Thieme

# RANK and RANKL Expression in Tumors of Patients with Early Breast Cancer

## RANK- und RANKL-Expression in den Tumoren von Patientinnen mit frühem Brustkrebs



**Authors**

Annika Behrens[1,2,3‡], Lena Wurmthaler[1,2,3‡], Felix Heindl[1,2,3], Paul Gass[1,2,3], Lothar Häberle[1,2,3,4], Bernhard Volz[1,5], Carolin C. Hack[1,2,3], Julius Emons[1,2,3], Ramona Erber[2,3,6], Arndt Hartmann[2,3,6], Matthias W. Beckmann[1,2,3], Matthias Ruebner[1,2,3], William C. Dougall[7,8], Michael F. Press[9], Peter A. Fasching[1,2,3], Hanna Huebner[1,2,3]

**Affiliations**

1 Department of Gynecology and Obstetrics, University Hospital Erlangen, Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU), Erlangen, Germany
2 Comprehensive Cancer Center Erlangen-EMN, University Hospital Erlangen, Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU), Erlangen, Germany
3 Bavarian Center for Cancer Research (BZKF), Erlangen, Germany
4 Biostatistics Unit, Department of Gynecology and Obstetrics, University Hospital Erlangen, Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU), Erlangen, Germany
5 Ansbach University of Applied Sciences, Ansbach, Germany
6 Institute of Pathology, Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU), Erlangen, Germany
7 Hematology and Oncology Research, Amgen, Inc., Seattle, WA, USA
8 Immunology in Cancer and Infection Laboratory, QIMR Berghofer Medical Research Institute, Herston, Queensland, Australia
9 Department of Pathology, Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, CA, USA

**Key words**

breast cancer, RANK, RANKL, prognosis, immunohistochemistry

**Schlüsselwörter**

Brustkrebs, RANK, RANKL, Prognose, Immunohistochemie

**received** 25.8.2023
**accepted** 15.10.2023
**published online** 22.11.2023

**Bibliography**

Geburtsh Frauenheilk 2024; 84: 77–85
**DOI** 10.1055/a-2192-2998
**ISSN** 0016-5751



Georg Thieme Verlag KG, Rüdigerstraße 14,
70469 Stuttgart, Germany

**Correspondence**

Prof. Dr. Peter A. Fasching
Department of Gynecology and Obstetrics, University Hospital Erlangen, Comprehensive Cancer Center Erlangen-EMN, Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU)
Universitätsstraße 21/23
91054 Erlangen, Germany
peter.fasching@uk-erlangen.de

Additional material is available at https://doi.org/10.1055/a-2192-2998.

**ABSTRACT**

**Introduction**

The receptor activator of nuclear factor-κB (RANK) pathway was associated with the pathogenesis of breast cancer. Several studies attempted to link the RANK/RANKL pathway to prognosis; however, with inconsistent outcomes. We aimed to further contribute to the knowledge about RANK/RANKL as prognostic factors in breast cancer. Within this study, protein expression of RANK and its ligand, RANKL, in the tumor tissue was analyzed in association with disease-free survival (DFS) and overall survival (OS) in a study cohort of patients with early breast cancer.

‡ These authors contributed equally.

**Patients and Methods**
607 samples of female primary and early breast cancer patients from the Bavarian Breast Cancer Cases and Controls Study were analyzed to correlate the RANK and RANKL expression with DFS and OS. Therefore, expression was quantified using immunohistochemical staining of a tissue microarray. H-scores were determined with the cut-off value of 8.5 for RANK and 0 for RANKL expression, respectively.

**Results**
RANK and RANKL immunohistochemistry were assessed by H-score. Both biomarkers did not correlate ($\rho = -0.04$). According to molecular subtypes, triple-negative tumors and HER2-positive tumors showed a higher number of RANK-positive tumors (H-score ≥ 8.5), however, no subtype-specific expression of RANKL could be detected. Higher RANKL expression tended to correlate with a better prognosis. However, RANK and RANKL expression could not be identified as statistically significant prognostic factors within the study cohort.

**Conclusions**
Tumor-specific RANK and RANKL expressions are not applicable as prognostic factors for DFS and OS, but might be associated with subtype-specific breast cancer progression.

## ZUSAMMENFASSUNG

**Einleitung**
Es gibt eine Verbindung zwischen dem Rezeptor-Aktivator des Nuklearfaktor-κB-(RANK-)Signalwegs und der Pathogenese von Brustkrebs. Mehrere Studien haben versucht, eine Assoziation zwischen dem RANK-/RANKL-Signalweg und der Krankheitsprognose herzustellen, aber die bisher erzielten Ergebnisse sind uneinheitlich. Ziel dieser Arbeit war es, weitere Kenntnisse zur Expression von RANK/RANKL als prognostischer Faktor beim Mammakarzinom zu sammeln. Dazu wurde die Proteinexpression von RANK und seines Liganden, RANKL, im Tumorgewebe einer Studienpopulation von Patientinnen mit frühem Brustkrebs analysiert und auf eine Assoziation mit dem krankheitsfreien Überleben (DFS) und Gesamtüberleben (OS) geprüft.

**Patientinnen und Methoden**
Analysiert wurden 607 Proben, die Patientinnen mit Primärtumoren und frühem Brustkrebs in einer bayerischen Brustkrebs-Fall-Kontroll-Studie entnommen wurden. Es wurde geprüft, ob es eine Korrelation zwischen RANK- und RANKL-Expression und DFS and OS gibt. Zur Quantifizierung der Expression wurden Tissue-Micro-Arrays einer immunhistochemischen Färbung unterzogen. Die H-Scores wurden bestimmt. Der jeweilige Cut-off-Wert war 8,5 für RANK- bzw. 0 für RANKL-Expression.

