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The significance of HOXB7 and IL17RB serum levels in prognosis of hormonally dependent breast cancer: A pilot study

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Abstract:	Purpose: Improved prognostication of a patient's outcome could allow for personalized treatment decisions in breast cancer. Homeobox B7 (HOXB7) and interleukin 17 receptor B (IL17RB) are proteins reportedly involved in the development of hormonal therapy resistance. Their prognostic value was previously investigated in tumor tissue but recent mass spectrometric detection of HOXB7 and IL17RB proteins in serum has prompted us to perform the first prognostic evaluation of their serum levels. Patients and Methods: The study included 81 premenopausal breast cancer patients that received adjuvant hormonal therapy. The median follow-up period was 61 months. HOXB7 and IL17RB serum protein levels were measured by quantitative sandwich ELISA and prognostically evaluated by Cox proportional hazards regression analysis. Results: HOXB7 protein was detected in 96.3% and IL17RB in 33.3% of serum samples. Higher levels of serum HOXB7 significantly associated with favorable disease outcome by prognosticating distant (by HR=0.04; P=0.001) and local recurrence (by HR=0.03, P=0.001). The recurrence rates in the HOXB7high and HOXB7low subgroups of patients (cut-off 81.5 pg/mL) were 0% and 17%, respectively. Serum IL17RB levels did not significantly associate with either local or distant events. The multivariate analysis highlighted estrogen receptor, histological grade, nodal status and HOXB7 as independent prognostic parameters. Conclusions: Our findings validate the previous mass-spectrometry data by showing that HOXB7 and IL17RB cellular proteins are detectable in serum by a standard ELISA assay. Furthermore, we show that HOXB7 serum levels are the relevant prognosticator of response to hormonal therapy.

The significance of HOXB7 and IL17RB serum levels in prognosis of hormonally dependent breast cancer: A pilot study

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Abstract

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Patients and Methods: The study included 81 premenopausal breast cancer patients that received adjuvant hormonal therapy. The median follow-up period was 61 months. HOXB7 and IL17RB serum protein levels were measured by quantitative sandwich ELISA and prognostically evaluated by Cox proportional hazards regression analysis.

Results: HOXB7 protein was detected in 96.3% and IL17RB in 33.3% of serum samples. Higher levels of serum HOXB7 significantly associated with favorable disease outcome by prognosticating distant (by *HR*=0.04; *P*=0.001) and local recurrence (by *HR*=0.03, *P*=0.001). The recurrence rates in the $HOXB7^{high}$ and $HOXB7^{low}$ subgroups of patients (cut-off 81.5 pg/mL) were 0% and 17%, respectively. Serum IL17RB levels did not significantly associate with either local or distant events. The multivariate analysis highlighted estrogen receptor, histological grade, nodal status and HOXB7 as independent prognostic parameters.

Conclusions: Our findings validate the previous mass-spectrometry data by showing that HOXB7 and IL17RB cellular proteins are detectable in serum by a standard ELISA assay. Furthermore, we show that HOXB7 serum levels are the relevant prognosticator of response to hormonal therapy.

Keywords: biomarker; breast cancer; hormonal therapy; HOXB7; IL17RB.

1. Introduction

Diagnosis, prognosis and treatment of cancer are the main issues in oncology. The clinical significance of recurrence prediction in breast cancer is in its potential to enhance patient survival and quality of life by enabling reliable early treatment decisions [1]. Patients reliably prognosticated at high risk of relapse would thereby receive more intense treatments, while those at low risk could be less intensely treated. Since the existing prognostic tools are still unreliable to support individual treatment decisions, this study was undertaken to evaluate novel breast cancer biomarkers. We focused on interleukin 17 receptor B (IL17RB) and homeobox B7 (HOXB7) as the proteins reportedly involved in the development of hormonal therapy resistance. Such resistance is manifested by relapse and progression of the disease and remains one of the major problems in breast cancer treatment.

Interleukins are a group of cytokines secreted by leukocytes and involved in an immune response. Several interleukins have been shown to have prognostic value in different types of human cancers and have been found to be engaged in hormonal therapy resistance [2, 3]. Within tumors, they affect the adaptive immune reaction, proliferation and survival of malignant cells, angiogenesis, metastasis and response to hormones and chemotherapeutic agents [4]. The IL-17 family of ligands and receptors shares minimal homology with other cytokines and is involved in the pathogenesis of a number of diseases such as psoriasis, rheumatoid arthritis and breast cancer [5]. IL17RB is the receptor for the pro-inflammatory cytokines IL17B and IL17E, but not for IL17A or IL17C. This receptor activates nuclear factor-kappa B (NF-κB), upregulates pro-inflammatory IL8 and regulates growth and differentiation of hematopoietic cells. Intratumoral T helper 17 cells produce IL17, the major mediator of the intratumoral inflammation which contributes to the progression of breast cancer [6].

Another protein involved in the hormonal therapy resistance is HOXB7 from the family of sequence-specific homeobox (HOX) transcription factors, with a role in embryogenesis and tissue differentiation. Although silenced in adult cells, it is widely re-expressed in cancer [7] and capable of activating several oncogenic pathways [8]. HOXB7 renders breast cancer MCF-7 cells resistant to tamoxifen [9] but prognosticates both favorable [10] and poor [8, 11, 12] disease outcomes in breast cancer. Increased expression of this gene is also associated with melanoma and ovarian carcinoma.

