Aus dem Institut für Pathologie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Tumorprogression und Ansprechen auf neoadjuvante Chemotherapie bei Brustkrebspatientinnen mit niedriger Hormonrezeptorpositivität

Tumorprogression and responsiveness to neoadjuvant chemotherapy in breast cancer patients with low hormone receptor positivity

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Abbreviations

AGO-B: Arbeitsgemeinschaft Gynäkologische Onkologie, Breast Group

ASCO: American Society of Clinical Oncology

BC: breast cancer

BRCA: breast cancer gene

CAP: College of American Pathologists

cDNA: complementary DNA

CI: confidence interval

CK: cytokeratin

CTLA4: cytotoxic T-lymphocyte-associated antigen 4

DCC: Dextran-coated charcoal

DCIS: ductal carcinoma in situ without invasive carcinoma

DDFS: distant-disease free survival

DFS: disease-free survival

DNA: deoxyribonucleic acid

EGFR: epidermal growth factor receptor

EIA: enzyme immunoassay

ER: estrogen receptor

ERBB2: erb-b2 receptor tyrosine kinase 2

ET: endocrine therapy

FFPE: formalin-fixed, paraffin-embedded tissue

FISH: fluorescence in situ hybridization

fmol: femtomoles

GBG: German Breast Group

HDI: human development index

HER2: human epidermal growth factor receptor 2

HR: hormone receptors

IHC: immunohistochemical

IRS: immunoreactive score

Ki67: (Nuclear antigen present only in the nuclei of cycling cells)

LAG-3: lymphocyte activation gene 3

LBA: ligand-binding assay

LC: lobular carcinoma

LPBC: lymphocyte-predominant breast cancer

mg: miligrams

mi: microscopic

mRNA: messenger RNA

NACT: neoadjuvant chemotherapy

NET: neoadjuvant endocrine therapy

NST: no special type

OS: overall survival

pCR: pathological complete response

PD-L1: programmed cell death ligand-1

PR: progesterone receptor

RNA: ribonucleic acid

RT-PCR: real-time reverse transcription polymerase chain reaction

SISH: silver stain hybridization in situ

TIL: tumour infiltrating lymphocytes

TNBC: triple-negative breast cancer

TNM: [primary tumor (T), regional lymph nodes (N), distant metastases (M)]

ypT0 ypN0: no microscopic evidence of residual viable tumour -invasive or noninvasive- in resected specimens of the breast and lymphatic nodes

ypT0/is ypN0: microscopic evidence non-invasive residual tumour in resected specimens of the breast, and no evidence of invasive tumour in lymphatic nodes

1. Abstracts

1.1 Abstract (English)

Background: According to current guidelines, patients with breast cancer (BC) with low levels (1-9%) of hormone receptor (HR) are eligible to receive endocrine adjuvant therapy. However, some data suggest that these tumours express a basal-like molecular phenotype associated with triple negative BC (TNBC) rather than an unequivocal luminal phenotype, represented by strong HR-positive BC. Adjuvant endocrine therapy is not offered to patients with TNBC. They are good candidates for neoadjuvant chemotherapy, showing increased pathological complete response (pCR) rates compared with non-TNBC. We aimed to evaluate the differences among patients with TNBC, HER2-negative with a low HR-expression, and HER2-negative tumours with strong HR-expression, regarding pCR and survival in two large cohorts from neoadjuvant clinical trials.

Methods: We compared negative [oestrogen (ER) and progesterone receptor (PR) <1%], low-positive (ER and/or PR 1-9%), and strong-positive (ER or PR 10-100%) HR-expression in neoadjuvant treated patients with HER2-negative BC (n=2765). End-points were pCR, disease-free survival (DFS), distant-disease free survival (DDFS), and overall survival (OS). Additionally, RNA sequencing on available tumour tissue samples from patients with low-HR expression (n=38) was performed.

Results: Ninety-four (3.4%) patients had low HR-positive tumours, 1769 (64.0%) had strong HR-positive tumours and 902 (32.6%) had TNBC. There were no significant differences in pCR rates between women with low HR-positive tumours (27.7%) and women with TNBC (35.5%). DFS and DDFS were also not different [for DFS hazard ratio 1.26, 95%-CI (confidence interval): 0.87-1.83, log-rank test p=0.951, for DDFS hazard ratio 1.17, 95%-CI: 0.78-1.76, log-rank test p=0.774]. Patients with strong HR-positive tumours had a significantly lower pCR rate (pCR 9.4%, odds ratio 0.38, 95%-CI:0.23-0.63), but better DFS (hazard ratio 0.48, 95%-CI: 0.33-0.70) and DDFS (hazard ratio 0.49, 95%-CI: 0.33-0.74) than patients with low HR-positive tumours. Molecular subtyping (RNA sequencing) of low HR-positive tumours classified these predominantly into a basal subtype (86.8%).

Conclusion: Patients with low HR-positive/HER2-negative tumours achieve higher pCR rates and show poorer survival, compared to patients with strong HR-positive/HER2 negative BC, akin to patients with TNBC. The majority of low HR-positive/HER2 negative tumours exhibit

a basal-like gene expression signature. Patients with low HR-positive/HER2-negative tumours might be regarded as candidates for therapy strategies targeting TNBC.

1.2 Abstract (Deutsch)

Hintergrund: Nach aktuellen Leitlinien sind Patientinnen mit Brustkrebs (BC) mit niedriger Expression (1-9%) der Hormonrezeptoren (HR) für eine endokrine Therapie geeignet. Unterschiedliche Daten deuten jedoch darauf hin, dass diese Tumoren häufig einen basalen molekularen Subtyp zeigen, der mit einem dreifach negativem BC (TNBC) assoziiert ist (HR <1%), und eher nicht einem eindeutigen luminalen Phänotyp (meist stark HR-positiv) entsprechen. Adjuvant endokrine Therapie wird zu Patientinnen mit TNBC nicht angeboten. Sie sind gute Kandidaten für eine neoadjuvante Chemotherapie und weisen im Vergleich zu Patienten ohne TNBC erhöhte pathologische Komplettremission (pCR)-Raten auf. Unser Ziel war es, die Unterschiede zwischen Patientinnen mit TNBC, HER2-negativen mit niedriger HR-Expression, und HER2-negativen Tumoren mit starker HR-Expression in Bezug auf pCR und Überleben in zwei großen neoadjuvanten klinischen Studienkohorten zu evaluieren.

Methoden: Wir verglichen negative [Östrogen (ER) und Progesteronrezeptor (PR) <1%], niedrig positive (ER und / oder PR 1-9%) und stark positive (ER oder PR 10-100%) HR-Expression in neoadjuvant behandelten Patientinnen mit HER2-negativem BC (n = 2765). Endpunkte waren pCR, das krankheitsfreie Überleben (DFS), das Fernkrankheits-freie Überleben (DDFS) und das Gesamtüberleben (OS). Eine RNA-Sequenzierung am verfügbaren Tumorgewebe mit niedriger HR-Expression (n = 38) wurde durchgeführt.

Ergebnisse: Vierundneunzig (3,4%) Patientinnen hatten niedrige HR-positive, 1769 (64,0%) hatten starke HR-positive Tumoren und 902 (32,6%) hatten TNBC. Es gab keine signifikanten Unterschiede in den pCR-Raten zwischen Frauen mit niedrigen HR-positiven Tumoren (27,7%) und Frauen mit TNBC (35,5%). DFS und DDFS unterschieden sich ebenfalls nicht (für DFS Hazard Ratio 1,26, 95% -CI: 0,87-1,83, Log-Rank-Test p = 0,951, für DDFS Hazard Ratio 1,17, 95% -CI: 0,78-1,76, Log-Rank-Test p = 0,774). Patientinnen mit starken HR-positiven Tumoren hatten eine signifikant niedrigere pCR-Rate (pCR 9,4%, Odds Ratio 0,38, 95% -CI: 0,23-0,63), aber eine bessere DFS (Hazard Ratio 0,48, 95% -CI: 0,33-0,70) und DDFS (Hazard Ratio 0,49, 95% -CI: 0,33-0,74) als Patienten mit niedrigen HR-positiven Tumoren. Die molekulare Subtypisierung (RNA-Sequenzierung) von Tumoren mit niedriger HR-Positivität klassifizierte diese überwiegend in einen basalen Subtyp (86,8%).

Schlussfolgerung: Patientinnen mit niedrig HR-positivem/HER2-negativem Mammakarzinom erreichen höhere pCR-Raten und haben ein schlechteres Überleben als Patienten mit stark HR-

positivem/HER2-negativem BC, ähnlich wie Patienten mit TNBC. Die Mehrheit der niedrig HR-positiven/HER2-negativen Tumoren weist eine basalähnliche Genexpressionssignatur auf. Patientinnen mit niedrig HR-positivem/HER2-negativem Mammakarzinom könnten als Kandidaten für Therapiestrategien gegen TNBC in Betracht gezogen werden.

2. Introduction

This work focuses on women diagnosed with primary breast cancer (BC), with no expression of human epidermal growth factor receptor 2 (HER2), treated with neoadjuvant chemotherapy (NACT) in two clinical trials in Germany. Of main interest hereby is the response of women with tumours showing low hormone receptor expression (1-9%) to the given therapy and their survival rates after 10 years of diagnosis. Current clinical guidelines cannot suggest a specific treatment regimen for this subgroup of patients.

2.1 Epidemiology of breast cancer

Female BC was the leading cause of global cancer incidence in 2020 (11.7% of all cancer cases) and represented an estimated of 2.3 million new cases-years. It is the fifth major cause of cancer mortality worldwide and the leading cause of cancer mortality among women, with a mortality rate of 13.6 per 100.000 population-years.[1]

In Germany, breast cancer is the most common cancer in women, with around 69.900 new cases annually. The incidence in 2018 was 112.6 per 100.000 population-years with a standardized mortality rate of 22.8 per 100.000 population-years.[2]

Globally, incidence rates of BC in countries with high human development index (HDI) are 88% higher than in countries with low or medium HDI (55.9 and 29.7 per 100.000 population-years respectively), but mortality rates among women in these countries are 17% higher.[1] These differences concerning incidence and mortality rates are in part due to the aging population, the level of access to good quality screening programs and resourceful treatments settings. They may also reflect the prevalence of the reproductive and hormonal risk factors associated with BC (early age at menarche, later age at menopause, advanced age at first birth, low parity, less breastfeeding, menopausal hormone therapy, oral contraceptives)[3] as well as the prevalence of lifestyle risk factors (alcohol intake, excess body weight, physical inactivity).[4]

Breast cancer in males continues to be rare, representing less than 1% of global diagnoses of BC.