**Ergebnisse**
RANK- und RANKL-Immunhistochemie wurden mithilfe des H-Scores beurteilt. Es gab für keinen der 2 Biomarker eine Korrelation ($\rho = -0{,}04$). Bei den Subtypen tripelnegativer Tumor und HER2-positiver Tumor fand sich eine höhere Anzahl RANK-positiver Tumoren (H-Score ≥ 8,5), aber es war keine subtypspezifische RANKL-Expression nachzuweisen. Eine höhere RANKL-Expression korrelierte tendenziell mit einer besseren Prognose. RANK- und RANKL-Expression waren aber in dieser Patientinnenpopulation nicht statistisch signifikante prognostische Faktoren.

**Schlussfolgerungen**
Die tumorspezifische RANK- und RANKL-Expression eignet sich nicht als prognostischer Faktor für DFS und OS, könnte aber mit der Krankheitsprogression bestimmter Brustkrebs-Subtypen assoziiert sein.

## Introduction

The receptor activator of nuclear factor-κB (RANK) pathway has been identified as the main regulatory mechanism of bone metabolism and physiology [1, 2]. Additionally RANK and its ligand, RANKL, have been linked to mammary gland development and the pathogenesis of breast cancer [3, 4, 5, 6]. The RANK pathway has been further connected with the *BRCA1-associated* pathogenesis of breast cancer [7, 8] and with checkpoint inhibition for the treatment of cancer cells [9]. Therefore, the RANK pathway is a target of many current therapeutic developments.

Since the development of denosumab, a human monoclonal anti-RANKL antibody, there is an increasing interest to investigate the RANK/RANKL and osteoprotegerin pathway and their interplay. Denosumab was initially approved for the treatment of osteoporosis, but is also used for the prevention of bone metastases and skeletal-related events in cancer patients in various studies [10, 11]. Recently, denosumab was tested in two large randomized trials investigating, whether treatment of early breast cancer patients with denosumab would result in an improved prognosis [12, 13, 14]. The ABCSG-18 study (NCT00556374) included postmenopausal patients with early hormone receptor-positive breast cancer. In this trial, the denosumab treatment was planned for five years with administration every six months. Disease-free survival (DFS) was one of the secondary objectives. Patients treated with denosumab had a higher probability of remaining disease-free (hazard ratio: 0.82 [95% CI: 0.69–0.98]) [13]. The other study (D-CARE, NCT01077154) included patients from all molecular subtypes with a high risk of recurrence. The primary endpoint was bone metastasis-free survival (BMFS) and DFS was one of the secondary endpoints. Neither BMFS (hazard ratio: 0.97;

95% CI 0.82–1.14) nor DFS (hazard ratio: 1.04; 95% CI: 0.91–1.19) was different between both randomization arms [14]. Subgroup analyses showed no association between menopausal status or hormone receptor status and denosumab treatment [14].

Although several studies have been published that try to link prognosis with the RANK pathway [15, 16, 17, 18, 19, 20], studies have been inconsistent with some not showing a prognostic effect. This could be linked to non-independent prognostic information parameters [19]. Differences in RANK-related prognosis could be due to an association of RANK expression with basal-like and triple-negative breast cancers (TNBCs) [15, 19]. Another study reported a protective effect of RANKL on prognosis, with low RANKL mRNA expression being associated with increased risk for relapse and bone metastases; however, again this was not statistically significant in multivariate analysis [20].

Therefore, the aim of this study was to investigate RANK and RANKL as prognostic factors in breast cancer. The primary study aim was to characterize RANK and RANKL expression in – to our knowledge – largest consecutive cohort of breast cancer patients and determine if there is a correlation with DFS. A secondary study aim is to determine if there is an association with overall survival (OS).

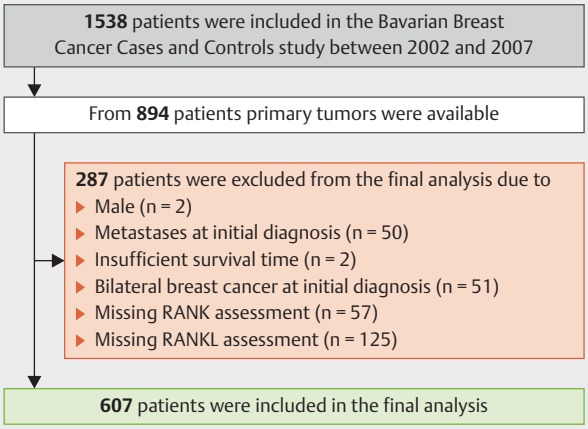
## Patients and Methods

### Patients

The Bavarian Breast Cancer Cases and Controls (BBCC) study is a case–control study aimed at investigating molecular and epidemiological breast cancer risk, prognostic, and predictive factors [17, 21, 22, 23, 24, 25, 26]. Patients were eligible if they were at least 18 years of age and had been diagnosed with invasive breast cancer. From 2002–2007, a total of 1538 patients were included in the study (▶ **Fig. 1**). Primary tumors were available from 894 of these patients for the construction of a tissue microarray (TMA) with 0.8 mm per dot. For this analysis, patient groups were excluded in the following hierarchical order: male patients (n = 2), patients with metastases at initial diagnosis (cM1, n = 50), insufficient survival time (n = 2), bilateral breast cancer at initial diagnosis (n = 51), missing RANK (n = 57) or RANKL assessment (n = 125). Therefore, the final sample size was 607. The ethics committee of the Medical Faculty of Erlangen University Hospital approved the study, and all patients provided written informed consent.

