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Stem cell markers predict prognosis in breast cancer overall (EpCAM), and in subgroups of large (CD133), triple-negative (CD44), and hormone receptor positive carcinomas (CD166)

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For my parents, who made this thesis possible

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Abbreviations

ALCAM…… Activated leucocyte adhesion molecule
BSA……. Bovine Serum Albumin
CD…… Cluster of differentiation molecule
CI……. Confidence interval
cm…… Centimeters
CT…… Computed tomography
PET-CT…… Positron emission tomography-computed tomography
DAPI…… 4′,6-Diamidino-2-phenylindole dihydrochloride
DCIS…… Ductal Carcinoma in Situ
EDTA…….. Ethylenediaminetetraacetic acid
EpCAM…… Epithelial cell adhesion molecule
ER……. Estrogen receptor
FFPE…… Formalin fixed, paraffin embedded
FISH……. Fluorescence in Situ Hybridization
H&E…….. Hematoxylin and eosin staining
Her2…….. Human epidermal growth receptor 2
HR…… Hazard ratio
IHC…….. Immunohistochemistry
LIN…… Lobular Intraductal Neoplasia
LN……. Lymph node
µm…… Micrometers
PBS……. Phosphate Buffered Saline
PR…….. Progesterone receptor
TMA……. Tissue microarray
TNM……. Tumor size, nodal status, distant metastases (staging system)
UDH……. Usual Ductal Hyperplasia
UICC…… Union for International Cancer Control

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1 Introduction and research goals

1.1 Etiology and clinical characteristics of breast carcinomas

Breast carcinoma is the most common cancer in women [64]. In 2016, 68.950 women developed breast cancer in Germany and 18.570 women died of it [102]. Despite enormous efforts in basic and clinical research, breast cancer still remains to be a disease with an unpredictable prognosis on an individual patient level. In order to improve this situation, prognostication instruments such as the PREDICT tool have been developed, using cell surface marker expression [78]. Furthermore, assays examining gene expression profiles, namely Oncotype DX and MammaPrint, have been developed to offer clinicians the possibility to use the variation of gene expression in breast cancer for therapy guidance [82, 114]. Despite their clinical prognostic capacities for specific patient groups, these diagnostic tools fall short in some breast cancer patient subgroups [21]. Hence, there is still a clinical need to effectively predict prognosis for all breast cancer patients on an individual level. This is the scope of this thesis. One major obstacle to accurate prognostics and hence effective, individualized therapy is the tumor heterogeneity of breast cancer. Recent evidence points to a subset of tumor cells, so called cancer stem cells, that drive tumor growth and heterogeneity as well as metastasis and therapeutic resistance [19, 131]. Multiple cell surface markers identifying cancer stem cells have been found in breast cancer tissue samples and cell lines [45]. There are contradicting results regarding the association of breast cancer stem cell markers with the prognosis of patients [61]. To further clarify the association of cancer stem cell markers with disease progression in breast cancer, a tissue microarray was constructed. Four cancer stem cell markers were tested for their association with clinical and histopathological parameters as well as survival. The prognostic impact of these stem cell markers on breast cancer specific subgroups were the focus of this thesis.

1.1.1 Epidemiology and clinical presentation

Cancer is expected to be the leading cause of death around the world in the 21st century [25]. The most common type of cancer and the leading cause of death from cancer worldwide in women is breast cancer.

In 2018 there have been 2.08 million estimated new breast cancer cases in women worldwide, as well as 626,700 estimated breast cancer deaths [64]. After a peak in breast cancer incidence in western countries in the 1990's, incidence and mortality have decreased in recent years. The decline in incidence of breast cancer is mainly due to reduced prescription of postmenopausal hormone therapy. The reduction in mortality on the other hand was mostly caused by an increase of mammography screenings, thus detecting cancer at an earlier stage, and improved treatment options [99].

Symptoms of breast cancer include pain in the breast, a palpable mass or nodules and nipple discharge. Although these signs are commonly associated with benign lesions, in a significant minority of cases they do occur due to malignant neoplasms [122].