2.2 Breast cancer intrinsic subtypes

Based on evaluation of gene expression patterns and their correlation with clinical outcome in the early 2000s, Sorlie, Perou and colleagues proposed five tumour subtypes for BC: luminal A, luminal B, normal-like, HER2-enriched, and basal-like.[5, 6] Basal-like and HER2-enriched BC subtypes were associated with the shortest survival times while luminal tumours (expressing hormone receptors) had a relatively favourable prognosis.

Because gene expression profiling is still not available to most patients and physicians, this molecular classification has been adapted based on immunohistochemical (IHC) biomarkers: estrogen receptor (ER), progesterone receptor (PR), HER2, and an assay to measure tumour proliferation (i.e. Ki-67). The classification system based on IHC assays was proposed in the St. Gallen International Breast Cancer Conference of 2011 and refined at the St. Gallen Consensus in 2013 [7, 8]

2.2.1 Immunohistochemical surrogates for molecular classification of breast cancer

Luminal (hormone receptor positive) breast cancer

Patients with carcinomas that express ER or PR and are negative for HER2 account for 70-75% of all BC cases. Luminal tumours are usually divided in luminal A-like or luminal B-like according to tumour grade, magnitude of receptor-expression and proliferation rates. In line with the molecular findings of these subclasses, luminal A-like neoplasms generally show lower grade, high levels of ER and PR and low levels of proliferation, being clinically less aggressive and more sensitive to endocrine therapy than to conventional chemotherapy. In contrast, luminal B-like tumours are typically ER-positive, variable for PR and with a higher tumour grade and proliferative rate, considered clinically more "aggressive" with a poorer prognosis, but better response to chemotherapy. [5, 6, 9]

There is no consensus about the exact threshold for the proliferation marker Ki67 to distinguish between cancer subtypes. In the St. Gallen International Breast Cancer Conference of 2021, the panel of experts supported a recommendation of the International Ki67 in Breast Cancer Working Group, which stated „patients whose tumours show <5% of proliferation should not receive chemotherapy, whereas for those with tumours evaluated as Ki67 >30% chemotherapy should be offered".[10]

Triple-negative breast cancer

BC patients with tumours being negative for the three markers (ER-, PR-, HER2) are classified as triple-negative breast cancer (TNBC). This subgroup comprises 15% of all BC cases. These patients are usually younger and have higher rates of local and distant recurrence and higher death rates within the first 5 years after diagnosis. Their tumours show very often molecular features of the basal-like molecular subtype, have high proliferation rates, aggressive clinical behavior and better response to regimens containing chemotherapy. Basal-like cancers frequently show mutations in the genes TP53 and BRCA1 and are characterized by constitutive expression of genes typically found in basal myoepithelial cells of healthy breast tissue. [11, 12]

Good correlation exists between the molecular subtype and the current trio of IHC-surrogates, however, terms like "triple negative"-, and "basal-like cancer" should not be interchanged.[13]

HER2 positive breast cancer

The molecular HER2-enriched subtype consists of tumours with overexpression and/or HER2 gene amplification by IHC/ fluorescence in situ hybridization (FISH). This subgroup comprises about 10-15% of all BC patients.[14] Patients with HER2 positive breast cancer experience a poor clinical outcome but are good candidates for anti-HER2 targeted therapy. Since the introduction of the targeted therapy the risk of relapse in the early stage was lowered and survival in the metastatic setting improved.

Patients whose tumours express HER2 and co-express ER or PR frequently present early recurrences, nodal metastasis and often have tumours that are low-positive or negative for PR. These tumours are less responsive to endocrine therapy compared to hormone receptor positive and HER2-negative tumours.[15] In the neoadjuvant setting, data from several clinical trials showed that pathological complete response (pCR) rates were significantly lower in HER2+/ER-positive than in HER+/ER-negative tumours, regardless of the type of anti-HER2 targeted therapy.[16, 17]

Preclinical models have suggested a bidirectional crosstalk between the HER2 and HR pathways, which seems to play an important role in the development of resistance to endocrine as well as to anti-HER2 therapy.[18, 19]

Table 1 summarizes the most important BC subtypes and the immunohistochemical surrogate markers in current clinical practice.

Table 1 Breast cancer subtypes and the immunhistochemical surrogate markers

Subtype	Luminal			HER2-Positive		TNBC
Biomarker	Luminal A	Luminal B		HER2-/ HR-positive	HER2-positive	
		Ki67 (high)[a]	PR <20%			
ER (%)	Positive	Positive	Positive	Positive	Negative	Negative
PR (%)	Positive	>20	<20	Positive	Negative	Negative
HER2	Negative	Negative	Negative	Positive	Positive	Negative
Ki67(%)[a]	Low	High	Any	Any	Any	Any

TNBC, triple-negative breast cancer; ER, estrogen receptor; PR, progesteron receptor; HER2, Human Epidermal growth factor Receptor-2.

a. There is no consensus about the threshold for Ki67. In general, patients with tumours evaluated as having <5% do not receive chemotherapy, whereas patients with tumors assessed as Ki67 >30% often receive chemotherapy. [10, 20]

Table created by Villegas, SL.

Research on the biology of BC allows to refine the BC-classification. Studies that included analyses of copy number alterations, DNA methylation, exome sequencing, and transcriptomics (among others methods), suggested up to 10-different subclasses of tumours, which correspond to a common clinical characteristic or outcome.[21-23] Importantly, this deeper and broader exploration identified a number of intermediate prognostic groups among ER-positive cases (i.e. three subgroups in luminal B and two subgroups in luminal A tumours)[21]. Although not all these subclasses are translated into clinically relevant subgroups, these findings emphasize the concept of BC-heterogeneity and reminds us that the routinely performed phenotypic characterisation may group cases together that not necessarily show an expected clinical behaviour.

2.3 Histological types of breast cancer

Up to 95% of breast carcinomas are adenocarcinomas and originate within the mammary ductal-lobular system.[24]

The most common subtype is the invasive carcinoma "of no special type" (NST), formerly called invasive ductal carcinoma, which accounts for up to 75% of all invasive BC. [25] The NST subtype includes all tumours which do not belong in any of the special histological variants. These BC special types comprise up to 25% of BC and consist of at least 17 distinct types according to the latest edition of World Health Organization's classification of breast tumours.[26] The most common special type is the invasive lobular carcinoma (LC), that constitutes up to 5-15% of all BC.[25] A distinctive common finding of LC is the lack of E-Cadherin expression.This

transmembrane protein normally mediates cell-cell adhesion and acts as an invasion suppressor factor. The absence of E-Cadherin positivity along with the histological features are currently used as a marker to distinguish LC from NST.[27]

It is important to emphasize that the terms "ductal" and "lobular" carcinoma do not imply histogenesis or site of origin within the breast anatomy. The presence of mammary stem cells was revealed in the mid 2000's by studies in rodents.[28] This finding made clear that stem cells give origin to all other epithelial cell types within the breast, taking different pathways and differentiating into luminal (meant for milk production) and myoepithelial cells (associated with milk ejection). In line with this concept, histological and genomic analyses have proposed two pathways for carcinoma origin, leading to luminal or basal breast cancer subtypes.[29]

2.4 Treatment of ER-positive breast cancer –an overview

2.4.1 Surgery and adjuvant therapies

Treatment of early BC includes a combination of local approaches (surgery or radiotherapy), systemic treatments (chemotherapy, endocrine therapy, and targeted therapy) and supportive measures. Breast conservation surgery with oncoplastic procedures (skin-sparing/nipple-sparing) is nowadays the gold standard for local treatment of early BC, regardless of tumour histology. Clear margins after surgery represent a successful result of this local therapy and for that reason, specimens undergo a careful pathological evaluation. Clear margins are currently defined as "no tumour on ink" for invasive cancer and 2 mm for intraductal carcinoma.[30, 31]

Post-operative whole-breast radiotherapy is still the standard of care after breast-conserving surgery to reduce the 10-years risk of recurrence.[32] It is also recommended for the axilla in cases with lymph-node involvement.

ER-positive cases represent the majority of BC and are not only heterogeneous with respect to phenotype and molecular signature but also with respect to relapse patterns and therapeutic responses (to endocrine-, and chemotherapy). Nonetheless, as a general principle, an adjuvant (after surgery) endocrine therapy (i.e. selective estrogen receptor modulators, selective estrogen receptor downregulators and aromatase inhibitors), should be used in all luminal-like cancers, taking into account the characteristics of the tumour and the patients preferences.

In postmenopausal women, the options include either tamoxifen or an aromatase inhibitor. Aromatase inhibitor is often preferred in cases with higher risk of recurrence, like BC stage II or III, additional HER2-positivity, high grade tumours or with high proliferation index. Classically, the duration of the endocrine treatment is five years, in which up to 50% of the

patients can be non-fully adherent due to side effects (including sexual, vasomotor, cardiovascular, musculoskeletal, cognitive, gastrointestinal, genitourinary, as well as higher rate of endometrial cancer and thromboembolic events).[33] Yet, an extension of this treatment up to 10 years has been recently suggested for stage II and III BC with positive node, based on the observations that BC recurrences continue to occur 20 years after diagnosis.[34] The additional 5-years extension with aromatase inhibitors prevented distant recurrence and secondary BC in high-risk patients, but without an improvement in overall survival.[35] Recent data from a clinical trial suggests that extended duration of adjuvant endocrine therapy beyond 7-8 years does not improve clinical outcome.[36]

2.4.2 Chemotherapy

BC cases with high ER-Expression (>50%) are considered highly endocrine responsive tumours and are usually associated with lesser absolute benefit of chemotherapy.[37] In contrast, cases with low ER-Expression (<10%) together with lack of PR-expression, tumour high grade or high proliferation index are associated with lower responsiveness to endocrine therapy.[37] Therefore, it is important to identify those patients with luminal cancers that would probably response well to endocrine therapy without any additional treatment and those patients who may be candidates for chemotherapy. To decide whether or not a patient is a candidate for chemotherapy, the anatomical disease stage remains a decisive criterion (i.e. it is likely to be administered to patients with stage III disease).[20] In contrast, there are groups of patients in which chemotherapy is more commonly avoided, like patients with lobular tumour-type and luminal-A cases with low grade score, negative lymph nodes or up to three affected axillary nodes. To determine if BC patients in between this case-spectrum can obtain additional benefit from chemotherapy, well-established multigene signature assays have been integrated into the decision-making process in clinical practice. In ER-positive, HER-negative disease with N0-N1 clinical stages and a low-risk signature, adjuvant chemotherapy could be safely omitted.[20, 38-40]

2.4.3 Neoadjuvant therapies

Neoadjuvant treatment (NACT) is recommended for stage II-III disease, TNBC and HER2-positive cases (tumours >2 cm).[20] In tumours smaller than 2cm but showing an aggressive phenotype like luminal-B, NACT is also indicated.[41] These therapies shrink the tumour, allow

higher proportions of breast and axillary conservation, and allow to assess an early in-vivo response to the systemic treatment.