### Data collection

Data collection has been described in detail elsewhere [27, 28]. In brief, all clinical and histopathological data were documented prospectively in an annually audited database. In addition, treatment procedures are audited annually as well, requiring treatment accordance with the German guidelines for more than 95% of the patients. Follow-up information regarding local recurrences, distant metastases, and death were provided for a minimum of 10 years after the initial diagnosis.




▶ **Fig. 1** Patient flowchart.

### Tissue analysis

The core biopsies were formalin-fixed, paraffin-embedded (FFPE). The tumor areas were marked on a hematoxylin-and-eosin (H & E) stained slide by an experienced pathologist. For constructing tissue microarrays (TMAs) cylindric tissue core biopsies (0.8 mm per core) of several sample donor blocks were re-embedded into a single microarray block at defined coordinates. The TMA was stained with anti-human RANK (N-1 H8; Amgen) or RANKL (M366; Amgen) mouse monoclonal antibodies or isotype-matched control mouse IgG as previously described [29]. Specificities of the RANK and RANKL antibodies have been reported before [30]. We scored the RANKL and RANK expression within the primary tumors individually, according to the semi-quantitative histochemical score (H-score) [31]. Immunohistochemical interpretation was performed by an experienced pathologist blinded to any sample identification. The percentage of RANKL positive tumor cells was multiplied by staining intensity: 0, negative; 1+, weak; 2+, moderate; and 3+, strong). The H-score was defined as the sum of all calculated tumor cell percentage/intensity products per TMA dot and ranged from 0 to 300. In this context, 300 represents 100% of cells having a strong staining intensity. The cohort was analyzed according to the previously published RANK cut-off H-scores of ≥ 8.5 as optimal DFS and OS [19]. This cut-off has been defined using an automated cut-off finder and showed significant association with pathological complete response, DFS and OS [19]. For RANKL a cut-off H-score of > 0 was used.

Molecular subtypes were defined as described earlier [32]. Data on tumor type, tumor grade, estrogen receptor (ER), progesterone receptor (PR), and HER2 status were received from the routine pathology reports. If the patient had an overexpression of HER2 of 3+ as assessed by immunohistochemistry or showed amplification of the *HER2* gene by fluorescence in situ hybridization, they were considered as HER2 positive. If they were HER2 negative and did not show an expression of estrogen receptor (ER) and progesterone receptor (PR), they were considered as triple negative. In case they were HER2 negative and showed an expression of either ER or

PR they were further divided into luminal A like (tumor grade of 1 or 2) and luminal B like (tumor grade of 3).

### Statistical considerations

DFS was defined as the time from the date of primary diagnosis to the earliest date of disease progression (distant metastasis, local recurrence, death from any cause) or the date of censoring. Patients who were lost to follow-up before the maximal observation time of 10 years or were disease-free after the maximal observation time were censored at the last date they were known to be disease-free or at the maximal observation time. OS was defined in a similar fashion.

The primary objective was to investigate whether the biomarkers RANK and RANKL have prognostic value in addition to well-known prognostic patient and tumor characteristics. A multivariable Cox regression model (basic model) was fitted with DFS and OS, respectively, as outcome and the following predictors: age at diagnosis (continuous), body mass index (continuous), tumor stage (ordinal, T1 to T4), lymph node status (categorical; N0, N1) and molecular class (categorical; TNBC, luminal A like, luminal B like, HER2). Detailed information for the factor molecular class can be found elsewhere [32]. The proportional hazards assumptions were checked for both outcomes, using the method of Grambsch and Therneau [33]. Where the proportional hazards assumption was violated, stratification for the according predictors was implemented in the models. Based on the results, the DFS model was stratified by lymph node status whereas the OS model was stratified by molecular class.

Subsequently, an additional Cox regression model (biomarker model) was fitted containing the biomarkers RANK (categorical; "low", < 8.5; "high", ≥ 8.5; [19]) and RANKL (categorical; "low", = 0; "high", > 0) and the predictors of the basic model. The biomarker model was compared with the basic model using a likelihood ratio test (LRT). A significant P value would indicate that biomarker information improves survival prognosis additionally to the considered prognostic factors. Adjusted hazard ratios (HRs) for RANK and RANKL were estimated using the biomarker model. Subjects with missing survival information and missing values in the biomarkers of interest (RANK and RANKL) were excluded from analysis. Missing values in other predictors were imputed as done in Salmen et al. [34].

As sensitivity analyses, unadjusted HRs were estimated using univariable Cox regression models for the biomarkers RANK and RANKL separately. Survival rates were estimated using the Kaplan-Meier product limit method. Furthermore, Spearman's correlation coefficient ρ between RANK and RANKL was calculated to assess the monotonic relationship between RANK and RANKL H-scores.

Molecular subtypes (TNBC, luminal A like, luminal B like, HER2 positive) were compared with regard to the binary variables RANK and RANKL, respectively, using the $\chi^2$ test. All of the tests were two-sided, and $p < 0.05$ was regarded as statistically significant. Calculations were carried out using the R system for statistical computing (version 3.4.0; R Development Core Team, Vienna, Austria, 2017).