As mentioned above, the prognostic value of intratumoral expression of HOXB7 and IL17RB has already been reported in the breast [13, 14] and other cancers [15]. However, there are also reports that even serum levels of several intracellular proteins exert diagnostic

or prognostic value [16, 17]. This was unexpected, given the previous understanding that serum exclusively contains secretory proteins. However, in the recent mass spectrometric study confirming the presence of HOXB7 and IL17RB in the blood [18], we found firm support by evidence to initiate the first prognostic investigation of these proteins in serum.

This study explored whether HOXB7 and IL17RB can be detected in serum using a standard sandwich ELISA assay. Furthermore, we aimed to evaluate the prognostic performance of HOXB7 and IL17RB serum levels in hormonally treated breast cancer patients.

2. Patients and methods

2.1. Patients

This retrospective study included 81 premenopausal women with a median age of 44 years. Clinicopathological characteristics of the patients at the time of primary diagnosis are shown in Table 1. Patients were diagnosed at different stages of the disease, but none of them had metastatic disease at the time of diagnosis. All patients were diagnosed and underwent surgical resection. Histological specimens were examined and classified according to the criteria of American Joint Committee on Cancer / Union International Contre le Cancer (AJCC/UICC) for TNM stage, histological type, tumor grade and receptor status.

All patients received adjuvant hormonal therapy based on estrogen receptor (ER) and/or progesterone receptor (PR) proportion scoring according to Allred et al. [19]. Endocrine therapy consisted of tamoxifen alone or a combination of tamoxifen and luteinizing hormone-releasing hormone (LHRH) agonist goserelin (Zoladex®). Taken together, the selection criteria were: hormone receptor-positivity, premenopausal status and hormone therapy. The median follow-up period was 61 months.

The prospective sample size calculation was based on a pilot experiment of 35 similar patients. The calculation parameters were: target power of 0.8, effect size by hazard ratio (HR) of 5, alpha 0.05, variability in standard deviations (SD) of 0.66 and the event rate of 20%. Variability was calculated for each feature as a distance between average values of the patient subgroups with and without the actual recurrence, expressed in SDs.

The required numbers were 35 patients with 7 events. The actual patient number was 81, with 9 distant events and 9 local events. The actual average SD distance between the subgroups with and without recurrence was 0.62 for distant metastasis and 0.58 for local events. The event rate was 11% for both local and distant events. The effect size for HOXB7 was 0.03 or 33.0 for local events and 0.04 or 25 for distant events. This resulted in the actual

power of 0.999 for prognostication of the local events and 1.0 for distant events. Calculations were performed by the two-sided stpower cox test (Stata/MP 13 software, StataCorp, College Station, TX, USA).

2.2 Ethical Issues

This non-interventional, retrospective and predictive study was approved by the Institutional Ethics committee of the Institute of Oncology and Radiology of Serbia (#4428/2-01). Written informed consent was obtained from the patients for this study. This study conforms to The Code of Ethics of the World Medical Association (Declaration of Helsinki), printed in the British Medical Journal (18th July 1964) and with its later amendments.

2.2. Measurement of HOXB7 and IL17RB levels in serum

Five milliliters of peripheral blood were taken from all patients postoperatively. Blood samples were centrifuged at 950 g for 10 min and serums were stored at ≤ −20 °C.

HOXB7 and IL17RB were determined by ELISA according to the manufacturer's instructions (MBS7606548 and MBS911729, Mybiosource.com, San Diego, CA, USA).

2.3. Measurement of hormone levels in serum

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

2.4. Prognostic performance evaluation

Categorization of the continuous values measured in serum was achieved by the outcome-oriented optimal cut-point selection by use of the log-rank test and the X-tile 3.6.1 software from Yale University (New Haven, CT, USA) [20]. Univariate Cox proportional hazards regression test was performed for comparison of the prognosticated and actual, local and distant events. The HR designates the effect size by Cox regression, corresponding to recurrence rates in high- and low-risk groups of patients (IBM SPSS Statistics for Windows version 23, IBM Corp. Chicago, IL, USA). Each feature satisfied the proportional hazards assumption based on the Schoenfeld residuals by phtest (Stata/MP 13 package, StataCorp, College Station, TX, USA). Multivariate stepwise Cox proportional hazards regression analysis was performed to test for independence of each prognostic factor. Variables

categorized by the outcome were added to a full model using the forward selection entry criterion of $P < 0.20$ in univariate analysis and removed using backward elimination by the selection stay criterion of $P < 0.05$ in IBM SPSS Statistics for Windows.

2.5. Validation

The *P*-values and confidence intervals (95%CI) of the obtained HRs were corrected for bias using the bootstrap internal validation in IBM SPSS Statistics version 23 for Windows. Bootstrap resample validation is a very powerful tool for testing model stability by constructing confidence intervals and calculating *P*-values. It was proposed in 1979 [21] but spread into a wide academic use much later as it largely depends on computing power. The used bootstrap variant of resampling with replacement produces new "surrogate" data sets with the same number of cases as the original data set. This is achieved by a random selection of observations from the original sample until the same number of observations is achieved, followed by calculation of prognostic estimates such as the 95%CI and *P*-value. The performed bootstrap is defined as “resampling with replacement” because the selected observations are not removed from the pool during resampling. Therefore, some measurements may be selected multiple times while certain observations may not appear in a resample. By creating 1000 different resamples the bootstrap does not increase information but offers a more stable estimate of the prognostic performance.