The most important parameter to measure the success of a neoadjuvant treatment is the achievement of a pathologic complete response (pCR). A pCR is defined as "no invasive and no in situ cancer residuals in breast or nodes", after pathological examination of the surgical specimen. This definition allowed to discriminate between patients with favorable and unfavorable outcomes. Achievement of pCR had no significant prognostic value in patients with luminal-A or luminal B/HER2-positive tumours, whereas in patients with luminal-B/HER2-negative, HER2-positive (HR-negative), and TNBC was prognostic for survival, making pCR a suitable surrogate end point for clinical trials including these intrinsic subtypes.[42]

In current clinical practice, postmenopausal patients with clinical stage II or III, and ER-positive/HER2-negative tumours could be eligible for 4-8 months of aromatase inhibitors as neoadjuvant endocrine therapy (NET).[41] The exact duration of treatment, the role of NET in premenopausal women and the actual impact on overall survival are still subjects under research. There is no validated pathological assessment or score after NET to be a surrogate endpoint for survival.[43]

2.5 Therapy options for TNBC

Although NACT represents the best first line treatment for TNBC, pCR rates among these patients range from 30% to 50%.[44, 45] In addition, patients with residual tumour burden after NACT have worse survival in case of TNBC compared to non-TNBC.[46] Consequently, for this cancer subtype therapeutics other than chemotherapy (i.e. immunotherapeutics) are of great interest.

In BC, the expression of inhibitory and co-stimulatory molecules (immune checkpoints) like programmed cell death ligand-1 (PD-L1), cytotoxic T-lymphocyte-associated antigen 4 (CTLA4), and lymphocyte activation gene 3 (LAG-3) is one of the mechanisms of tumour cells to evade immune cells in the tumour microenvironment. PD-L1 is a one of the ligands for the Programmed death 1 (PD-1) protein, a T-cell coinhibitory receptor. When this interaction was blocked in cancer cells in the early 2000's, immune function in vitro was enhanced, promoting an antitumour immune response.[47] Subsequentially, clinical trials with PD1 antibodies demonstrated tumour regression in colon, renal and lung cancers, and melanoma. Research about PD-L1 expression in BC found that although it was generally rare, it was markedly

enriched in basal-like tumours and correlated with levels of tumour infiltrating lymphocytes (TIL).[48]

In early-stage BC, trials in the neoadjuvant setting demonstrated that the addition of an anti-PDL1 (durvalumab) or anti-PD1 (pembrolizumab) agent to standard chemotherapy improved the rate of pCR in TNBC.[45, 49]

There are also promising, new agents like the anti-trop2 antibody–drug conjugate (IMMU132)[50] and anti-LIV1 antibody–drug conjugate (SGN-LIV1), that showed high response rates in advanced, refractory TNBC.[51]

Several, ongoing research projects are studying many more immunotherapeutic approaches, including new checkpoint blockades, checkpoint inhibitors combined with poly (ADP-Ribose) polymerase (PARP) inhibitors, combination with new targeted therapy involving PI3K/AKT/mTOR and MEK pathways, immune induction strategies, tumor antigen vaccines, dendritic cell activators, and adjuvants that activate innate immunity, among others.

The whole treatment approach of BC is decisively based on the histopathological and molecular characteristics of the tumour. That is the reason why accurate measurement and correct evaluation of hormone receptors expression represent a cornerstone in BC. As molecular testing is not universally accessible yet, immunohistochemical assessment of ER, PR and HER2 as surrogates, will continue to be the gold standard for diagnosis and treatment of BC patients.[20]

3. Evaluation of receptors -surrogates for tumour subtypes

3.1 Hormone receptors -the most important biomarker in breast cancer

The ER and PR are part of a large family of nuclear receptors that act as transcription factors, with impact on proliferation of breast epithelial cells when activated. The biological events driven by ER-pathway are directly related to BC development and progression. Around 65% to 75% of BC express ER.

Some years after the discovery of the estrogen receptor by Dr. Elwood Jensen and Dr. Herbert Jacobson[52] using radioactive markers in 1958, J. Gorski developed a reliable test for the presence of ER, that estimated cytosol receptor content by "sucrose density gradient centrifugation", using radioactive estrogen as a marker.[53] According to this test, using fresh or frozen homogenized tumour tissue of primary or metastatic BC, patients had "positive or negative uptake patterns", based on the magnitude of sedimentation peaks in the experiment. With this method, clinical correlations were made in the early 1970´s, showing that BC patients

with tumours lacking ER, had very little chance to response to ablative-endocrine therapy (adrenalectomy, oophorectomy, hypophysectomy) and that some –but not all- who possessed estrogen-binding proteins, would benefit from such treatments.

In the mid 1960´s Dextran-coated charcoal (DCC) assays were developed to measure insulin in serum by separating insulin from insulin-antibody complexes, due to the property of absorbing small molecules and not its binding-form.[54] In the following decade, ligand-binding assays (LBA) using DCC method were developed to quantitatively measure ER and PR in breast cancer tissue, [55, 56] enabling many studies describing the prognostic and predictive value of hormone receptors.[57, 58]

By 1977, Jensen and colleagues[52] had developed monoclonal antibodies targeting ER which facilitated later techniques like ER enzyme immunoassay[59] and IHC in formalin-fixed, paraffin-embedded tissue (FFPE).[60]

Later on, comparison studies about the new available methods to measure ER began to confirm that the results were highly correlated to those obtained using the LBA/DCC method.[59, 61] The new IHC method was easier to perform, required much less tissue and was less expensive, which contributed to the establishment of IHC as the new gold standard, which remains nowadays.

3.2 Scoring systems and threshold for hormone receptor positivity

Several semi-quantitative scoring systems have been developed to assess HR-expression on FFPE specimens. Almost all of them base the scoring on the proportion of tumour cells stained and the staining intensity. Among these, the Immunoreactive Score (IRS) is widely used in Germany.[62] It is the product of the percentage of positive nuclei of tumour cells in five gradations (0 positive tumour cells positive=0; <10%=1; 10-50%=2; 51-80%=3; >80%=4) and the proportion of the staining intensity in four gradations (no staining=0, weakly positive=1, moderate=2, strongly positive=3). Score results range from 0-12.

Another widely used scoring system is the Allred score (modified quick score).[63] It uses the same intensity gradation of IRS system (0–3) and adds the proportion of tumour cells stained into six categories (no staining=0, <1%=1;1-10%=2; 11-33%=3; 34-66%=4; and 67-100% of the cells stained=5). The result is limited to a range of 0 to 8, with a score of >2 considered positive.

The threshold for HR-positivity was not a result of prospective validations but it was set according to correlation values to response to endocrine therapy in retrospective analyses.

Responsiveness to antihormonal therapy of patients with tumours expressing low HR-levels (1-9%) was interpreted as "uncertain" as stated by the International Expert Consensus on the primary therapy of early Breast Cancer in 2005.[64] Before 2010, BC cases with ER or PR <10% on IHC were commonly classified as HR-negative.

The cut-off point for HR-positivity was lowered to 1% in 2010, according to guidelines proposed by the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP),[65] meant to offer a chance to patients to benefit from anti-hormone therapies. Since then a breast cancer case is considered positive for hormone receptors (ER or PR), when at least 1% of tumour cells show nuclear staining on IHC-assays. The previous group of patients considered as having tumours negative for HR is now consequently eligible for endocrine therapy. Nonetheless, after the change of cut-off, evidence in the adjuvant setting suggested that these patients, in common with HR-negative cases, do not appear to benefit from endocrine therapy.[66] At the molecular level, cases with low HR-expression were also comparable with HR-negative tumours.

The most recent ASCO/CAP guidelines regarding a threshold for HR-positivity continued recommending 1% as the cut-off but proposed that cases with 1-10% expression of HR should be classified as "low or weakly positive".[67]

Due to an open debate about best threshold for HR-positivity and the lack of guidelines for specific treatment of low HR-positive, the treatment approach of these cases poses a challenge in clinical practice.

4. Study aims

The aim of our study was to evaluate how patients with low HR-positive tumours responded to NACT in comparison to patients with TNBC and strong HR-positive tumours. We further aimed to analyze survival rates differences among these groups of patients after 10 years of diagnosis.

Additionally, RNA sequencing was performed to identify gene expression patterns and molecular subtypes within the group of tumours with low HR-levels.

Specifically, we evaluated clinical outcomes of patients with low HR-positive/HER2-negative BC compared to patients with TNBC and patients with strong HR-positive/HER2-negative BC in the neoadjuvant setting, regarding rates of pathological complete response, disease-free survival, distant-disease free survival, and overall survival of patients with early breast cancer, all treated with neoadjuvant chemotherapy in large clinical trials.

5. Patients and Methods

5.2 Clinical trial cohorts

To evaluate the response to NACT and survival rates of patients with low HR-positive tumours in comparison to patients with TNBC and strong HR-positive tumours, a statistical analysis was performed on data of participants of the randomized clinical trials GeparQuinto[16] and GeparSepto[68] led by the German Breast Group (GBG) and Arbeitsgemeinschaft Gynäkologische Onkologie, Breast Group (AGO-B). A total of 2765 women with histologically confirmed primary BC through core biopsy was included (figure 1). Patients from GeparQuinto trial received neoadjuvant treatment with four cycles of epirubicin plus cyclophosphamide, followed by four cycles of docetaxel. In the GeparSepto trial, patients were treated with nab-paclitaxel or paclitaxel for four cycles, followed by epirubicin plus cyclophosphamide for four cycles. The treatment period was between 18-24 weeks. Inclusion criteria and detailed clinical trials methods have been published.[16, 68, 69]