▶**Table 1** Baseline characteristics of study population. Mean (standard deviation, SD) or median (interquartile range, IQR) where appropriate are shown for continuous characteristics and frequency (percentage) for categorical characteristics.

| Characteristic | | |
|---|---|---|
| Age at diagnosis (years) | Mean (SD) | 57.5 (12.4) |
| BMI | Median (IQR) | 25.3 (22.65–28.58) |
| Lymph node status | N0 | 352 (58.0) |
| | N1 | 255 (42.0) |
| Tumor stage | T1 | 324 (53.4) |
| | T2 | 226 (37.2) |
| | T3 | 29 (4.8) |
| | T4 | 28 (4.6) |
| Molecular class | TNBC | 86 (14.2) |
| | Luminal A | 226 (37.2) |
| | Luminal B | 224 (36.9) |
| | HER2 | 71 (11.7) |
| RANK H-score | Median (IQR) | 0 (0–10) |
| RANK H-score | < 8.5 | 444 (73.1) |
| | ≥ 8.5 | 163 (26.9) |
| RANKL H-score | Median (IQR) | 0 (0–0) |
| RANKL H-score | 0 | 540 (89.0) |
| | > 0 | 67 (11.0) |

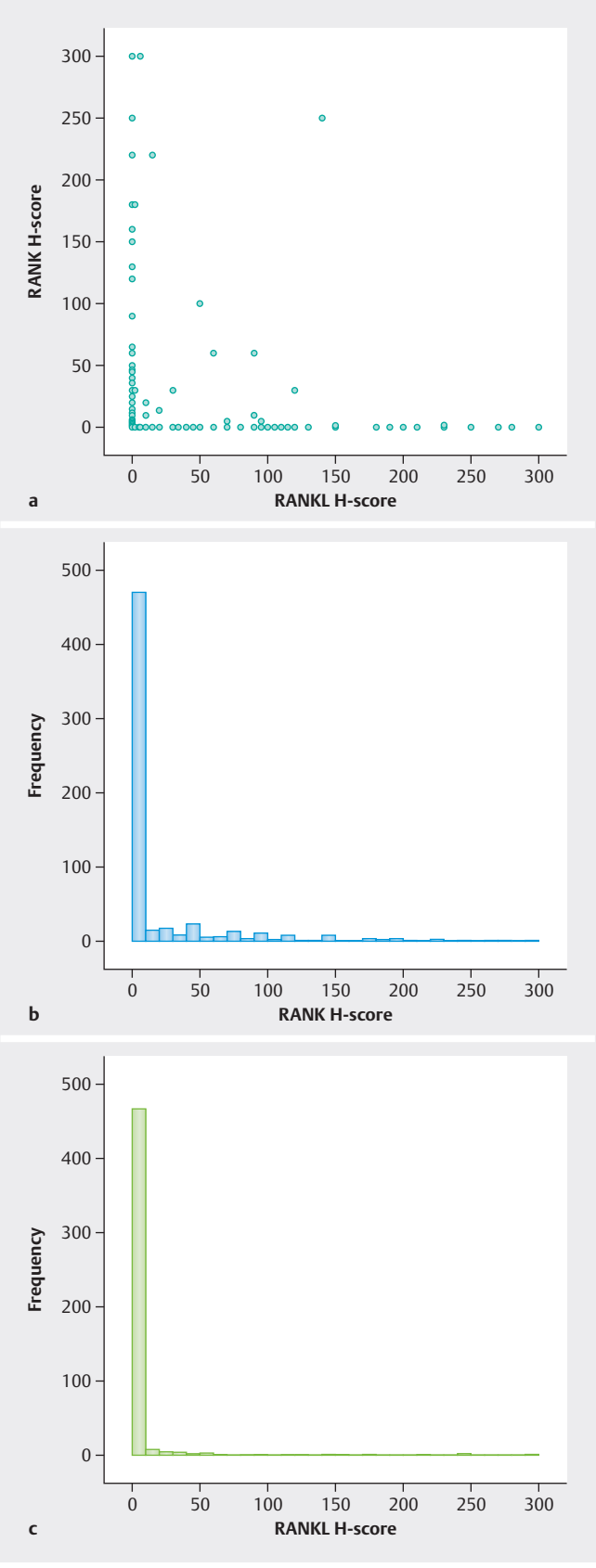
Abbreviations: BMI = body mass index; SD = standard deviation; IQR = interquartile range; TNBC = triple-negative breast cancer

## Results

The total patient population for the analysis was 607 patients with primary and early breast cancer. Mean age was 57.5 (± 12.4) years and most patients had a tumor size of pT1 (n = 324; 53.4 %). With regard to the molecular subtype most patients were either luminal A like (n = 226; 37.2 %) or luminal B like (n = 224; 36.9 %). All patient and tumor characteristics are presented in ▶ **Table 1**.

RANK and RANKL immunohistochemistry were assessed by H-score. Both biomarkers did not correlate (ρ = − 0.04, ▶ **Fig. 2a**). Their distribution is shown in ▶ **Fig. 2b** and ▶ **Fig. 2c**. Most tumors did not have a RANK or RANKL expression at all. An H-score over 0 was seen in 205 patients (33.8%) for RANK and in 67 patients (11.0%) for RANKL. With the previously published cut-off of 8.5 for RANK we saw 163 patients (26.9%) above that cut-off.

With regard to the distribution of RANK and RANKL H-scores according to molecular subtypes, there was a higher frequency of tumors with a RANK H-score ≥ 8.5 in TNBC (52.8 %) and in HER2 positive tumors (40.8 %), while these figures were 17.0 % and 22.2 % in patients with luminal A like and luminal B like tumors, respectively. For RANKL no statistically significant differences could be found across the molecular subtypes (▶ **Table 2**, Supplementary **Fig. S1**, online).




▶ **Fig. 2** Distribution and correlation of the biomarkers RANK and RANKL. **a** Scatterplot RANK H-score and RANKL H-score, Spearman's rank correlation, ρ = − 0.04. **b** Distribution of RANK H-score. **c** Distribution of RANKL H-score.

