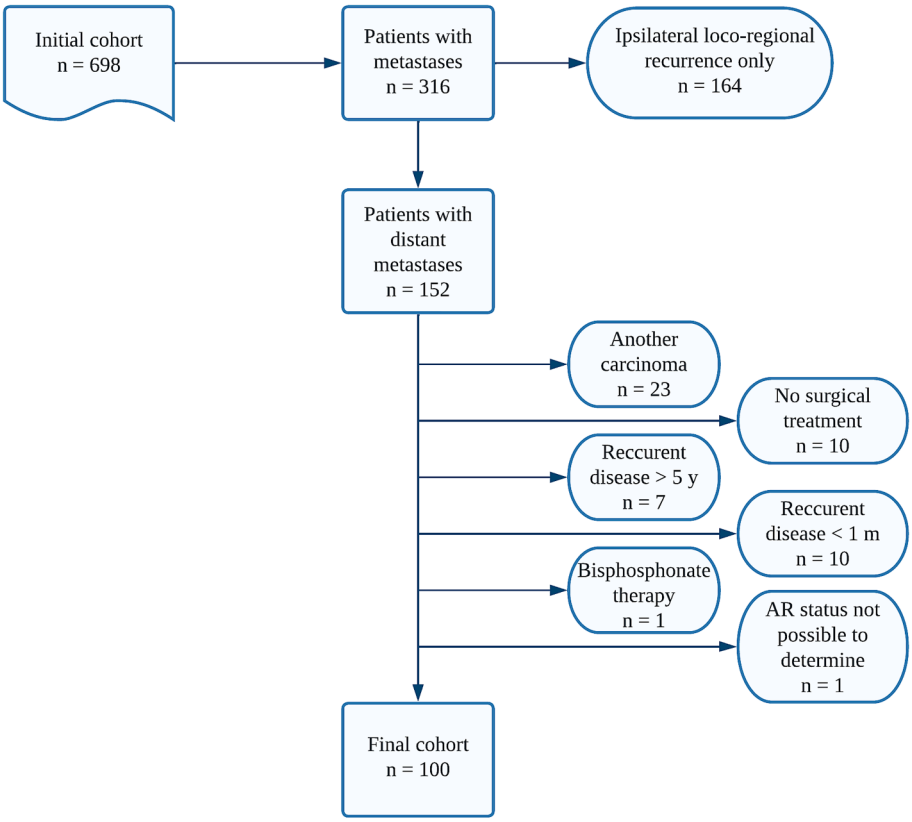

Fig. 1. Flowchart for the identification of eligible patients.

Table 1
Clinical and histopathological characteristics of patients.

Characteristic	N (%)
T-stage	
1	21 (21 %)
2	53 (53 %)
3	17 (17 %)
4	9 (9 %)
N-stage	
0	47 (47 %)
1	31 (31 %)
2	8 (8 %)
3	10 (10 %)
Unknown	4 (4 %)
Tumour Grade	
1	0
2	10 (10 %)
3	79 (79 %)
Unknown	11 (11 %)
Level of sTILs*	
0 (None)	25 (25 %)
1 (Mild)	31 (31 %)
2 (Moderate)	19 (19 %)
3 (Strong)	13 (13 %)
Unknown	12 (12 %)
AR expression (%)	
Absent (0)	65 (65 %)
Present (1–100)	35 (35 %)
Intensity of AR staining*	
0 (None)	65 (65 %)
1 (Mild)	7 (7 %)
2 (Moderate)	14 (14 %)
3 (Strong)	14 (14 %)

AR: Androgen receptor; T: tumour stage, sTILs: stromal tumour-infiltrating lymphocytes, N: Node stage.
*The level of sTILs and the intensity of AR staining were both assessed semi-quantitatively.

There was a trend towards more intense staining in samples with higher proportion of nuclei with expression of AR (Fig. 4). In 25 (25 %) of our patients no sTILs were identified in a primary tumour (Table1).

3.3. Association between AR, sTILs and bone metastases

The logistic regression model demonstrated a good fit to our data, as indicated by an R^2 McF value of 0.39. As shown in Table 2, neither the proportion of nuclei expressing AR (OR 1.0, 95 % CI 0.96 – 1.03, p = 1.0) nor the intensity of AR expression in primary breast tumours (for strong expression OR 0.71; 95 % CI 0.02 – 21.14, p = 1.0) exhibited any significant association with the development of bone metastases. All levels of sTILs in primary tumours displayed a strong association with a reduced risk of bone metastases (mild infiltration with stromal TILs: OR 0.01, 95 % CI < 0.01 – 0.08, p = 0.01). Our findings remained consistent after exclusion of patients with metachronous bone metastases.