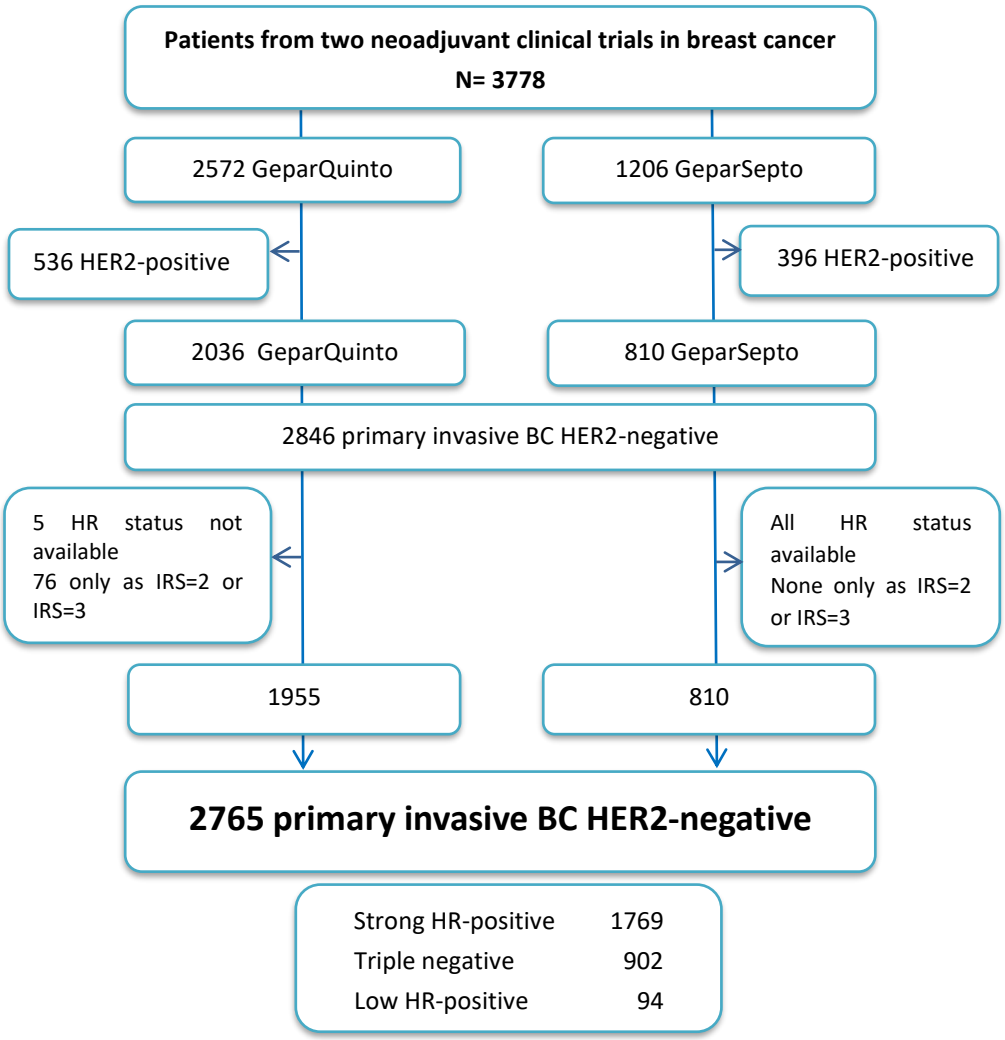
Written informed consent for participation, data collection and data use for translational studies was obtained from all patients and the respective ethics committees approved all trials.

Patients with HER2-positive tumours (n=1205) were excluded from the analysis.

Methods of the present work were published in:

Villegas SL, Nekljudova V, Pfarr N, Engel J, Untch M, Schrodi S, Holms F, Ulmer HU, Fasching PA, Weber KE, Albig C, Heinrichs C, Marme F, Hartmann A, Hanusch C, Schmitt WD, Huober J, Lederer B, van Mackelenbergh M, Tesch H, Jackisch C, Rezai M, Sinn P, Sinn BV, Hackmann J, Kiechle M, Schneeweiss A, Weichert W, Denkert C, Loibl S. Therapy response and prognosis of patients with early breast cancer with low positivity for hormone receptors - An analysis of 2765 patients from neoadjuvant clinical trials. Eur J Cancer. 2021;148:159-70. doi: 10.1016/j.ejca.2021.02.020.[70]

Figure 1 Study Profile: Overview of included and excluded samples from GBG/AGOB clinical trials



HER2: human epidermal growth factor receptor 2; HR: Hormone receptor; IRS=immunoreactive score[62]

Source: Figure 1 of publication:
Villegas SL, Nekljudova V, Pfarr N, Engel J, Untch M, Schrodi S, Holms F, Ulmer HU, Fasching PA, Weber KE, Albig C, Heinrichs C, Marme F, Hartmann A, Hanusch C, Schmitt WD, Huober J, Lederer B, van Mackelenbergh M, Tesch H, Jackisch C, Rezai M, Sinn P, Sinn BV, Hackmann J, Kiechle M, Schneeweiss A, Weichert W, Denkert C, Loibl S. Therapy response and prognosis of patients with early breast cancer with low positivity for hormone receptors - An analysis of 2765 patients from neoadjuvant clinical trials. Eur J Cancer. 2021;148:159-70. doi: 10.1016/j.ejca.2021.02.020.

5.3 Evaluation of biomarkers

For GeparQuinto trial, local pathology evaluation on HR-status, proliferation index and pathologic response to treatment registered in the clinical trial database was used. For GeparSepto trial, HR-status was centrally assessed by the Institute of Pathology, Charité University (Berlin, Germany). Immunohistochemical staining for hormone receptors was performed on the VENTANA BenchMark XT automated slide stainer according to the manufacturer's instructions. Specific rabbit monoclonal primary antibody against human ER

(clone SP1, Ventana) and monoclonal mouse anti-human progesterone receptor (Clone PgR 636, Autostainer Dako) were used.

The evaluation of positive controls on slide (presence of HR-positive, non-neoplastic ductal-lobular epithelium) is part of the central assessment. The Institute of Pathology that performed the assays participates of internal and external quality control and quality assurance programs.

HR-status was recorded as percentage of positive tumour cells (with any intensity) or as immunoreactive score (IRS).[62] For the IRS, staining intensity and proportion of stained tumour cells are both scored. Staining intensity negative=0; weak=1; moderate=2; strong=3. Percentage of stained tumour cells: 0% positive=0; 1–9% positive=1; 10–50% positive=2; 51–80% positive=3; >80% positive=4. Both values are multiplied resulting in an IRS score between 0 and 12, where 12 represents a marker strongly expressed in >80% of tumour cells. Examples of the different expression levels of ER are shown in figure 2a-d.

5.3.1 Definition of HR-subgroups

HR-negative tumours were defined as ER and PR=0% or as an IRS=0.

Tumours with low HR-positivity were defined as ER=1-9% or IRS=1 and PR=0-9%. Cases with ER-negativity (ER=0%) and PR low-positivity (PR=1-9%) were also included in this category.

Strong HR-positive tumours were those expressing ER≥10% or scored as IRS=4-12 regardless PR, or PR unequivocal strong positive (≥10% or IRS=4-12) regardless ER.

An overview of the definition of HR-subgroups is shown in table 2.

Table 2 Definition of HR-subgroups according to ER-status and PR-status

Hormone receptor status		**ER**		
		Negative (<1%)	**Low positive (1-9%)**	**Strong positive (>10%)**
PR	**Negative (<1%)**	Negative	Low HR positive	Strong HR positive
	Low positive (1-9%)	Low positive	Low HR positive	Strong HR positive
	Strong positive (>10%)	Strong HR positive	Strong HR positive	Strong HR positive

ER; estrogen receptor; PR: progesterone receptor; HR: Hormone receptor

Table created by Villegas, SL

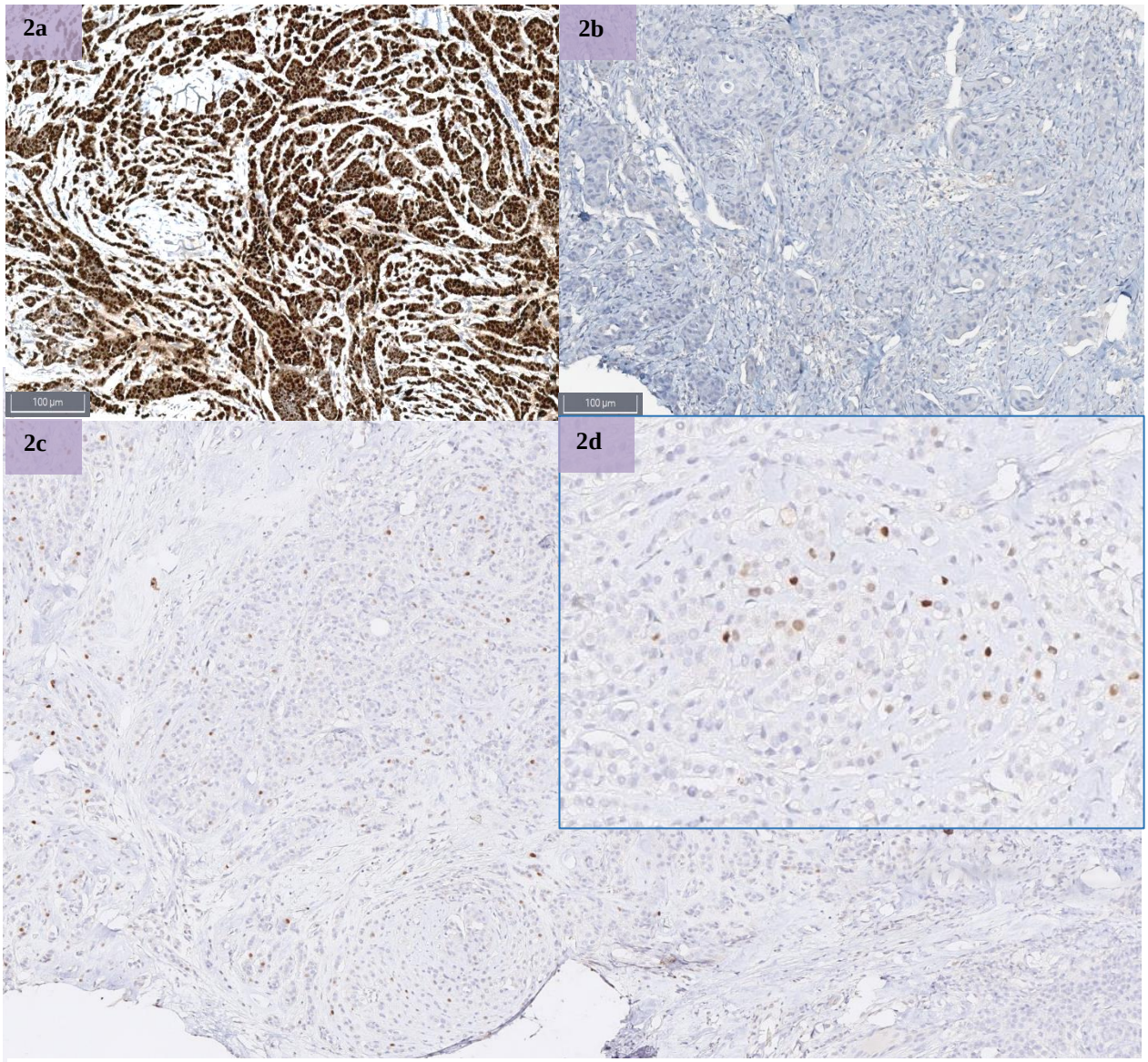


Figure 2a-d. Photomicrographs of representative invasive breast cancer tissue immunostained for estrogen receptor (ER positive cells showed a dark brown nuclear signal), study cases.

2a: Using this field, the case would get a total immunoreactivescore (IRS) of 12 (>80% of tumour cells staining intensity score [3]); 2b: ER=0% tumour cells staining, IRS=0; 2c: Invasive breast cancer with low ER-positivity by 1-9% of tumour cells staining regardless intensity; 2d: magnification on the case with low ER-positivity. Figure created by Villegas, SL.