With regard to prognosis, the addition of biomarkers RANK and RANKL to established prognostic factors did not significantly improve the prediction of DFS (p = 0.55, likelihood ratio test, LRT). The DFS hazard ratio for RANK expression ≥ 8.5 vs. expression < 8.5 in the multivariable model was 1.11 (95 % CI: 0.78–1.59), while the hazard ratio for RANKL was 0.77 (95 % CI: 0.44–1.37) for patients with an expression > 0 vs. those without expression. Unadjusted analyses yielded similar results (▶ **Table 3**). Kaplan-Meier curves for DFS are presented in ▶ **Fig. 3 a** and ▶ **Fig. 3 b**.

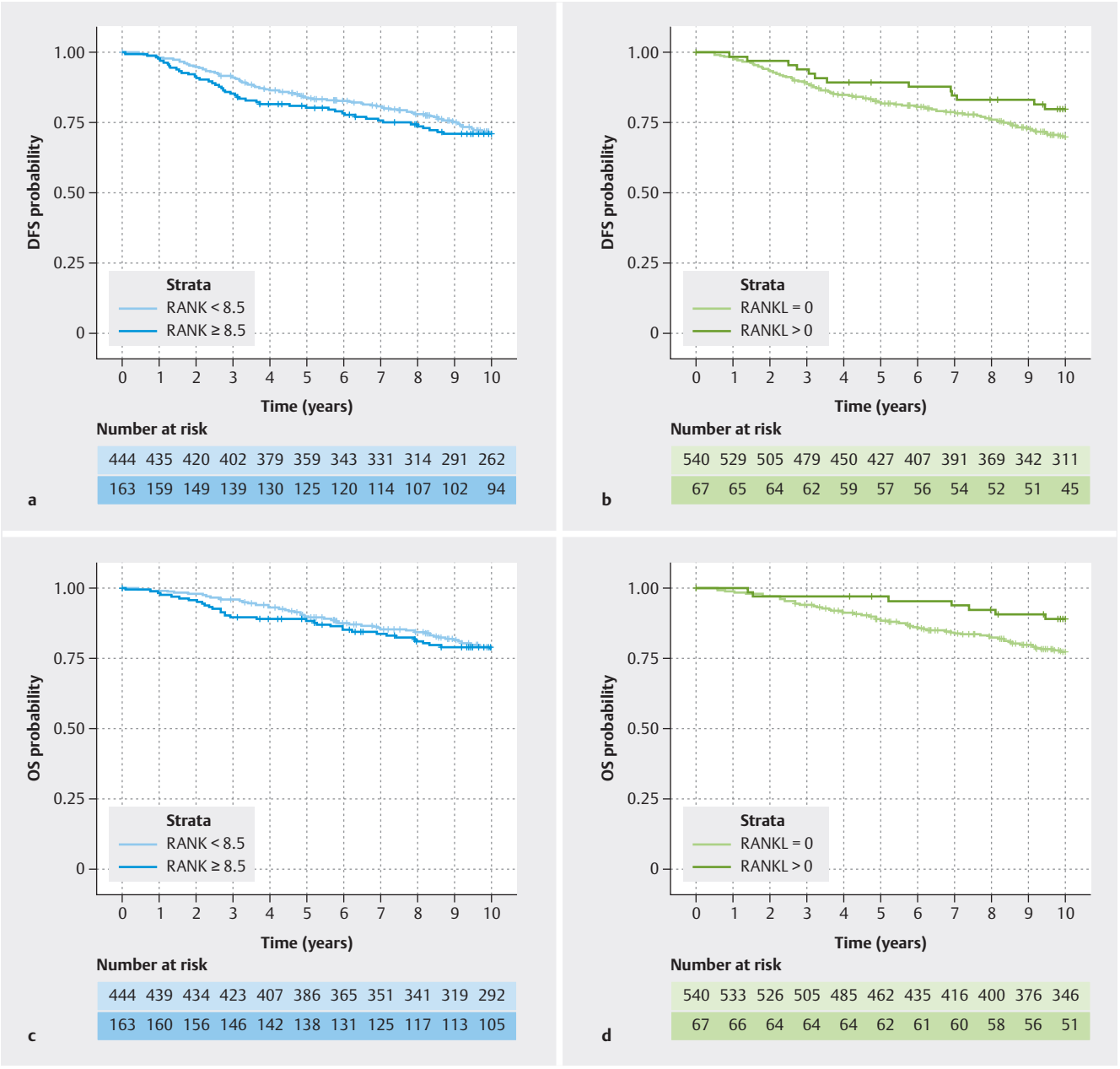
With regard to OS, RANK and RANKL did not significantly improve prediction of OS when considered with established prognostic factors (p = 0.28, LRT, ▶ **Table 3**). The OS hazard ratio for RANK expression ≥ 8.5 vs. expression < 8.5 in the multivariable model was 1.02 (95% CI: 0.67–1.55), while the hazard ratio for RANKL was 0.56 (95% CI: 0.26–1.21) for patients with an expression > 0 vs. those without expression. Unadjusted analyses yielded similar results for the biomarker RANK (▶ **Table 3**). For RANKL; however, the unadjusted HR was 0.45 (95% CI: 0.21–0.96, p = 0.04, ▶ **Table 3**). Kaplan-Meier curves for OS are presented in ▶ **Fig. 3 c** and ▶ **Fig. 3 d**.

## Discussion

In this retrospective cohort study, we did not show a clear prognostic effect of RANK or RANKL expression assessed by immunohistochemistry in the primary tumor on DFS or OS. However, there seemed to be a trend that RANKL expression might be associated with a more favorable prognosis for OS. RANK was expressed more frequently in triple negative and HER2 positive tumors in our cohort, while RANKL expression did not differ across molecular subtypes.