4. Discussion

Bone metastases are an important cause of morbidity and mortality in women with TNBC [2]. In our study we did not find a significant association between the nuclear expression of AR and development of bone metastases. However, there was a very strong association between the absence of sTILs and an increased risk for bone recurrence in patients with TNBC. Therefore, patients with early TNBC and absent sTILs in their primary tumours might particularly benefit from adjuvant treatment with bisphosphonates and other new investigational therapies.

In our study 45 % of patients with TNBC presented with bone metastases at the time of their distant recurrence. This proportion is slightly higher than previously reported in similar larger studies which showed that 22.9 % – 36.1 % of patients with TNBC present with bone metastases [1,11]. Furthermore, in our cohort of patients AR was expressed in 35 % of primary tumours and this proportion is consistent with previous

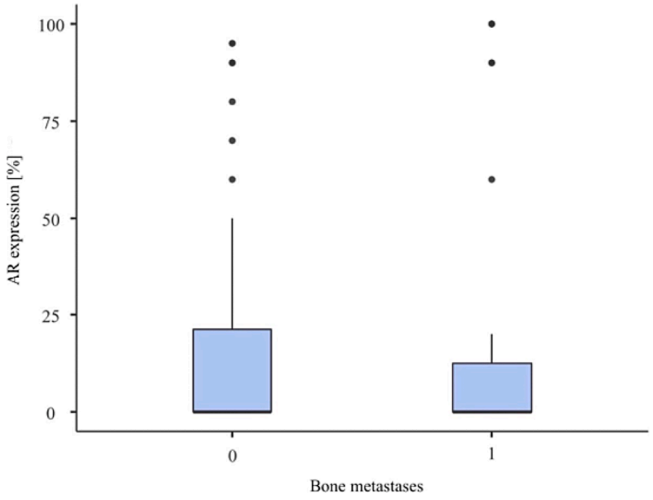

Fig. 3. Distribution of nuclear expression of AR in patients without (0) and with (1) bone metastases.

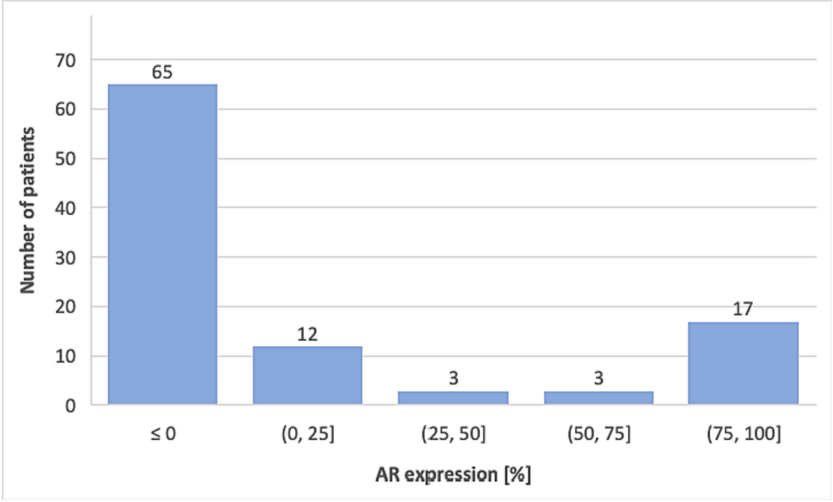

Fig. 2. Distribution of the nuclear expression of AR in cancer cells.

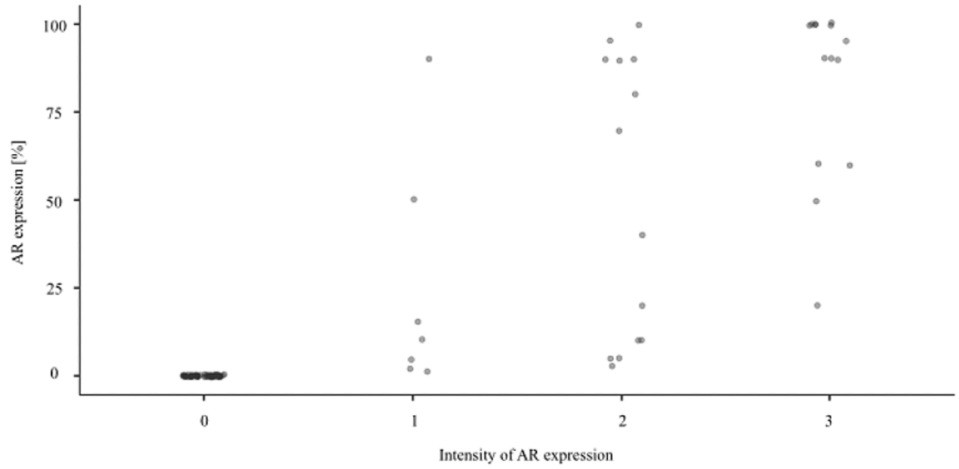

Fig. 4. Correlation between the expression of AR and the intensity of AR staining. The intensity of AR staining is defined as 0 = negative reaction, 1 = weak reaction, 2 = moderate reaction, 3 = strong reaction.