3. Results

The average serum HOXB7 level for the whole patient group was 107 pg/mL, ranging from 13.7 – 1070 pg/mL, while the average serum IL17RB level was 535 pg/mL, ranging from 174 – 4393 pg/mL. For HOXB7, 96.3% of the samples were above the sensitivity level of 5 pg/mL and 80.2% were within the detection range of 31.2 – 1000 pg/mL, while for IL17RB, 33.3% of the samples were above the sensitivity threshold of 156 pg/mL and 24.7% were within the detection range of 625 – 40000 pg/mL.

Table 1 presents the clinicopathological parameters of the patient group. The prognostic performance of clinicopathological characteristics, HOXB7 and IL17RB, is presented in Table 2. Grade, nodal and hormone receptor (ER, PR) status exerted prognostic significance only when distant metastases and local relapses were separated as events. In contrast, age and HOXB7 were prognostically significant in all instances, either when local and distant events were accounted together or separately (Table 2). HOXB7 provided the most pronounced HRs in univariate analysis (0.03-0.04), while IL17RB did not provide significant prognostic

performance by any endpoint (Table 2). HRs below or above 1.0 indicate a prognostic association with good or poor disease outcome, respectively. For instance, HR of 0.03 obtained for HOXB7 indicated that patients with HOXB7 serum protein levels above the outcome-oriented threshold exerted a 33-fold lower risk of incurring an event in comparison to patients with lower HOXB7 values (Table 2). Thereby, the $HOXB7^{high}$ subgroup included 29 patients with levels over the cut-off point of 81.5 pg/mL and a homogeneous absence of distant and local recurrences, with a 0% rate (Figure 1). The recurrence rate in the $HOXB7^{low}$ subgroup (HOXB7 below 81.5 pg/mL) of 52 patients was 17%. According to that, Kaplan-Meier estimator plots illustrate the prognostic association of HOXB7 by considering the distant events (Figure 2). The upper solid line represents the low recurrence-risk $HOXB7^{high}$ subgroup with 29 patients, while the lower dotted line indicates the high recurrence-risk $HOXB7^{low}$ subgroup with 52 patients, based on outcome-oriented cut-off point of 81.5 pg/mL. Consequently, high serum levels of HOXB7 indicate lower recurrence risk. It is important to note that Figure 1 presents numerical HOXB7 raw values measured in serum, before categorization, while the Kaplan-Meier plot presented in Figure 2 is based on the categorized HOXB7 values.

Taken together, the results presented in Table 2 and Figures 1 and 2 are compatible with the association of HOXB7 with the low recurrence risk. The prognostic value of HOXB7 and IL17RB was independent of the type of hormone therapy, as we found that the statistics in the subgroup of 59 patients treated only with tamoxifen was virtually unchanged (not shown) in comparison to the results presented in Table 2 for the whole patient group.

The average measured HOXB7 concentration was 43 pg/mL in patients with a local or distant recurrence and 118 pg/mL in the remaining patients without any recurrence. IL17RB, which did not reach prognostic significance, presented the average value for patients with a local or distant recurrence of 730 pg/mL, while for patients without any disease recurrence the average value was 479 pg/mL.

HOXB7 and IL17RB levels did not significantly correlate neither mutually nor with any of the clinicopathological parameters by Spearman's rank-order correlation test (Table 3). This result indicated that HOXB7 and IL17RB serum levels were parameters independent of available clinicopathological parameters.

The multivariate Cox proportional hazards regression analysis of the metastasis risk included age, LH, ER, PR, grade, nodal status, tumor size, stage and HOXB7 since they satisfied the forward entry criterion of $P \leq 0.2$ in the univariate analysis by distant events (Table 2). This analysis highlighted nodal status, ER, grade and HOXB7 as the independent

prognostic factors (Table 4). Multivariate analysis was performed by considering distant events because these are the most relevant for disease outcome, and the distant events also allowed the inclusion of the majority of clinicopathological parameters (Table 2).

4. Discussion

This study was based on the recent finding of many intracellular and membrane proteins, including HOXB7 and IL17RB, in human serum [18]. We report the first prognostic evaluation of HOXB7 and IL17RB serum levels in breast cancer. The serum is clinically exceptionally important as a sampling source because it allows repeated measurements. HOXB7 and IL17RB were prognostically evaluated in the hormone receptor-positive patient group due to their known involvement in resistance to hormonal therapy.

The intratumoral expression of HOXB7 and IL17 RB was previously reported to associate with metastasis risk, while this study made the first attempt to measure HOXB7 and IL17RB in serum [10, 11, 14]. Our detection of these proteins in the circulation is in agreement with the previous mass spectrometry studies which identified both HOXB7 and IL17RB in serum [18]. Many other transcription factors and membrane receptors have been detected in circulation [17, 18] but it remains unclear whether these circulatory proteins are modified in comparison to their intracellular counterparts. The function of HOXB7 and IL17RB in circulation is also uncertain since proteins may exert different functions in intracellular and extracellular microenvironments [22].