Similar to our results, it was shown elsewhere that neither RANK nor RANKL expression in young breast cancer patients was associated with DFS after a 65 month follow-up [35]. However, they found a correlation between high RANKL expression in the primary tissue and expression of genes involved in pathways related to the mammary gland development, bone resorption, T-cell proliferation and the regulation of chemotaxis [35]. A study similar to ours with a similar sample size showed a comparable result with regard to positively assessed tumors [19]. In this study 27% of all tumors showed a RANK H-score ≥ 8.5, while our study had a positivity rate of 26.9%. With regard to RANKL they reported positive tumors in 6% of the cases, while we had H-scores over 0 in 11% of all patients. Similar to our study, a higher frequency of high RANK expression was seen in TNBC patients [19, 35] and in patients with HER2 positive tumors [19]. Concerning prognosis, the previous study showed a significant effect of high RANK expression on prognosis in the univariate analysis which was not maintained in a multivariable model.

More recently, in a third, smaller study with a cohort of 87 TNBC patients analyzed in a comparable manner to our study, a worse relapse-free and OS rate was shown in correlation with high RANK and RANKL levels in TNBC patients [36]. Most likely these described effects are attributable to the association of RANK expression with triple negativity.




▶ **Fig. 3** Kaplan-Meier curves for DFS of patients. **a** With low (< 8.5) and high (≥ 8.5) RANK H-score values, **b** with low (= 0) and high (> 0) RANKL H-score values. Kaplan-Meier curves for OS of patients. **c** With low (< 8.5) and **d** high (≥ 8.5) RANK H-score values.

▶**Table 2** Comparing the expression of RANK and RANKL H-scores (binary) over the molecular classes.

| | Molecular Class | | | | P |
|---|---|---|---|---|---|
| | TNBC | Luminal A | Luminal B | HER2 positive | |
| RANK H-Score < 8.5 | 42 (47.2) | 195 (83) | 165 (77.8) | 42 (59.2) | < 0.0001 |
| RANK H-Score ≥ 8.5 | 47 (52.8) | 40 (17) | 47 (22.2) | 29 (40.8) | |
| RANKL H-Score ≤ 0 | 80 (89.9) | 215 (91.5) | 180 (84.9) | 65 (91.5) | 0.130 |
| RANKL H-Score > 0 | 9 (10.1) | 20 (8.5) | 32 (15.1) | 6 (8.5) | |

▶**Table 3** Unadjusted and adjusted hazard ratios for RANK H-score and RANKL H-score for the outcomes DFS and OS.

| Outcome | Biomarker | Unadjusted HR (95 % CI) | P value | Adjusted* HR (95 % CI) | P value |
|---|---|---|---|---|---|
| DFS | RANK | 1.08 (0.77,1.52) | 0.64 | 1.11 (0.78,1.59) | 0.56 |
| | RANKL | 0.64 (0.36,1.12) | 0.12 | 0.77 (0.44,1.37) | 0.38 |
| OS | RANK | 1.03 (0.69,1.54) | 0.92 | 1.02 (0.67,1.55) | 0.93 |
| | RANKL | 0.45 (0.21,0.96) | 0.04 | 0.56 (0.26,1.21) | 0.14 |

CI = confidence interval

* HRs Hazard ratios are adjusted for age at diagnosis, BMI, tumor stage, lymph node status, molecular class, RANK H-score, RANKL H-score

Different from the second and the third described study we saw a trend of patients with RANKL expression having a better prognosis than patients with no RANKL expression. Further a similar result was published with a study that analyzed RANK and RANKL with quantitative PCR of mRNA [20]. In that study with 814 patients, expression levels were dichotomized at the mean expression level and patients with a high RANKL expression had a better DFS, OS and bone metastasis free survival in the univariate analysis [20]. Similar to our study this effect was not maintained after adjustment for other prognostic factors, indicating that the effect might have been caused by confounding effects; however, neither of the above studies nor our study found an association between RANKL expression and tumor or patient characteristics [19, 20]. In contrast to our study, analyses evaluating serum instead of tumor-specific expression levels of RANK/RANKL, revealed an association of high RANKL, progesterone and soluble decoy receptor osteoprotegerin (OPG) serum levels with increased risk for development and/or progression of breast cancer [37, 38, 39]. However, it still needs to be investigated whether serum levels of RANK and RANKL or even levels of OPG have an impact on breast cancer prognosis regardless of other well described prognostic factors.

The present study has a number of limitations. First, the cohort analyzed was a retrospective group of breast cancer patients with heterogeneous treatments, although they were treated in accordance with established therapy guidelines. In addition, the number of cases with a TNBC subtype was quite low compared to luminal A and luminal B like breast cancer types and thus, a subtype-specific analysis was not reasonable. Nevertheless, our observations regarding the prognostic value of RANK and RANKL – even when not significant – showed the expected effect direction.

## Conclusion

With this retrospective cohort study investigating the prognostic impact of RANK and RANKL on DFS and OS by immunohistochemistry of the primary tumor, we further contribute to the knowledge of RANK and RANKL as prognostic factors in breast cancer.

In summary, we could confirm that RANK and RANKL tumor expression does not have a significant effect on OS or DFS after adjustment for other prognostic factors in our cohort of primary breast cancer cases, even though a trend for better OS and DFS for patients with present RANKL expression could be observed.

## Supplementary Material

**Supplementary Fig. S1:** Comparing the expression of RANK and RANKL H-scores (binary) over the molecular classes.


### Fundings

Bavaria California Technology Center (www.bacatec.de)

BCRP 12IB-0155 | California Breast Cancer Research Program

108295 | Dr. Mildred Scheel Foundation of German Cancer Aid

### Acknowledgement

Research for the present study was supported by a grant from the Bavaria California Technology Center (www.bacatec.de) and a grant from the California Breast Cancer Research Program (BCRP 12IB-0155 to MFPress). Peter A. Fasching was funded by a grant from the Dr. Mildred Scheel Foundation of German Cancer Aid (Deutsche Krebshilfe e. V.).