Table 2
Results of the logistic regression model exploring the independent predictive value of the selected variables for bone recurrence in TNBC.

Variable	OR	95 % CI Lower limit	95 % Upper limit	P-value	Adjusted* P-value
AR expression [%]	1.00	0.96	1.03	0.82	1.00
Intensity of AR staining	–	–	–	–	–
0 (None)(REF.)	0.93	0.04	19.30	0.96	1.00
1 (Weak)		0.02	9.38	0.63	1.00
2 (Moderate)	0.48	0.02	21.14	0.84	1.00
3 (Strong)	0.71				
T-stage					
1 (REF.)	–	–	–	–	–
2	0.45	0.10	2.08	0.30	1.00
3	0.11	0.01	1.25	0.08	0.61
4	0.04	<0.01	0.60	0.02	0.20
N-stage					
1 (REF.)	–	–	–	–	–
2	6.22	1.28	30.19	0.02	0.21
3	3.33	0.21	52.59	0.39	1.00
4	1.23	0.08	19.71	0.88	1.00
Level of sTILs					
0 (None) (REF.)	–	–	–	–	–
1 (Mild)	0.01	<0.01	0.08	<0.01	0.01
2 (Moderate)	0.01	<0.01	0.24	<0.01	0.01
3 (Intense)	0.02	<0.01	0.21	<0.01	0.02

AR: Androgen receptor; OR: Odds ratio; sTILs: stromal tumour-infiltrating lymphocytes.
*P-values are corrected with the Holm's method for multiple comparisons.

reports [7,12,13]. Neither the proportion of AR positive cancer cells nor the intensity of AR positive reaction were significantly associated with bone recurrence in our study (Table 2). Studies have shown differences in the expression of specific biomarkers between primary tumours and CTCs in different cancers, including breast cancer, most likely due to the heterogeneity of the primary cancer and the accumulating genetic instability [14,15]. In a study of 140 patients with different metastatic breast cancer subtypes de Kruijff et al. [16] showed a discrepancy in both directions in the expression of AR between CTCs and primary tumours in 58 % of patients. Similarly, smaller but still substantial discrepancy in the expression of AR between CTCs and primary tumours was reported by Li et al. [17] in a cohort of patients with ER-positive breast cancers. Moreover, CTCs can originate from metastatic sites rather than solely from the primary tumour in TNBC [18]. Several studies showed that in up to one third of patients with breast cancer there was a discrepancy between the AR status of metastases and primary tumours [19,20]. As compared to the primary tumour tissue an analysis of the CTCs and/or circulating tumour DNA (ctDNA) might be a better strategy to identify a potential biomarker for the development of bone metastases in TNBC.

In our study 25 % of women had no sTILs in their primary tumours. This is in line with the report of a pooled analysis of 2,148 patients with early TNBC where 77 % of patients had at least 1 % of sTILs in primary tumours [21]. Although our primary aim was not to seek an association between sTILs and bone recurrence we found that the absence of stromal infiltration with TILs is strongly associated with bone recurrence in TNBC. Interestingly, all levels of stromal infiltration with TILs, including mild infiltration strongly decreased the risk for bone recurrence (Table 2). It is now well established that lower levels of sTILs are associated with shorter disease-free survival (DFS) and overall survival (OS) in women with early-stage TNBC who are treated with neo/adjuvant anthracyclines with or without taxanes [21–23]. Furthermore, patients with absent sTILs but expressed AR may have particularly aggressive TNBC. In a study by Mangia et al. [24] patients with sTILs-/AR + phenotype had shorter DFS than those with sTILs-/AR-; in contrast, patients with sTILs+/AR + had an excellent outcome. Preclinical models showed that reduced expression of the Catenin Delta 1 (CTNND1) in a primary tumour, which is a protein involved in cell adhesion and signalling, promotes development of bone metastases in TNBC through the activation of the CXCR4/CXCL12 axis, which subsequently leads to activation of the PI3K/AKT/HIF-1α pathway and accelerated infiltration by neutrophiles to inhibit the cytotoxic T-cell response in bone metastases [25]. Similarly, survival data of the TNBC cohort from The Cancer Genome Atlas Program (TCGA) showed that patients with lower expression of CTNND1 in primary tumours were associated with a higher rate of recurrence to the bone, shorter distant metastases-free survival and OS as compared with patients with a higher expression of CTNND1 [25]. It is currently not known how expression of CTNND1 correlates with sTILs and AR in TNBC. It is well known that the level of sTILs tends to be lower in metastases than in primary tumours of patients with TNBC, which is consistent with the concept that cancer develops higher immunosuppression with growth and metastasizing [23,25–27]. In a study comparing immunophenoscore (IPS), a measure of immunogenicity of metastases from different site showed that bone, brain and liver metastases showed lower IPS score (i.e., lower immunogenicity) than lung metastases, irrespective of the origin of the primary tumour [28]. While sTILs have a strong prognostic value their predictive role in women with early TNBC is less clear. Although higher levels of sTILs are associated with a higher rate of pathologic complete response (pCR) a substantial proportion of patients with absent or very low sTILs can still