HOXB7 has been mainly reported as tumor-promoting with high intratumoral levels associated with a poor disease outcome in breast and colorectal carcinoma [11, 23, 24]. Our current data may, therefore, seem in contradiction to the previous studies as we report the significant association of high HOXB7 serum levels with the favorable outcome. However, this is not the case because none of the previous studies measured HOXB7 in serum and it is unlikely that HOXB7 in serum fully reflects its intratumoral content which was measured in previous studies [11, 23, 24]. Also, it is possible that HOXB7 could have different functions in various cellular contexts. Only one previous study [10] reported the results in accordance with ours, but it was also related to intratumoral expression of HOXB7. Comparing the expression levels of different HOX genes among the different types of cancer tissues, Makiyama et al. [10] found, that the expression level of HOXB7 was lower in lymph node metastasis-positive cancer tissues than in the negative cancer tissues. It can be also speculated, that the existing detectable level of HOXB7 released into the circulation is in large part a marker of increased malignant cell death and thus prognosticates favorable outcome. This

possibility is supported by the fact that nucleosomes, which presumably contain HOXB7, are often released by necrosis or apoptosis [25, 26] and circulating nucleosomes are known to be elevated in the sera of cancer patients [27]. Alternatively, HOXB7 might have been released into extracellular space by healthy cells throughout the body and viable tumor cells through an active process such as exosome or microvesicle release [28]. The unconventional protein secretion represents an additional active mechanism by which intracellular proteins can be delivered onto the plasma membrane or extracellular matrix [29]. In the case of high serum HOXB7 deriving mostly from viable intratumoral cells, the association with low metastasis risk reported here might have been mediated through basic fibroblast growth factor (bFGF) upregulation. This possibility is based on the fact that HOXB7 is known to upregulate bFGF [30], which has previously been shown to associate with favorable breast cancer outcome by us [31] and others [32, 33].

Serum levels of a number of cytokines [3] and their soluble receptors [34] provide significant prognostic value in breast cancer. However, the membrane receptors such as IL17RB were not expected in serum and were therefore measured only intratumorally [35]. However, we were able to detect IL17RB protein in serum by a standard sandwich ELISA test. Similarly to HOXB7, the circulatory IL17RB might equally have originated from necrotic and/or apoptotic cells or activated viable cells of various types that are known to produce and shed membrane microvesicles or exosomes into their surroundings. The biological role of these structures is poorly understood, but may include secretory processes, immunomodulation, coagulation and intercellular communication [36]. Interestingly, although IL17RB promoted chemoresistance in tumor epithelial cell lines [37] and its expression in tumor tissue associated with poor prognosis [35], the serum levels of IL17RB did not associate either with local or distant recurrences. The reason for that might be the low detectability of IL17RB in serum samples, as IL17RB protein was detected in only 33.3% of samples by ELISA. In contrast, HOXB7 protein was detected in 96.3% of samples. Lower detectability of IL17RB might reflect the lower sensitivity of its ELISA assay (156 pg/mL) in comparison to HOXB7 (5 pg/mL).

We tested HOXB7 and IL17RB proteins in the hormone receptor-positive patient group due to their implication in resistance to hormonal therapy [9, 38]. HOXB7 thereby physically interacts with ERα [9], and the HOXB7–ERα complex enhances transcription of many ERα target genes, including HER2 [39]. In this study, serum levels of HOXB7 and IL17RB did not correlate with tumor ER levels neither with serum estradiol levels. Moreover, the absence of a significant correlation of HOXB7 with any other clinicopathological

parameters suggested an independent prognostic value of HOXB7 and this was confirmed by multivariate analysis. On the contrary, the absence of correlation between IL17RB and clinicopathological parameters may be explained by IL17RB serum levels that were below the sensitivity of the ELISA test in most of the patients (67%). Due to hormone receptor-positivity of breast cancer, these patients were treated with adjuvant endocrine therapy such as LHRH and/or tamoxifen [40]. LHRH agonists are recommended in younger breast cancer patients as they induce temporary ovarian suppression and thus preserve ovarian function from the toxic effects of chemotherapy [41] in premenopausal women who are at high risk of relapse [42]. However, in spite of the effectiveness of hormonal therapy, ER+ breast cancers still exert high recurrence rates, in large part due to the phenomenon of resistance to hormonal therapy.

4.1.Limitations and advantages of the study

The main limitation of this study was its sample size of 81 patients, although it exceeded the sample size requirement. This study investigated the performance of HOXB7 and IL17RB in the prognostic model limited to their serum levels in breast carcinoma patients with and without recurrences because it was a pilot study primarily directed to check if it is possible to detect intracellular or membrane proteins in circulation. The fact that there was a significant difference in average HOXB7 levels between the groups of patients with and without recurrences (118 pg/mL versus 43 pg/mL) is indicative per se; the same refers to IL17RB levels. According to that, we assumed that this was a convenient model, however, additional validation by studies in a larger patient group and involvement of healthy controls would be needed to further characterize the prognostic clinical validity of the analysis performed in this study.

Advantages of this study include an improvement of the statistical reliability by using bootstrap as a bias-correction method. The advantages further include the use of a breast cancer patient group which is uniformly positive for ER and PR. Such design has reduced group inhomogeneity which could have masked the prognostic performance of HOXB7 or IL17RB. Furthermore, prognostic factors measured in serum provide an important advantage in clinical use by enabling repeated measurements.