### Conflict of Interest

P.A.F. reports personal fees from Novartis, grants from BioNTech, personal fees from Pfizer, personal fees from Daiichi Sankyo, personal fees from AstraZeneca, personal fees from Eisai, personal fees from Merck Sharp & Dohme, grants from Cepheid, personal fees from Lilly, personal fees from Pierre Fabre, personal fees from SeaGen, personal fees from Roche, personal fees from Hexal, personal fees from Agendia, personal fees from Gilead.
P.G. has received honoraria from Novartis, MSD, and AstraZeneca.
J.E. has received honoraria from Eisai, Pfizer, and Novartis.
A.H. has received honoraria for lectures or consulting/advisory boards for Abbvie, Agilent, AstraZeneca, Biocartis, BMS, Boehringer Ingelheim, Cepheid, Diaceutics, Gilead, Illumina, Ipsen, Janssen, Lilly, Merck, MSD, Nanostring, Novartis, Pfizer, Qiagen, QUIP GmbH, Roche, Sanofi, 3DHistotech and other research support from AstraZeneca, Biocartis, Cepheid, Gilead, Illumina, Janssen, Nanostring, Novartis, Owkin, Qiagen, QUIP GmbH, Roche, Sanofi.

M.F.P. declares advisory board membership for Biocartis, Cepheid, Lilly USA; reports a consultant role for AstraZeneca, Eli Lilly & Company, Merck, Novartis, and Zymeworks; reports ownership interest in TORL Biotherapeutics; reports fee-for-service agreements from 1200 Pharma, Ambrx, TORL Biotherapeutics, TRIO, TRIO-US, and Zymeworks.
The remaining authors declare that they do not have a conflict of interest.

## References

[1] Lacey DL, Boyle WJ, Simonet WS et al. Bench to bedside: elucidation of the OPG-RANK-RANKL pathway and the development of denosumab. Nat Rev Drug Discov 2012; 11: 401–419. doi:10.1038/nrd3705

[2] Simonet WS, Lacey DL, Dunstan CR et al. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell 1997; 89: 309–319. doi:10.1016/s0092-8674(00)80209-3

[3] Fata JE, Kong YY, Li J et al. The osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development. Cell 2000; 103: 41–50. doi:10.1016/s0092-8674(00)00103-3

[4] Gonzalez-Suarez E, Jacob AP, Jones J et al. RANK ligand mediates progestin-induced mammary epithelial proliferation and carcinogenesis. Nature 2010; 468: 103–107. doi:10.1038/nature09495

[5] Joshi PA, Jackson HW, Beristain AG et al. Progesterone induces adult mammary stem cell expansion. Nature 2010; 465: 803–807. doi:10.1038/nature09091

[6] Schramek D, Leibbrandt A, Sigl V et al. Osteoclast differentiation factor RANKL controls development of progestin-driven mammary cancer. Nature 2010; 468: 98–102. doi:10.1038/nature09387

[7] Sigl V, Owusu-Boaitey K, Joshi PA et al. RANKL/RANK control Brca1 mutation-driven mammary tumors. Cell Res 2016; 26: 761–774. doi:10.1038/cr.2016.69

[8] Nolan E, Vaillant F, Branstetter D et al. RANK ligand as a potential target for breast cancer prevention in BRCA1-mutation carriers. Nat Med 2016; 22: 933–939. doi:10.1038/nm.4118

[9] Ahern E, Smyth MJ, Dougall WC et al. Roles of the RANKL-RANK axis in antitumour immunity – implications for therapy. Nat Rev Clin Oncol 2018; 15: 676–693. doi:10.1038/s41571-018-0095-y

[10] Amgen Inc. Highlights of Prescribing Information Xgeva (Denosumab). 2013. Accessed March 09, 2019 at: https://www.accessdata.fda.gov/drugsatfda_docs/label/2013/125320s094lbl.pdf

[11] Amgen Inc. Highlights of Prescribing Information Prolia (Denosumab). 2018. Accessed March 09, 2019 at: https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/125320s186lbl.pdf

[12] Coleman R, Finkelstein DM, Barrios C et al. Adjuvant denosumab in early breast cancer (D-CARE): an international, multicentre, randomised, controlled, phase 3 trial. Lancet Oncol 2020; 21: 60–72. doi:10.1016/S1470-2045(19)30687-4

[13] Gnant M, Pfeiler G, Steger GG et al. Adjuvant denosumab in postmenopausal patients with hormone receptor-positive breast cancer (ABCSG-18): disease-free survival results from a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet Oncol 2019; 20: 339–351. doi:10.1016/S1470-2045(18)30862-3

[14] Coleman RE, Finkelstein D, Barrios CH et al. Adjuvant denosumab in early breast cancer: First results from the international multicenter randomized phase III placebo controlled D-CARE study. J Clin Oncol 2018; 36: 501. doi:10.1200/JCO.2018.36.15_suppl.501

[15] Santini D, Schiavon G, Vincenzi B et al. Receptor activator of NF-kB (RANK) expression in primary tumors associates with bone metastasis occurrence in breast cancer patients. PLoS One 2011; 6: e19234. doi:10.1371/journal.pone.0019234

[16] Zhang L, Teng Y, Zhang Y et al. Receptor activator for nuclear factor kappa B expression predicts poor prognosis in breast cancer patients with bone metastasis but not in patients with visceral metastasis. J Clin Pathol 2012; 65: 36–40. doi:10.1136/jclinpath-2011-200312