Seventy-six cases from GeparQuinto trial assessed only as IRS=2 or IRS=3 (and missing data on exact percentage of positive cells) were excluded from the evaluation, since these scores could contain cases with >10% of positive cells with weak staining.

HER2-expression was evaluated based on immunohistochemistry (anti-HER2/neu rabbit monoclonal primary antibody, clone 4B5) and when needed, through silver stain hybridization in situ (SISH) on the VENTANA BenchMark XT automated slide stainer, according to ASCO/CAP guidelines.[71] Ki67 was assessed using the antibody MIB-1 (Dako), evaluated as

positive when a nuclear staining was present and reported in percentage. For this analysis, a cut-off of 20% positive cells was used.[68] Baseline clinico-pathological data were extracted from the study databases. When central data was missing it was substituted with local data.

5.4 Clinical endpoints

Clinical endpoints were pathological complete response (pCR), disease-free survival (DFS), distant-disease free survival (DDFS), and overall survival (OS). Standard definitions for breast cancer clinical trial endpoints were applied.[72] A pCR was defined as no microscopic evidence of residual viable, invasive or non-invasive tumour in surgical specimens of the breast and lymph nodes after neoadjuvant treatment (ypT0 ypN0). An exploratory analysis with a pCR definition including cases with residual non-invasive tumour was also performed (ypT0/is ypN0).

DFS was defined as the time interval between randomization and any disease recurrence, secondary malignancy, or death due to any cause, which ever occurred first. DDFS was defined as the time from randomization to any distant disease recurrence, any secondary malignancy, or death due to any cause. OS was defined as the time interval between randomization and death due to any cause.

5.5 Statistical analysis

Pearson´s chi-squared test was performed to compare baseline clinico-pathological parameters (age, menopausal status, tumour stage, nodal status, tumour grade, histological tumour type, therapy regimen, Ki67 level, tumor infiltrating lymphocytes) and pCR between HR-subgroups. A logistic regression model for endpoint pCR was performed on pooled data from all trials (valid n=2692). Survival probabilities (OS, DFS, DDFS) were calculated with the Kaplan-Meier method and were compared using the log-rank test. DFS sub-analysis was performed based on pCR status (yes vs. no). Cox regression was used to model DFS, DDFS and OS after exclusion of one censored case before the first event (valid n=2691). The regression models by HR-expression level (low-positive, negative, strong-positive) were adjusted for age (<40, 40-50, 51-70, >70 years), tumour stage (T1 vs. T2 vs. T3 vs. T4), nodal status (negative, positive), tumour grading (G1 vs. G2 vs. G3), histological type (no special type, lobular, other), and clinical trial in categorical form (GeparQuinto, GeparSepto) as a surrogate for differing neoadjuvant treatments. For DFS, DDFS, and OS analyses the model was additionally adjusted by pCR after NACT. Categories of tumor infiltrating lymphocytes were not included in the models as this variable was not systematically recorded by the time of patient recruitment of

the clinical trials. Statistical evaluation was carried out using SPSS Statistics 19 (IBM Corporation, Somers, NY, USA). All tests were two-sided and p-values <0.05 were considered as significant. In all regression models for both cohorts low HR-positive patients comprise the reference category; odds and hazard ratios are reported for strong HR-positive versus low HR-positive or HR-negative versus low HR-positive.

5.6 RNA Expression analysis of tumors with low HR expression

A total of 47 tumors with low-HR expression were selected from the clinical trial cohorts, based on the availability of tissue samples in the GBG biobank. RNA sequencing was successfully performed in 38 (80.9%) of these tumors (figure 3). This analysis was performed by cooperation partners of the project from the Technical University of Munich.

Briefly, to quantify gene expression from FFPE tissue samples, a modified 3' mRNA-seq approach adapted from the mcSCRB-seq method was used.[73, 74] Total RNA was extracted from FFPE tissue sections and 25 ng of total RNA was used as input for 3' mRNA-seq. In the first step, polyA+ RNAs were selected during cDNA synthesis through annealing to a poly-dT oligo containing an unique molecular identifier (UMI), well-barcode and Illumina adapter sequence. Second strand synthesis was achieved by template switch RT-PCR using a template switch oligo containing an Illumina adapter sequence. Samples were pooled after cDNA synthesis, afterwards cDNAs were purified and amplified. The sequencing libraries were sequenced on a NextSeq550Dx Instrument. Gene expression counts were generated using the zUMIs pipeline[75] and further statistical analysis was performed in R version 3.6.3 using the DESeq2 version 1.24.0 package[76] for normalization and the sva version 3.32.1 package[77] for batch correction. Molecular subtypes were assigned using the AIMS version 1.16.0 package.[78]

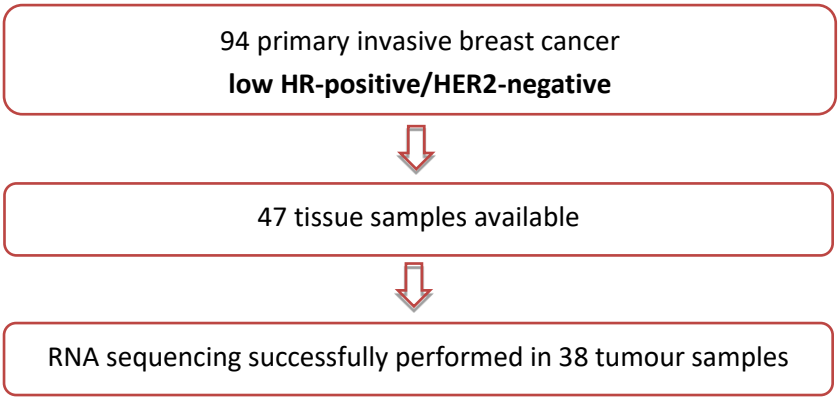


Figure 3. Low HR-positive cases analyzed by RNA-Sequencing

Source: Figure 1 of publication:

Villegas SL, Nekljudova V, Pfarr N, Engel J, Untch M, Schrodi S, Holms F, Ulmer HU, Fasching PA, Weber KE, Albig C, Heinrichs C, Marme F, Hartmann A, Hanusch C, Schmitt WD, Huober J, Lederer B, van Mackelenbergh M, Tesch H, Jackisch C, Rezai M, Sinn P, Sinn BV, Hackmann J, Kiechle M, Schneeweiss A, Weichert W, Denkert C, Loibl S. Therapy response and prognosis of patients with early breast cancer with low positivity for hormone receptors - An analysis of 2765 patients from neoadjuvant clinical trials. Eur J Cancer. 2021;148:159-70. doi: 10.1016/j.ejca.2021.02.020.

6. Results

6.1 Baseline parameters of clinical trial cohorts

Median age of patients was 49 (range 21-78 years). Most of women were premenopausal (59%), had carcinomas of no special type (81.7%), clinical tumour stage 1-2 (76.7%) and clinical nodal status 0-1 (95%).

Ninety-four (3.4%) patients had low HR-positive, 1769 (64.0%) strong HR-positive and 902 (32.6%) HR-negative tumours. The pCR rate after NACT across clinical trials was 18.6%.

Time of follow-up of patients with HR status available (n=2765) was 89.3 months (range 87.2-91.3).

Information about tumor infiltrating lymphocytes was available for 1229 cases. Of those, 18.1% were classified as lymphocyte-predominant BC.

Frequencies of clinical tumour stage and tumour grade, clinical nodal status, baseline Ki67 (categorical), tumor infiltrating lymphocytes as well as pCR rate were not significantly different between low HR-positive and HR-negative subgroups.

Most of the included results of the present work were published in:

Villegas SL, Nekljudova V, Pfarr N, Engel J, Untch M, Schrodi S, Holms F, Ulmer HU, Fasching PA, Weber KE, Albig C, Heinrichs C, Marme F, Hartmann A, Hanusch C, Schmitt WD, Huober J, Lederer B, van Mackelenbergh M, Tesch H, Jackisch C, Rezai M, Sinn P, Sinn BV, Hackmann J, Kiechle M, Schneeweiss A, Weichert W, Denkert C, Loibl S. Therapy response and prognosis of patients with early breast cancer with low positivity for hormone receptors - An analysis of 2765 patients from neoadjuvant clinical trials. Eur J Cancer. 2021;148:159-70. doi: 10.1016/j.ejca.2021.02.020.[70]

6.2 Outcome analysis – pCR and survival analysis

After NACT, 9.4% of patients with strong HR-positive BC achieved a pCR, while among those with HR-negative and low HR-positive tumours, pCR rates were 35.5% and 27.7%, respectively (figure 4). Also, in the multivariate model there was no statistically significant

difference in pCR between patients with low HR-positive and HR-negative tumours (OR: 1.47, 95%-CI: 0.89-2.42, p=0.132).

Because an increase of pCR in GeparSepto study was associated with the administration of nab-paclitaxel,[68] an additional analysis excluding the patients that received this drug (n=407) was performed. The pCR rate remained significantly higher in patients with low-HR tumours in comparison to patients with strong HR-positive tumours. This interpretation is also valid when using ypT0/is for the definition of pCR.[70]

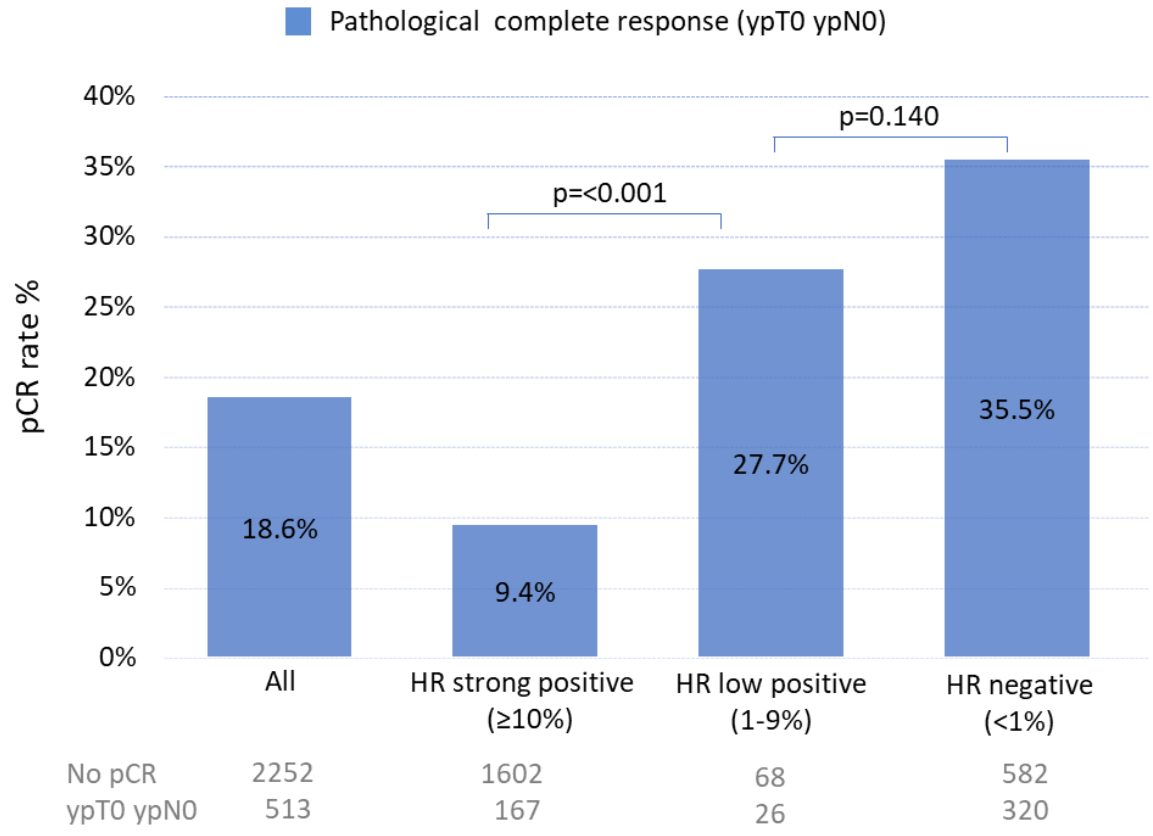


Figure 4. Pathological complete response (pCR; ypT0 ypN0) across breast cancer patients with different expression levels of hormone receptor (HR) from the neoadjuvant clinical trials; N=2765