5. Conclusions

In conclusion, our findings validate the previous mass-spectrometry data by showing that HOXB7 and IL17RB cellular proteins are detectable in serum by a standard ELISA

assay. The origin of the serum HOXB7 and IL17RB remains unclear, but the involvement of active or passive release from malignant tumor cells seems plausible. This study further advances in the understanding of the field by showing that HOXB7 as the intratumoral prognostic biomarker also exerts significant prognostic value when measured in serum by ELISA. HOXB7 seems to be a relevant predictor of response to hormonal therapy since higher levels of serum HOXB7 significantly associated with favorable disease outcome. It needs to be emphasized, that the observed prognostic power of HOXB7 was in line with the ER as the main determinant of hormonally-dependent breast cancer. Although this conclusion should be tested in a larger group of patients, this promotes the potential clinical relevance of HOXB7 in breast cancer prognosis especially because ELISA is widely used and inexpensive method in routine clinical laboratory practice and serum is an easily available source of biomarkers.

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The Author Contribution

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Declaration of Competing Interest

The authors declare no conflict of interest.

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Jacek Nikliński, Editor-in-Chief

Advances in Medical Sciences

RE: Submission of the revised manuscript to the *Advances in Medical Sciences*

February 12, 2021

Dear Professor Nikliński,

Enclosed we send you the revised manuscript entitled "The significance of HOXB7 and IL17RB serum levels in prognosis of hormonally dependent breast cancer: A pilot study".

Changes within the revised manuscript text which were introduced as a response to suggestions by the Reviewers are marked in red font colour. For clarification, we have introduced several minor textual changes. These changes are marked by underlining.

We found the Reviewers' suggestions very helpful. Below we address their comments in detail.

Changes according to the comments of Reviewer 1

1) With majority of the research reporting HOXB7 as a poor prognostic marker in various cancers, it is an interesting observation that this study reports higher levels of serum HOXB7 is significantly associated with favourable disease outcome. While the study has a decent sample size to be considered for a pilot study, the outcome of the study warrants higher sample size to validate their findings with certain level of confidence.

Author response: In the original version of the manuscript we have used the sample size calculation to objectively assess the needed patient numbers in order to achieve sufficient level of prognostic confidence. Thereby, the current size of the patient group in this study already warrants the statistically sufficient

level of confidence as it exceeded the statistical sample size requirements by more than two-fold. Prospective power analysis is the most widely accepted tool for the sample size estimation. The actual power of 0.999 for prognostication of the local events and 1.0 for distant events is at the maximum achievable levels, thus indicating the high reliability of obtained statistical analysis results. This has been described in full detail within the original *2.1. Patients* subsection of the Patients and Methods section:

"The prospective sample size calculation was based on a pilot experiment of 35 similar patients. The calculation parameters were: target power of 0.8, effect size by hazard ratio (HR) of 5, alpha 0.05, variability in standard deviations (SD) of 0.66 and the event rate of 20%. Variability was calculated for each feature as a distance between average values of the patient subgroups with and without the actual recurrence, expressed in standard deviations.

The required numbers were 35 patients with 7 events. The actual patient number was 81, with 9 distant events and 9 local events. The actual average SD distance between subgroups with and without recurrence was 0.62 for distant metastasis and 0.58 for local events. The event rate was 11% for both distant and local events. The effect size for HOXB7 was 0.03 or 33.0 for local events and 0.04 or 25 for distant events. This resulted in the actual power of 0.999 for prognostication of the local events and 1.0 for distant events".

2) With regards to the IL17RB - except for the fact that it was detected in serum, findings of the study is greatly limited by the sensitivity of the ELISA. With its detection observed only in 33% of the already limited sample size chosen for the study, it is understandable that there are no significant clinical correlations reported for the marker.

Author response: We fully agree with this remark of Referee 1 and now we also mention it in the manuscript text (Discussion section, page 11, lines 16-19).

3) The shortcomings of this study could be largely improved by increasing sample size with equal representation across the patient groups and it may also help in confirming the potential clinical relevance of the serum markers in the study.

Author response: This point of Reviewer 1 has already been elaborated in response to the point 1 above. Briefly, the sample size used in this study is already sufficient to establish the prognostic significance of the serum HOXB7, based on the performed prospective sample size calculation. Irrespectively of the current sample size, further studies in external groups are needed to establish the clinical relevance of serum HOXB7.

Changes according to the comments of Reviewer 3

Authors in the present study have shown that standard ELISA can detect HOXB7 and IL17RB proteins in patient sera where HOXB7 acts as a good prognostic marker for hormonal therapy. Small sample size affects the validity of this study and leads to bias in clinical decision-making. Overall, research method is simple and the content of the study lacks innovation. Following are the concerns with this manuscript.

Author response: We disagree with the remark of Reviewer 2 mentioning that this study lacks innovation. The original version of the manuscript mentions that this is the first study measuring protein levels of HOXB7 and IL17RB in serum (Abstract, line 6; Introduction section, page 3, lines 13-14; Discussion section, page 9, lines 3-10). This study is of high importance for prognostic advancements in cancer also because serum allows easy sampling and repeated measurements.

1) Please add graphs for HOXB7 ELISA to highlight the significance.