achieve pCR when treated with neoadjuvant chemotherapy. In a study by Denkert et al. [29] 50 % and 31 % of patients with TNBC who had ≥60 % and 0–10 % of TILs in stroma of primary tumours achieved a pCR when treated with neoadjuvant chemotherapy, respectively. In the GeparNuevo trial, which evaluated addition of the immune checkpoint inhibitor durvalumab to the chemotherapy patients with higher levels of sTILs had a higher rate of pCR irrespective of the treatment type, indicating that sTILs are not predictive for response to durvalumab [30]. In summary, patients with immunologically cold tumours, especially those who do not achieve pCR with neoadjuvant systemic therapy might be at increased risk for bone recurrence. These patients have a poor prognosis and might be candidates for adjuvant treatment with bisphosphonates and for new investigational agents.

We acknowledge several limitations of our study. Firstly, the sample size of our cohort was relatively small, which means we cannot exclude an association between the tissue AR expression and development of bone metastases with certainty. An analysis of the expression/signalling of AR in the CTCs and/or ctDNA might lead to different conclusions. Secondly, we observed that a slightly higher proportion of our patients with advanced TNBC had bone metastases as compared to other studies, suggesting that some of our patients with bone lesions may not have metastatic disease to the bones. This highlights a need for careful interpretation of the nature of bone lesions in women with advanced TNBC. Thirdly, we included patients with TNBC who developed their recurrence during the first five years after surgery. However, patients with TNBC rarely develop late recurrence (i.e. more than five years after surgery) and they were not included into our study. Fourthly, sensitivity and specificity of the IHC may vary depending on the specific antibody used [31]. Studies using polyclonal antibodies have reported higher rates of AR-positive breast cancer, while the use of monoclonal antibodies that do not recognize certain truncated variants of AR may lead to false negatives [32]. We used the monoclonal antibody SP107 which binds to the N-terminal domain of the AR and also identifies some truncated forms of AR, such as AR-V7, but not variants truncated in the N-terminal domain [31]. Fifthly, the assessment of AR expression using alternative methods could yield different results. Tumours that are classified as AR negative by the IHC may express AR mRNA when using the quantitative reverse transcription-polymerase chain reaction (qRT-PCR); on the other hand, expression of AR in tumour tissue does not necessarily mean that the protein is functional [19]. Finally, the evaluation of sTILs in our study was conducted semi-quantitatively. The assessment of sTILs according to the recommendations from the International Breast Cancer Immuno-Oncological Biomarkers Working Group may add some granularity in the understanding of the association between sTILs and bone recurrence [33]. However, as patients with absent sTILs had a substantially higher risk for bone recurrence as compared to those with only mild sTILs it is unlikely that an alternative method for the assessment of the sTILs would lead to different conclusions of our study.

In conclusion, nuclear expression of AR in primary tumour does not appear to be associated with development of bone metastases in patients with TNBC. However, due to the small sample size an association between AR and development of bone metastases cannot be excluded with certainty. Unexpectedly, we found that patients with absent sTILs have an increased susceptibility for the development of bone metastases and are consequently prone to more aggressive course of metastatic disease. Our findings highlight a need for larger studies to investigate a role of AR, sTILs and other tissue and liquid biopsy biomarkers for bone recurrence in patients with TNBC.

CRediT authorship contribution statement

Petra Ilenič: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Ajda Herman:** Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Erik Langerholc:** Conceptualization, Formal analysis, Methodology, Writing – review & editing. **Barbara Gazić:** Conceptualization, Formal analysis, Investigation, Resources, Writing – review & editing. **Boštjan Šeruga:** Conceptualization, Methodology, Resources, Formal analysis, Writing – original draft, Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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