Source: Figure 2 of publication:
Villegas SL, Nekljudova V, Pfarr N, Engel J, Untch M, Schrodi S, Holms F, Ulmer HU, Fasching PA, Weber KE, Albig C, Heinrichs C, Marme F, Hartmann A, Hanusch C, Schmitt WD, Huober J, Lederer B, van Mackelenbergh M, Tesch H, Jackisch C, Rezai M, Sinn P, Sinn BV, Hackmann J, Kiechle M, Schneeweiss A, Weichert W, Denkert C, Loibl S. Therapy response and prognosis of patients with early breast cancer with low positivity for hormone receptors - An analysis of 2765 patients from neoadjuvant clinical trials. Eur J Cancer. 2021;148:159-70. doi: 10.1016/j.ejca.2021.02.020.

In the multivariate analyses (adjusted for age, clinical tumour stage, clinical nodal status, grading, histological type, pCR status and neoadjuvant study), there was no statistically significant difference regarding DFS, DDFS and OS between patients with low HR-positive and HR-negative tumours (for DFS hazard ratio 1.26, 95%-CI (confidence interval): 0.87-1.83,

log-rank test *p*=0.951, for DDFS hazard ratio 1.17, 95%-CI: 0.78-1.76, log-rank test *p*=0.774; for OS hazard ratio 1.10, 95%-CI: 0.71-1.72, log-rank test *p*=0.618).

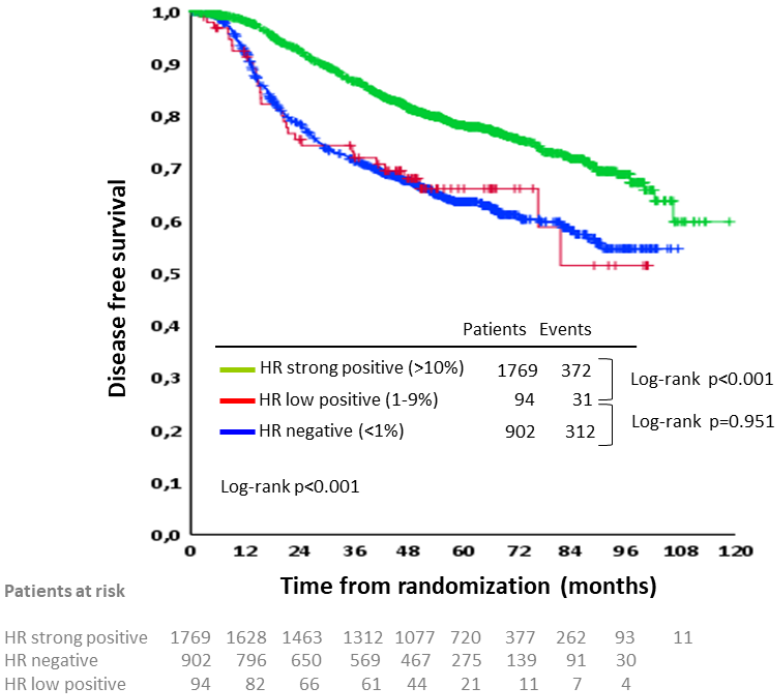
The differences in risk between patients with low HR-positive tumours and those with strong HR-positive tumours remained significant in the multivariate analysis as well. There was a higher probability of relapse, distant relapse, and death for women with tumours that had low expression of HR in comparison to women with strong HR-positive breast cancer (hazard ratio for DFS 0.48, 95%-CI: 0.33-0.70; for DDFS 0.49, 95%-CI: 0.33-0.74; for OS 0.38, 95%-CI: 0.24-0.60). Patients with strong HR-positive tumours had a significantly lower pCR rate (pCR 9.4%, odds ratio 0.38, 95%-CI:0.23-0.63) than patients with low HR-positive tumours.

Survival curves of the three HR-subgroups are depicted in figure 5.

When DFS was analyzed within categories of pCR (yes/no), no differences were found between the HR-subgroups for patients who achieved a pCR after NACT (log-rank test p=0.141; with only one event in the low HR-positive subgroup). In contrast, in the non-pCR group, patients with low HR-positive and HR-negative tumours showed a higher probability of DFS event compared to patients with strong HR-positive tumours (p<0.001).

Variation of pCR definition in some settings, allows for the inclusion of carcinoma in situ after surgery (ypT0/is ypN0). When performing the analysis with this definition, there were 98 additional cases achieving a pCR (59 patients with strong HR-positive, 36 with HR-negative and 3 with low HR-positive tumours). The significant results regarding the similarity of low-HR and HR negative tumors did not change.

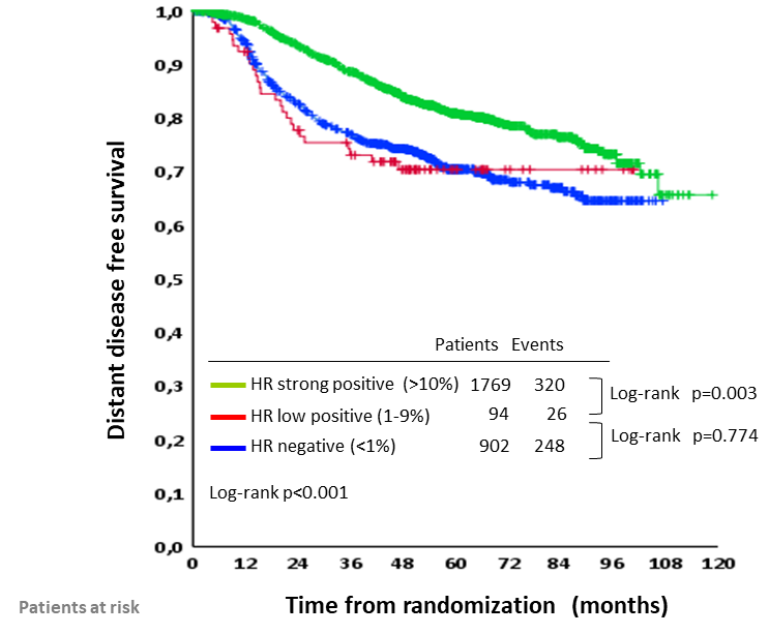
A



Patients at risk

HR strong positive	1769	1628	1463	1312	1077	720	377	262	93	11
HR negative	902	796	650	569	467	275	139	91	30	
HR low positive	94	82	66	61	44	21	11	7	4	

B



Patients at risk

HR strong positive	1769	1634	1479	1339	1108	740	392	275	97	12
HR negative	902	811	684	608	507	295	155	102	32	
HR low positive	94	83	68	62	46	23	12	9	5	

Figure 5a: Disease-free survival (DFS) stratified by HR-subgroups (strong HR positive, low HR positive, HR negative). N=2765;

Figure 5b: Distant-disease free survival (DDFS) stratified by HR-subgroups (HR strong positive, HR low positive, HR negative). N=2765

Source: Figure 3 of Publication:

Villegas SL, Nekljudova V, Pfarr N, Engel J, Untch M, Schrodi S, Holms F, Ulmer HU, Fasching PA, Weber KE, Albig C, Heinrichs C, Marme F, Hartmann A, Hanusch C, Schmitt WD, Huober J, Lederer B, van Mackelenbergh M, Tesch H, Jackisch C, Rezai M, Sinn P, Sinn BV, Hackmann J, Kiechle M, Schneeweiss A, Weichert W, Denkert C, Loibl S. Therapy response and prognosis of patients with early breast cancer with low positivity for hormone receptors - An analysis of 2765 patients from neoadjuvant clinical trials. Eur J Cancer. 2021;148:159-70. doi: 10.1016/j.ejca.2021.02.020.

6.3 RNA expression profiling of tumours with low hormone receptor expression

RNA sequencing was successfully performed on 38 tumours with low HR expression. Assignment of intrinsic molecular subtypes based on gene expression signatures classified 33 (86.8%) tumours as basal subtype, 4 (10.5%) as HER2 and 1 (2.6%) as normal subtype.

7. Discussion

7.1 Summary of major findings

This analysis of the two large cohorts of neoadjuvant treated patients (GeparQuinto and GeparSepto), shows that BC patients whose tumours exhibit a low HR-positive/HER2-negative marker profile and patients with TNBC do not differ in terms of pathological complete response, overall survival, disease-free survival, and distant disease-free survival. In contrast, in all these aspects they are very distinct to patients with strong HR-positive/HER2-negative tumours, who showed worse pCR rates but better survival probabilities.

7.2 The findings on the current state of research

The proportion of low HR-positive cases found in the neoadjuvant clinical trials is comparable to reported values in previous studies on adjuvant treated patients.[66] In this retrospective study, patients with low HR-positive and HR-negative tumours also showed similar survival rates. Since then, retrospective studies on ER-low positivity have consistently showed that this low-ER group does not have a significant better prognosis than triple-negative cases after adjuvant treatments and that they even share similar pathological parameters. [79-83]

In the neoadjuvant setting, a higher probability of achieving a pCR was also reported in patients with HER2-negative primary invasive BC, stage II and III with low percentage of ER-expression and non-randomly treated with NACT.[84] Another study involving 41 patients with low ER-expression according to H-score, reported higher rates of pCR, recurrence and death in ER-negative and low ER-positive cases compared with the strong ER-positive subgroup.[85] Recently, a study that included 165 patients treated with NACT, who had HER2-negative primary BC, found comparable pCR rates among ER-negative and ER-low positive subgroups.[86] A meta-analysis on low-ER positivity including twelve retrospective cohort studies concluded that low-ER patients had a more similar outcome to those with ER-negative disease regarding DFS and OS. In this analysis, neoadjuvant treated patients with low ER-

positive tumours exhibited higher pCR when contrasted with patients with ER-positive tumours, comparable to those with ER-negative cancer.[87]

Table 3 shows an overview of studies on low HR-positivity in HER2-negative breast cancer in the adjuvant or neoadjuvant setting.