Author response: In response to this remark of Reviewer 2, we now include the new Figure 1 as a plot showing continuous values of HOXB7 in serum for each individual patient. The textual description of the new figure is now also included (page 17). This new Figure 1 does not duplicate the existing Kaplan-Meier plot shown in Figure 2 because this plot presents the categorized values for HOXB7.

2) Did the authors analyze the prognostic value of IL17RB?

Author response: The prognostic performance of IL17RB has been presented in the original version of the manuscript in Table 2. The presented statistical evaluation indicates that IL17RB did not exert statistically significant performance in prognostication of disease recurrence in breast cancer, with the respective *P*-values of 0.18, 0.66 and 0.14 for distant+local, distant and local metastases. This prognostic evaluation of IL17RB is now further accentuated in the text (Results section, page 8, lines 9-10).

3) Please add number at risk at tick marks in the KM plot.

Author response: These numbers have now been added in Figure 2 according to this request of Reviewer 3.

4) There are some grammatical and typological errors in the manuscript, which needs to be corrected and rewritten.

Author response: In response to this request of Reviewer 3, the entire manuscript has been corrected by Grammarly and a native English speaker.

Changes according to the comments by Editor

First of all, please make sure to point out - in the text - the STRENGTHS and the NOVELTY of your study.
Author response: According to this suggestion of the Editor, we have now expanded the explanation of strengths and novelty of the current study (Abstract, lines 2-7; Introduction section, page 2, lines 2-11 and Discussion section, page 12, lines 13-18).

We hope that you will find our manuscript now after its revision acceptable for publication in *Advances in Medical Sciences*. We look forward to your response at your earliest convenience.

Yours sincerely,

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The Author Contribution

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Data Collection: Jelena Milovanović

Statistical Analysis: Marko Radulovic

Data Interpretation: Marko Radulovic, John Greenman, Jelena Milovanović

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Literature Search: Nataša Todorović-Raković, Marko Radulovic

Funds Collection: Marko Radulovic

Declaration of Competing Interest

The authors declare no conflict of interest.

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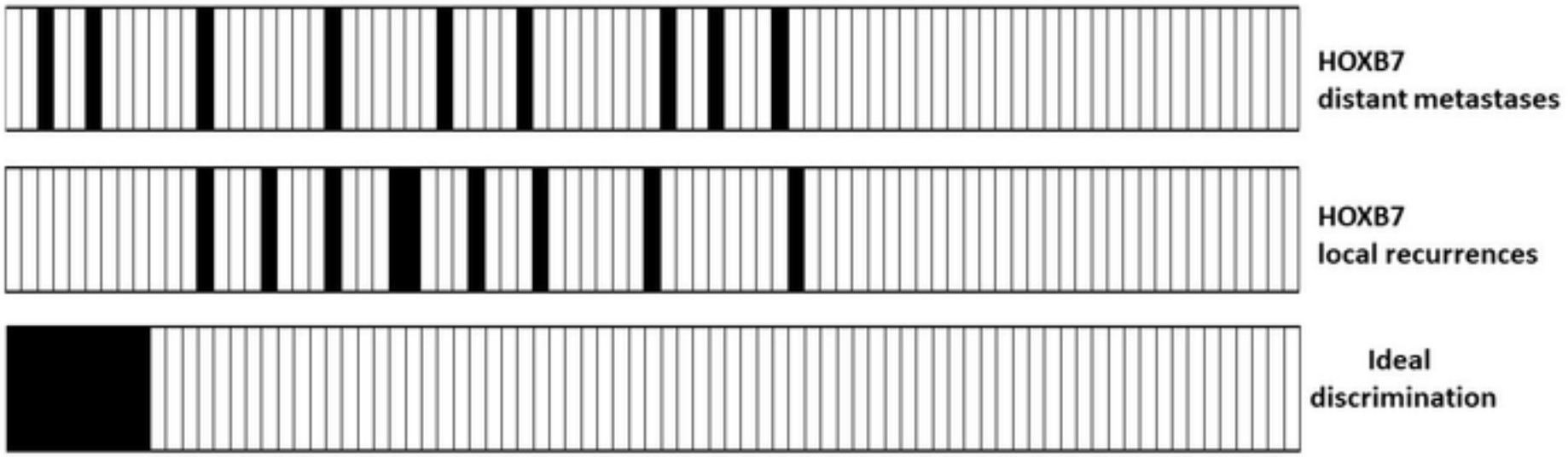