[17] Hein A, Bayer CM, Schrauder MG et al. Polymorphisms in the RANK/RANKL genes and their effect on bone specific prognosis in breast cancer patients. Biomed Res Int 2014; 2014: 842452. doi:10.1155/2014/842452

[18] Park HS, Lee A, Chae BJ et al. Expression of receptor activator of nuclear factor kappa-B as a poor prognostic marker in breast cancer. J Surg Oncol 2014; 110: 807–812. doi:10.1002/jso.23737

[19] Pfitzner BM, Branstetter D, Loibl S et al. RANK expression as a prognostic and predictive marker in breast cancer. Breast Cancer Res Treat 2014; 145: 307–315. doi:10.1007/s10549-014-2955-1

[20] Timotheadou E, Kalogeras KT, Koliou GA et al. Evaluation of the Prognostic Value of RANK, OPG, and RANKL mRNA Expression in Early Breast Cancer Patients Treated with Anthracycline-Based Adjuvant Chemotherapy. Transl Oncol 2017; 10: 589–598. doi:10.1016/j.tranon.2017.05.006

[21] Fasching PA, Loehberg CR, Strissel PL et al. Single nucleotide polymorphisms of the aromatase gene (CYP19A1), HER2/neu status, and prognosis in breast cancer patients. Breast Cancer Res Treat 2008; 112: 89–98. doi:10.1007/s10549-007-9822-2

[22] Haberle L, Wagner F, Fasching PA et al. Characterizing mammographic images by using generic texture features. Breast Cancer Res 2012; 14: R59. doi:10.1186/bcr3163

[23] Heusinger K, Jud SM, Haberle L et al. Association of mammographic density with hormone receptors in invasive breast cancers: results from a case-only study. Int J Cancer 2012; 131: 2643–2649. doi:10.1002/ijc.27515

[24] Heusinger K, Loehberg CR, Haeberle L et al. Mammographic density as a risk factor for breast cancer in a German case-control study. Eur J Cancer Prev 2011; 20: 1–8. doi:10.1097/CEJ.0b013e328341e2ce

[25] Rauh C, Hack CC, Haberle L et al. Percent Mammographic Density and Dense Area as Risk Factors for Breast Cancer. Geburtshilfe Frauenheilkd 2012; 72: 727–733. doi:10.1055/s-0032-1315129

[26] Schrauder M, Frank S, Strissel PL et al. Single nucleotide polymorphism D1853N of the ATM gene may alter the risk for breast cancer. J Cancer Res Clin Oncol 2008; 134: 873–882. doi:10.1007/s00432-008-0355-9

[27] Brennan M, Gass P, Haberle L et al. The effect of participation in neoadjuvant clinical trials on outcomes in patients with early breast cancer. Breast Cancer Res Treat 2018; 171: 747–758. doi:10.1007/s10549-018-4829-4

[28] Beckmann MW, Brucker C, Hanf V et al. Quality assured health care in certified breast centers and improvement of the prognosis of breast cancer patients. Onkologie 2011; 34: 362–367. doi:10.1159/000329601

[29] Wood CE, Branstetter D, Jacob AP et al. Progestin effects on cell proliferation pathways in the postmenopausal mammary gland. Breast Cancer Res 2013; 15: R62. doi:10.1186/bcr3456

[30] Branstetter DG, Nelson SD, Manivel JC et al. Denosumab induces tumor reduction and bone formation in patients with giant-cell tumor of bone. Clin Cancer Res 2012; 18: 4415–4424. doi:10.1158/1078-0432.CCR-12-0578

[31] McCarty KS, jr, Miller LS, Cox EB et al. Estrogen receptor analyses. Correlation of biochemical and immunohistochemical methods using monoclonal antireceptor antibodies. Arch Pathol Lab Med 1985; 109: 716–721

[32] Wunderle M, Pretscher J, Brucker SY et al. Association between breast cancer risk factors and molecular type in postmenopausal patients with hormone receptor-positive early breast cancer. Breast Cancer Res Treat 2019; 174: 453–461. doi:10.1007/s10549-018-05115-6

[33] Grambsch PM, Therneau TM. Proportional Hazards Tests and Diagnostics Based on Weighted Residuals. Biometrika 1994; 81: 515–526

[34] Salmen J, Neugebauer J, Fasching PA et al. Pooled analysis of the prognostic relevance of progesterone receptor status in five German cohort studies. Breast Cancer Res Treat 2014; 148: 143–151. doi:10.1007/s10549-014-3130-4

[35] Azim HA, jr, Peccatori FA, Brohee S et al. RANK-ligand (RANKL) expression in young breast cancer patients and during pregnancy. Breast Cancer Res 2015; 17: 24. doi:10.1186/s13058-015-0538-7

[36] Reyes ME, Fujii T, Branstetter D et al. Poor prognosis of patients with triple-negative breast cancer can be stratified by RANK and RANKL dual expression. Breast Cancer Res Treat 2017; 164: 57–67. doi:10.1007/s10549-017-4233-5

[37] Sarink D, Schock H, Johnson T et al. Circulating RANKL and RANKL/OPG and Breast Cancer Risk by ER and PR Subtype: Results from the EPIC Cohort. Cancer Prev Res (Phila) 2017; 10: 525–534. doi:10.1158/1940-6207.CAPR-17-0125

[38] Kiechl S, Schramek D, Widschwendter M et al. Aberrant regulation of RANKL/OPG in women at high risk of developing breast cancer. Oncotarget 2017; 8: 3811–3825. doi:10.18632/oncotarget.14013

[39] Park SS, Uzelac A, Kotsopoulos J. Delineating the role of osteoprotegerin as a marker of breast cancer risk among women with a BRCA1 mutation. Hered Cancer Clin Pract 2022; 20: 14. doi:10.1186/s13053-022-00223-3