Over the last decade, different studies on this topic have repeatedly suggested that low HR-positive tumours show more similarities to triple-negative than to strongly HR-positive tumours at the molecular level, showing frequently basal-like profiles rather than luminal ones.[81, 88, 89] In concordance with this evidence, our molecular subtyping based on gene expression data of 38 low HR-positive tumours classified most of these cases as basal subtype.

Table 3 Overview of studies on low HR-positivity in HER2-negative breast cancer in the adjuvant or neoadjuvant setting

Literature	**N – Design**	**Number of ER-low cases and (cut-off for low-HR definition)**	**Outcome**	**Conclusion/ *Comment**
Deyarmin et al., 2013[81]	N=1238 Gene expression analysis	54 (1-10%) 26 available for gene expression	-	62% classified as basal-like and 27% as HER2-enriched.
Yi et al., 2014[66]	N=9639 Retrospective, response to endocrine treatment and survival	250 (1-9%)	OS, RFS, DRFS (5 years)	Patients with Low-ER had worse survival rates than did patients with ER-positive ≥10% tumours. They do not appear to benefit from endocrine therapy.
Gloyeske et al., 2014 [83]	N=731 Retrospective, pCR after NACT	49 (H-Score 1-100) 18 received NACT	pCR	pCR was achieved by six (33%) patients with low ER-positive BC. *No comparative analysis regarding strong ER-positive and ER-negative.
Balduzzi et al., 2014 [79]	N=1424 Retrospective, survival	124 (1-10%)	OS, DFS (5 years)	No differences in OS or DFS among HR-negative and Low HR-positive. 110 cases received endocrine therapy with no significant effect on DFS.
Fujii et al., 2017[84]	N=3055 Cut-off analysis for likelihood of pCR after NACT and benefit of adjuvant hormonal therapy	171 (1-9%) 43 received adjuvant hormonal therapy	OS, TTR (3,9 years)	Cut-off of ER-expression below which pCR was likely was 9.5%. No differences of pCR and TTR rates among ER-negative and Low ER-positive. Adjuvant hormonal therapy was not significantly associated with TTR among Low ER-positive.
Landmann et al., 2018[85]	N=327 Retrospective, pCR after NACT.	41 (H-Score 1-100)	pCR, OS, DFS (2,8 years)	No differences of pCR-, OS-, DFS-rates among Low ER-positive and ER-negative.
Villegas et al., 2021[70]	N=2765 Retrospective, pCR after NACT und survival	94 (1-9%)	pCR, OS, DFS, DDFS (10 years)	No differences of pCR and survival rates among HR-negative and Low HR-positive. Higher pCR and worse survival among patients with low HR-

				expression compared to strong HR-positive.
Dieci et al., 2021[86]	N=406 Retrospective, pCR after NACT and survival	42 (1-9%) 24 patients received NACT	pCR, OS, DRFS (4,8 years)	No differences in pCR rates among patients with Low ER-positive and ER-negative tumours. No differences in OS, DRFS according to ER expression levels. *Residual carcinoma in situ was included in pCR definition.
Paakkola et al., 2021[87]	N=7791 12 retrospective cohort studies Systematic review and metaanalysis (pCR and survival)	499 (1-10%) 7/12 studies provided pCR data according to ER levels.	pCR, OS, DFS	Higher pCR among patients with low ER-expression level compared to strong ER-positive. Patients with Low ER-positive had worse DFS and OS compared to patients with strong ER-positive. No differences in DFS or OS between Low ER-positive and ER-negative subgroups.

DRFS=Distant-recurrence-free-survival; RFS=relapse free survival; OS=overall survival; TTR=time to recurrence; DFS=disease free survival; NACT= neoadjuvant chemotherapy; pCR=pathological complete response.
Table created by Villegas, SL.

7.3 The debate about low-HR positivity in breast cancer

The search for the best cut-off points for oestrogen receptor assays that could predict response to endocrine therapy began in the 1970s, when DCC biochemical assays were used for the quantification of oestradiol-binding protein in tumour tissue. With this method, the fresh-frozen tumour tissue had to be homogenized and the cytosol had to be extracted by centrifugation to perform the LBA involving incubation with radioactively labeled estradiol. Separation of receptor-bound estradiol from the unbound fraction was achieved by DCC. Unbound receptor content was calculated with the multipoint analysis (Scatchard plot)[90] and expressed as femtomoles of estradiol bounded per mg of extracted cytosol tissue protein (fmol/mg). The threshold to determine ER- and PR-positivity at that time was having at least 3 and 5 fmole/mg of protein of specific binding sites, respectively.[91]

In the following decade, the low-positivity concept for ER was for the first time presented in a systematic overview carried out by Early Breast Cancer Trialists' Collaborative of all available randomized trials on adjuvant endocrine and cytotoxic therapy covering 1985-1990.[92] A negative measurement and measurements of 1-9 fmole receptor protein per mg of cytosol protein were considered as "ER poor", while patients with tumours classified as having 10 or more fmole/mg (or described as "positive" in the original clinical trial) were classified as ER-positive. The benefit of tamoxifen, which was clinical standard since 1977, was confirmed for

patients with ER-positive tumours, especially with node-positive disease. A “still significantly favourable effect” was described for cases classified as “ER poor”, especially for women >50-years-old (3311 patients).[92] An overview-analysis by the Trialists' Collaborative group in 1992 about systemic treatment in early BC, including 133 clinical trials, [93] identified a threshold of 10 fmole/mg for ER positivity as a common practice in most laboratories.

A large metanalysis that analyzed data of >20,000 women with tumours assessed as having <10 fmol ER/mg protein (based on LBA/DCC assay) who received endocrine therapy, revealed that these patients had a prognosis comparable with those who did not receive any endocrine therapy, so no significant benefit was apparent.[94]

Based on the good concordance found in comparative studies of LBA/DCC and IHC methods, [63, 95] and the observation that the great benefit of endocrine therapy was predicted in patients with more than 10fmol/mg cytosol protein,[63, 91] a cut-off of 10% was assumed for IHC procedures and it was widely used for HR-positivity in the early 2000’s.[96]

In 2009, after analyzing the available evidence about levels of HR-expression and response to endocrine therapy, the St. Gallen International Breast Cancer Conference stated „any positive level of ER-expression is considered sufficient to justify the use of endocrine adjuvant therapy in almost all patients”.[97]. In 2010, the ASCO/CAP guidelines proposed that all BC cases should be considered HR-positive when the tumours showed at least 1% of nuclear staining for ER or PR.[65]

The decision to lower the threshold for HR-positivity to 1% might be mostly driven by the work of Harvey et. al [63], who compared the agreement between LBA/DCC and IHC methods, and also by the results of Viale et al.,[98] who centrally reviewed HR expression levels in the BIG 1-98 trial comparing letrozole and tamoxifen.

Harvey and colleagues performed a retrospective, univariate cut-point analysis of 777 patients that received any adjuvant endocrine therapy alone (n=517) or combined with chemotherapy (n=260), according to DFS as outcome. In this pooled analysis, the cutoff was set at IHC score greater than 2 with the Allred score, so the lowest possible positive score was 3, that corresponded to as few as 1% to 10% weakly staining tumour cells. This group of patients represented 5.1% of all included patients (n=38) and showed a better response to endocrine therapy, compared to patients with HR-negative tumours. However, no information was provided whether these patients belonged to the group of treatment in combination with chemotherapy. The proposed cut-off was subsequently applied to a subgroup of patients that only received endocrine therapy, with significant DFS benefit.

In the study of Viale et. al, patients with ER-expression levels between 1-9% (n=44) showed an intermediate 5-year-DFS survival curve between ER-negative (n=63) and ER-positive patients (n=3489) in the Kaplan-Meier analysis, suggesting some responsiveness to the adjuvant endocrine treatment. Nonetheless, hazard ratios are informed as significant based only on categories ER-positive vs. ER-negative and no specific statistical test or *p-values* among the subgroups of low-ER and ER-negative were provided.

Most likely, there are no studies perfectly designed to answer the question of best cut-off to define positivity for hormone receptors. A few retrospective analyses indicate that BC patients with low HR-levels could benefit from endocrine therapy, but emergent evidence during the last decade points out to a distinct biological and clinical behavior of low HR-positive cancers in comparison to strong HR-positive types (see table 3). This prompted a reexamination of the data about the optimal threshold for ER-positivity by international experts.

After re-reviewing the available evidence in 2020, ASCO/CAP guidelines granted that tumours with low ER-expression may be biologically distinct from tumours with high ER-expression (>10%) and that the threshold at 1% for ER-positivity might not uniformly predict differences in prognosis or prediction of therapy response. Nonetheless, even though the available data supporting potential benefit of endocrine therapy in cases with 1-10% ER-positivity was limited, they considered that due to the relatively low toxicity of the antihormone therapy and aiming to reduce false-negative results, ≥ 1% nuclear ER-staining by IHC should be maintained as the threshold for ER-positivity.[67]

The new recommendation in the 2020 treatment guidelines update was that, cases with 1-10% staining should be reported as “ER Low Positive”, with a comment mentioning the more limited clinical data and heterogeneous behavior and biology of this cancer subgroup.[67] Finally, the guideline continued to recommend analyzing the pros and cons of endocrine therapy for these low ER-positive cases individually. Interestingly, there was no agreement on best cut-off for HR-positivity for initiation of endocrine therapy during the last St. Gallen International Breast Cancer Conference in 2021.[20]

Currently, no treatment guidelines or consensus exist suggesting a particular regimen for the treatment of this group of patients. Some ongoing phase I-II clinical trials including patients whose tumours show low ER-expression, are testing the benefit of novel therapies (including MEK inhibitor, Olaparib and Afatinib) either alone or in combination with standard endocrine therapy.

The evaluation of the best threshold for HR-positivity remains a challenge due to low frequency of low HR-positive cases, lack of prospective studies based on IHC, and the necessary inclusion

of non-randomized treated patients in retrospective analysis. It is likely that molecular assays testing functional protein level can help overcome this problem in the future.