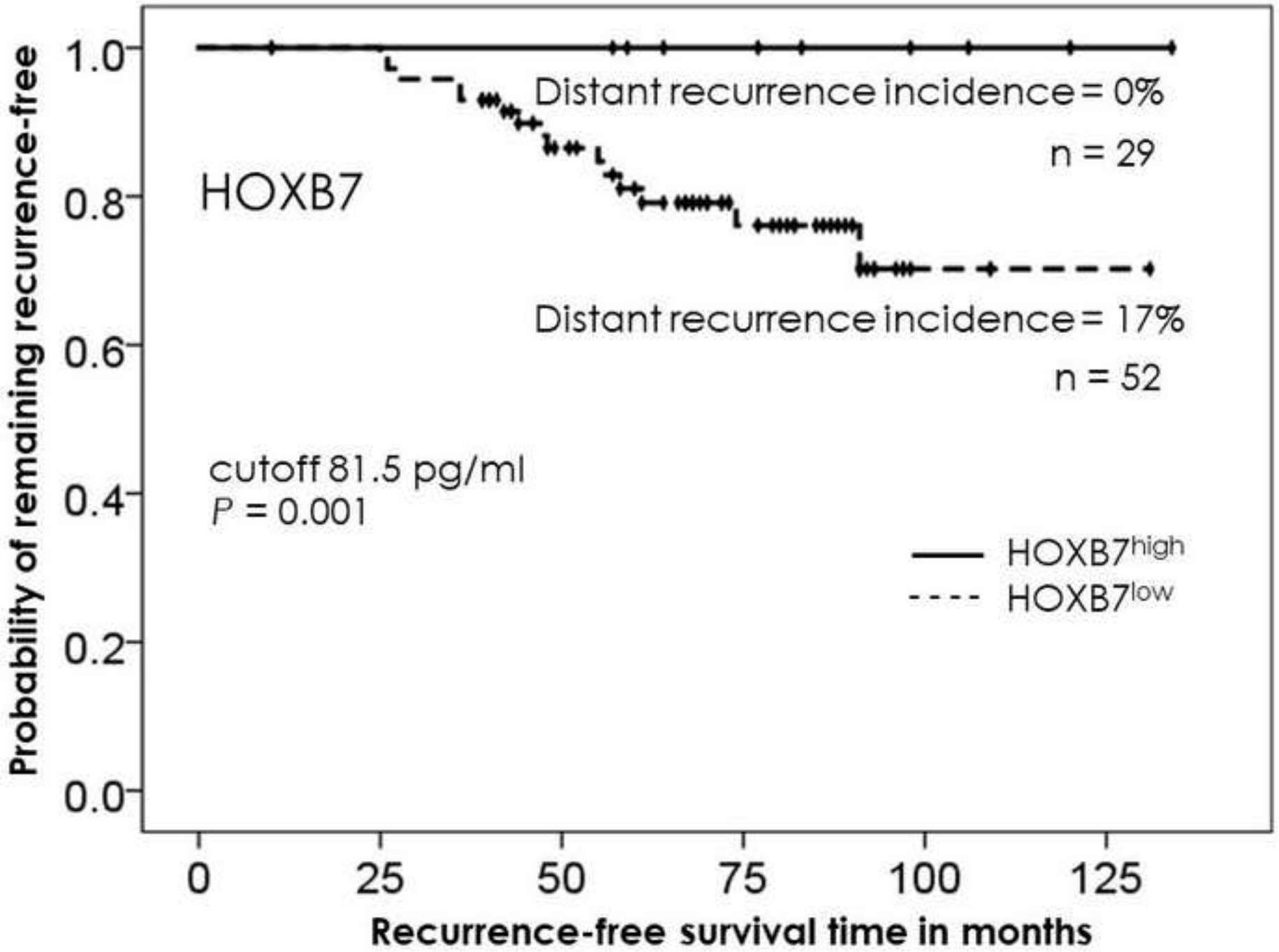

Figure Legends

Figure 1. Illustration of the obtained prognostic performance for HOXB7. Continuous values of HOXB7 for each patient are ordered sequentially from the lowest (left) to the highest (right). Patients with the actual occurrence of distant or local relapses are marked in black, while white fields indicate patients without a relapse. An ideal discrimination with AUC=1.0 is shown for orientation. The graph indicates that increasing values of HOXB7 indicate lower risk of either distant or local events.

Figure 2. Kaplan-Meier prognostic analysis of HOXB7 serum concentrations. The upper solid line represents the $HOXB7^{high}$ patient subgroup, while the lower dotted line indicates the $HOXB7^{low}$ patient subgroup. A wider separation between the upper and lower curves indicates better prognostic performance. *P*-values were calculated by the Cox proportional hazards regression test. Local and distant recurrence incidences were 0% for the $HOXB7^{high}$ patient subgroup and 17% for the $HOXB7^{low}$ patient subgroup. Classification of patients into $HOXB7^{low}$ and $HOXB7^{high}$ subgroups was performed using the outcome-oriented categorization approach. Calculation was performed by considering local and distant recurrences.

Table 1. Clinicopathological parameters at the time of primary diagnosis

Parameter	*Number of patients*	*%*
Age (years)		
< 44 (median)	37	46
≥ 44	44	54
Recurrence		
distant	9	11
local	9	11
distant + local	18	22
Menopausal status		
premenopausal	81	100
postmenopausal	0	0
Tumor size (cm)		
≤ 2	37	46
2 – 5	28	34
> 5	16	20
Nodal status		
N0	38	47
N+	43	53
Histological type		
Invasive ductal	37	46
Invasive lobular	26	32
Other types	18	22
Stage		
1	23	28
2	34	42
3	24	30
Histological grade		
G1	13	15
G2	59	73
G3	3	4
data not available	6	8
Hormone therapy		
Tamoxifen	59	73
Tamoxifen + Goserelin	22	27
Estrogen receptor status		
ER^{low}	15	19
ER^{high}	66	81
Progesterone receptor status		
PR^{low}	15	18
PR^{high}	59	73
data not available	7	9
***HER2* status**		
HER2−	62	76
HER2+	18	22
data not available	1	1
HOXB7*		
$HOXB7^{low}$	52	64
$HOXB7^{high}$	29	36
IL17RB*		
$IL17RB^{low}$	58	72
$IL17RB^{high}$	23	28

*Subgroup sizes refer to the outcome-oriented cut-off points of 81.5 pg/mL for HOXB7 and 235.1 pg/mL for IL17RB.