7.4 Strengths and limitations of the study

A detailed survival analysis according to endocrine adjuvant therapy in the present work was not possible and represents a limitation of the study. This therapy was not provided by the clinical trials and its administration was not systematically documented during the follow-up. Other limitations are the lack of information on exact percentage of ER/PR staining from 76 cases in the GeparQuinto trial (2.6% of total cases included), making it necessary to exclude those cases from the analysis. It is crucial to emphasize that regardless of the scoring system used to assess levels of HR expression, an exact percentage of positivity should be always documented. Probably, application of validated digital imaging analyses and artificial intelligence technology can soon help with this task, increasing accuracy and effectiveness in biomarker assessment. [99]

A re-evaluation of hormone receptor levels on archive material was not performed. BRCA status of the study population was unknown and a comparison in this important aspect between TNBC and low-HR positive/HER2-negative cases was not possible. Due to the small number of low-HR cases available for RNA-sequencing, we were not able to classify these according to the previously published TNBC subtypes.

To avoid a critical confounding factor, HER2-positive cases were excluded from the analysis, since ER-positive/HER2-positive early breast cancers are known to have a lower rate of pCR compared to ER-negative/HER2-positive cases, regardless the type of anti-HER2 targeted therapy used.[16, 17] However, further analyses of the subgroup of patients with low HR-positive/HER2-positive tumours would be highly interesting, as these receptor pathways are proven to set a bidirectional crosstalk. Epigenetic analysis of 23 cases based on DNA-Methylation, showed that samples with low HR-positivity lacking HER2-amplification, clustered with TNBC reference specimens. In contrast, most tumours with low HR-expression and HER2-amplification were grouped with HR-positive cancers.[100]

This study is based on large neoadjuvant clinical trials cohorts in Germany. Information about breast cancer patients with low HR-positive tumours in the neoadjuvant setting is scarce and this study provides important clinical information for this subgroup of patients. The statistical analyses between HR-subgroups allowed a detailed comparison among patients with different

levels of HR-expression. Another strength of the study is the 10 years-survival analysis that represents to our knowledge the longest period of analysis on this topic.

7.5 Implications for clinical practice and future research

The profile of patients included in the analysis differs to some extent from that of BC patients on a population-based level. Patients in the clinical trial cohorts had a lower median age at diagnosis (49 years) and a slightly higher proportion of HR-negative tumours (30%). In 2018, the median age of disease onset for BC in Germany was 64 years.[2] But around 30% of patients were younger than 55 years of age at the time of diagnosis.[2] The results of the study are valuable for this population since younger patients are most at risk of relapse and mortality,[2] due to higher prevalence of aggressive tumour subtypes, like TNBC.[101]

Our results support the view that patients with tumours exhibiting low levels of HR (1-9%) may respond akin patients with TNBC regarding neoadjuvant chemotherapy. It also indicate that new therapy options for TNBC should be evaluated in patients with tumours with <10% positivity of estrogen or progesterone receptors. This is especially important since breakthrough therapies in the immune oncology field focusing on TNBC have been approved and more of these clinical trials are being conducted exclusively in HR-negative tumours.

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9. Statutory Declaration
Eidesstattliche Versicherung und Anteilserklärung

„Ich, Sonia Villegas, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: Tumorprogression und Ansprechen auf neoadjuvante Chemotherapie bei Brustkrebspatientinnen mit niedriger Hormonrezeptorpositivität (Tumorprogression and responsiveness to neoadjuvant chemotherapy in breast cancer patients with low hormone receptor positivity) selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen werden von mir verantwortet.

Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Meine Anteile an etwaiger Publikation zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Betreuer/in, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

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Datum Unterschrift

Anteilserklärung an den erfolgten Publikationen

Sonia Villegas hatte folgenden Anteil an der Publikation:

Publikation 1: [Sonia L Villegas, Valentina Nekljudova, Nicole Pfarr, Jutta Engel, Michael Untch, Simone Schrodi, Frank Holms, Hans-Ulrich Ulmer, Peter Fasching, Karsten Weber, Christian Albig, Clemens Heinrichs, Frederik Marmé, Arndt Hartmann, Claus Hanusch, Wolfgang D Schmitt, Jens Huober, Bianca Lederer, Marion van Mackelenbergh, Hans Tesch, Christian Jackisch, Mahdi Rezai, Peter Sinn, Bruno V Sinn, John Hackmann, Marion Kiechle, Andreas Schneeweiss, Wilko Weichert, Carsten Denkert and Sibylle Loibl], [Therapy response and prognosis of patients with early breast cancer with low positivity for hormone receptors ean analysis of 2765 patients from neoadjuvant clinical trials], [European Journal of Cancer], [2021]

Liste der Beiträge im Einzelnen:

- Mitkonzeption der Studie, Erarbeitung der genauen Fragestellung.
- Mitkonzeption und Formulierung des statistischen Analyseplans.
- Schreiben der benötigten Programmierungs-Syntaxen zur Durchführung der Statistik mit der Software SPSS 2018. Behebung der Fehler in Syntaxen nach Validation von Koautorin.
- Bearbeitung der daraus resultierenden Outputs, Erstellung und Editierung aller Tabellen und Abbildungen in der Publikation.
- Dokumentation der genauen Schritte für die Ablesung der Daten aus den Outputs und Erstellung der Tabellen und Abbildungen.
- Vollständige Verfassung der Abschnitte „Introduction“, „Evaluation of receptors“, und „Low HR-positivity”, „Results“ and “Discussion”.
- Mitverfassung des Abschnitts „Patients and methods“.
- Auswahl und Analyse aller bibliographischen Referenzen
- Koordination der Kommunikation mit KoautorInnen und der Besprechungen der Ergebnisse.

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Unterschrift, Datum und Stempel des erstbetreunden Hochschullehrers

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Unterschrift der Doktorandin

10. Publication

10.1 Auszug aus der Journal Summary List (ISI Web of Knowledge)*

Journal Data Filtered By: Selected JCR Year: 2019 Selected Editions: SCIE,SSCI
Selected Categories: "ONCOLOGY" Selected Category Scheme: WoS
Gesamtanzahl: 244 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
21	Journal of the National Comprehensive Cancer Network	6,912	9.316	0.020020
22	CANCER TREATMENT REVIEWS	9,427	8.885	0.017800
23	Cancer Immunology Research	6,969	8.728	0.026440
24	LEUKEMIA	25,819	8.665	0.048640
25	Blood Cancer Journal	2,800	8.023	0.010400
26	ONCOGENE	66,303	7.971	0.068320
27	Clinical and Translational Medicine	1,349	7.919	0.003280
28	npj Precision Oncology	500	7.717	0.001520
29	BIOCHIMICA ET BIOPHYSICA ACTA-REVIEWS ON CANCER	5,650	7.365	0.007800
30	CANCER LETTERS	34,162	7.360	0.044450
31	EUROPEAN JOURNAL OF CANCER	32,241	7.275	0.048170
32	Gastric Cancer	5,525	7.088	0.010730
33	JOURNAL OF EXPERIMENTAL & CLINICAL CANCER RESEARCH	9,316	7.068	0.014540
34	Therapeutic Advances in Medical Oncology	1,894	6.852	0.004260
35	Molecular Oncology	6,378	6.574	0.013820
36	CANCER AND METASTASIS REVIEWS	6,247	6.400	0.005940
37	Cancers	10,442	6.126	0.018740
38	Oncogenesis	2,775	6.119	0.007750
39	STEM CELLS	20,554	6.022	0.024110
40	npj Breast Cancer	814	6.000	0.003590
41	JOURNAL OF PATHOLOGY	16,307	5.979	0.017910

10.2 Publication

Villegas SL, Nekljudova V, Pfarr N, Engel J, Untch M, Schrodi S, Holms F, Ulmer HU, Fasching PA, Weber KE, Albig C, Heinrichs C, Marmé F, Hartmann A, Hanusch C, Schmitt WD, Huober J, Lederer B, van Mackelenbergh M, Tesch H, Jackisch C, Rezai M, Sinn P, Sinn BV, Hackmann J, Kiechle M, Schneeweiss A, Weichert W, Denkert C, Loibl S. Therapy response and prognosis of patients with early breast cancer with low positivity for hormone receptors - An analysis of 2765 patients from neoadjuvant clinical trials. Eur J Cancer. 2021 Vol. 148 Pages 159-170

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11. Curriculum Vitae

"Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht."

"My curriculum vitae will not be published in the electronic version of my dissertation for data protection reasons."

12. Publication list

Jurmeister P, Weber K, **Villegas S**, Karn T, Untch M, Thieme A, Müller V, Taube E, Fasching P, Schmitt WD, Marmé F, Stickeler E, Sinn BV, Jank P, Schem C, Klauschen F, van Mackelenbergh M, Denkert C, Loibl S, Capper D. DNA methylation profiling identifies two distinct subgroups in breast cancers with low hormone receptor expression, mainly associated with HER2 amplification status. Clin Epigenetics. 2021 Oct 3;13(1):184. doi: 10.1186/s13148-021-01176-5. PMID: 34602069; PMCID: PMC8489064.

Impact factor of Clinical Epigenetics in 2021: 6.55

Villegas SL, Nekljudova V, Pfarr N, Engel J, Untch M, Schrodi S, Holms F, Ulmer HU, Fasching PA, Weber KE, Albig C, Heinrichs C, Marmé F, Hartmann A, Hanusch C, Schmitt WD, Huober J, Lederer B, van Mackelenbergh M, Tesch H, Jackisch C, Rezai M, Sinn P, Sinn BV, Hackmann J, Kiechle M, Schneeweiss A, Weichert W, Denkert C, Loibl S. Therapy response and prognosis of patients with early breast cancer with low positivity for hormone receptors - An analysis of 2765 patients from neoadjuvant clinical trials. Eur J Cancer. 2021 Vol. 148 Pages 159-170

Impact factor of European Journal of Cancer in 2020: 9.16

Xiao Y, Rabien A, Buschow R, Amtislavskiy V, Busch J, Kilic E, **Villegas SL**, Timmermann B, Schütte M, Mielke T, Yaspo ML, Jung K, Meierhofer D. Endocytosis-Mediated Replenishment of Amino Acids Favors Cancer Cell Proliferation and Survival in Chromophobe Renal Cell Carcinoma. Cancer Res. 2020 Dec 15;80(24):5491-5501. doi: 10.1158/0008-5472.CAN-20-1998. Epub 2020 Oct 28. PMID: 33115803.

Impact factor of Cancer Research in 2020: 12.70

Villegas SL, Darb-Esfahani S, von Minckwitz G, Huober J, Weber K, Marmé F, Furlanetto J, Schem C, Pfitzner BM, Lederer B, Engels K, Kümmel S, Müller V, Mehta K, Denkert C, Loibl S. Expression of Cyclin D1 protein in residual tumor after neoadjuvant chemotherapy for breast cancer. Breast Cancer Res Treat. 2018 Feb;168(1):179-187. doi: 10.1007/s10549-017-4581-1. Epub 2017 Nov 25. PMID: 29177689

Impact factor of Breast Cancer Research and Treatment in 2018: 3.61

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