Abbreviations: ER, estrogen receptor; ER^{low}, ER Allred proportion score < 3; ER^{high}, ER Allred proportion score ≥ 3; PR, progesterone receptor; PR^{low}, PR Allred proportion score < 3; PR^{high}, PR Allred proportion score ≥ 3; *HER2*; human epidermal growth factor receptor 2; *HER2*–, HER2 gene not amplified; *HER2*+, HER2 gene amplification; HOXB7, homeobox B7; IL17RB, Interleukin-17 receptor B.

Table 2. Prognostic performance of clinicopathological parameters together with HOXB7 and IL17RB [a]

	Distant and local events		Distant metastasis		Local recurrence	
	HR 95% CI[b]	P-value[b]	HR 95% CI[b]	P-value[b]	HR 95% CI[b]	P-value[b]
Age	0.25 0.08-0.64	**0.001***	0.16 0.003-0.73	**0.004***	0.15 0.002-0.66	**0.001***
Estradiol	0.33 0.03-1.01	0.08	0.65 0.03-2.43	0.57	0.28 0.02-1.28	0.13
LH	0.72 0.28-2.46	0.50	0.46 0.08-1.09	0.19	0.61 0.11-3.9	0.44
FSH	0.98 0.20-5.31	0.98	0.63 0.10-3.0	0.47	0.98 0.23-6.4	0.99
ER	0.23 0.03-1.03	0.09	0.04 0.03-0.04	**0.001***	0.47 0.03-0.48	0.38
PR	0.39 0.03-1.49	0.16	0.17 0.004-0.82	**0.02***	0.72 0.02-3.5	0.67
Histological grade	1.56 0.60-2.2	0.60	3.0 1.2-14.0	**0.01***	0.37 0.04-0.83	**0.02***
Nodal status	1.2 0.53-1.87	0.83	27.0 2.31-33.1	**0.001***	0.62 0.1-28.2	0.55
Tumor size	1.03 0.61-1.61	0.89	1.5 0.79-2.72	0.09	0.47 1.3-2.0	0.80
HER2	0.71 0.03-2.2	0.60	1.0 0.03-4.3	0.96	0.43 0.03-2.1	0.27
Stage	1.1 0.25-2.9	0.91	2.7 0.46-19.7	0.12	0.26 0.02-1.4	0.14
IL17RB	1.94 0.56-5.5	0.18	0.62 0.03-2.9	0.66	2.5 0.43-12.2	0.14
HOXB7	0.04 0.04-0.05	**0.001***	0.04 0.04-0.05	**0.001***	0.03 0.03-0.05	**0.001***

[a] Univariate Cox proportional hazards regression test, based on categorized parameter data
[b] bootstrap corrected
* P≤0.05

Bolding indicates statistical significance.

Abbreviations: LH, luteinizing hormone; FSH, follicle-stimulating hormone; ER, estrogen receptor; PR, progesterone receptor; *HER2*, human epidermal growth factor receptor 2; IL17RB, Interleukin-17 receptor B; HOXB7, homeobox B7.

Table 3. Correlations between HOXB7, IL17RB and the major clinicopathological parameters [a]

	HOXB7	IL17RB	Grade	Nodal status	Tumor size	LH	FSH	E2
IL17RB	-0.19							
Grade	-0.02	0.11						
Nodal status	0.03	0.07	**0.76***					
Tumor size	-0.09	0.15	**0.76***	**0.38***				
LH	-0.06	-0.07	0.13	0.02	0.16			
FSH	-0.02	0.03	-0.11	-0.05	-0.11	**0.65***		
E2	0.05	0.08	0.03	-0.12	0.09	-0.17	**-0.28***	
ER	-0.03	0.11	-0.17	-0.17	-0.14	-0.10	-0.05	0.14

[a] Measured continuous numerical values were used for calculation of Spearman's coefficients except for N status and disease grade which are inherently categorical

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

Bolding indicates statistical significance.

Abbreviations: IL17RB, Interleukin-17 receptor B; HOXB7, homeobox B7; LH, luteinizing hormone; FSH, follicle-stimulating hormone; E2, estradiol; ER, estrogen receptor.

Table 4. Multivariate Cox proportional hazards regression analysis of the prognostic features [a,b,c]

Parameter	P-value*	HR 95% CI*
ER	**0.03**	0.00 1x10(-6) – 0.01
Histological grade	**0.002**	47.3 1.4– 14.4
Nodal status	**0.05**	2.5 0.88 – 15.3
HOXB7	**0.03**	0.00 0.0 – 0.82

[a] Cox multivariate stepwise regression was performed by the forward selection entry criterion of $P<0.2$ and the backward elimination criterion of $P<0.05$. Only the remaining features are thus presented in this Table.
[b] Analysis was performed on the basis of distant metastases as events
[c] Performed by use of categorized data
* bootstrap corrected

Bolding indicates statistical significance.

Abbreviations: ER, estrogen receptor; HOXB7, homeobox B7.