As the disease progresses, the systemic spread of cancer poses the greatest threat to the patients. Metastatic disease is the most important cause of morbidity and mortality in breast cancer patients. Due to the detection and treatment of breast cancer in early, locally contained stages, survival has improved [160]. In most countries around the world the five-year survival of breast cancer patients was above 80% between 2005 and 2009; in Germany it has been 85.3% [9].

1.1.2 Diagnosis, grading and staging of breast cancer

The diagnosis of breast cancer is established through a number of different diagnostic modalities and an interdisciplinary approach. The initial clinical examination is followed by a mammogram, sonography or a combination of both imaging techniques. In case of a suspicious finding, a tissue biopsy is warranted to determine the histology [206]. In case a carcinoma is detected, the histology of the tumor is evaluated and classified into a grading system according to Elston and Ellis [62]. Furthermore, the expression of immunohistochemistry (IHC) markers, namely estrogen (ER), progesterone (PR) and Her2 receptors, is quantified and included into the prognostic considerations and used to explore therapeutic options [121]. In addition to the evaluation of the tumor itself, physical examination and imaging studies are used to search for regional and distant metastases [166].

The results of the various diagnostic modalities are then used to stage breast cancer according to the TNM-classification of the Union for International Cancer Control (UICC) and the American Joint Committee on Cancer (AJCC) and to determine the prognosis and further management of the disease [41].

In the recent past, molecular stratification of breast cancer has become increasingly important as a parameter for prognostic prediction and therapeutic intervention (Table 1) [77]. However, despite significant efforts the inter- and intratumor heterogeneity of breast cancer remains an unsolved problem in clinical oncology [143] due to the vast genetic diversity of breast cancer beyond the intrinsic subtypes [179].

	Luminal A-like	**Luminal B-like, Her2 negative**	**Luminal B-like, Her2 positive**	**Her2 positive**	**Triple-negative**
ER	**+**	**+**	**+**	-	-
PR	**+**	PgR - and any Ki67	any PgR	-	-
Her2	-	-	**over-expressed**	**over-expressed**	-
Ki67	Low	**Ki67 high** and any PgR	any Ki67	any Ki67	any Ki67

Table 1 Clinico-pathologic surrogate parameters for the intrinsic subtypes used in molecular stratification of breast cancer [77]

1.1.3 Treatment

After the initial diagnosis of breast cancer, staging of the disease is warranted to provide the patient with the ideal treatment option available. Staging for patients without clinical signs of advanced disease is focused on the assessment of locoregional invasion. Ductal carcinoma in situ (DCIS) and invasive carcinoma are initially treated with breast conserving surgery, if possible. During surgery, the sentinel lymph node is biopsied and axillary lymph node dissection may be necessary in case the carcinoma has metastasized to the sentinel node. Locoregional staging is conducted according to the pTNM system of the UICC, which includes tumor size and the occurrence of lymph node metastases. In most cases breast conserving surgery should be followed by whole-breast-radiotherapy to reduce the risk of local recurrence.

Systemic therapy is chosen according to receptor expression and intrinsic subtype of the breast cancer and can be used in the adjuvant or neo-adjuvant setting.

Patients with estrogen receptor expressing carcinomas should be treated with anti-hormone therapy including Tamoxifen or aromatase inhibitors.

Her2-neu positive carcinomas can be targeted with Trastuzumab and all breast cancer patients, with some exceptions, should receive anthracyclin- and/or taxane-based chemotherapy regimens [175].

Patients presenting with advanced disease, including locally advanced disease or distant metastases, have to be staged with bone-, computed tomography (CT)- or positron emission tomography-computed tomography (PET-CT)- scans. Just as primary tumors of the breast, metastatic lesions should be biopsied and assessed, if easily accessible. Treatment options for patients with advanced disease have to be explored with regard to patient specific factors such as biological age, co-morbidities and previous treatment as well as carcinoma specific factors such as hormone- and Her2-neu receptor expression [32].

1.1.4 Etiology

The etiology of breast cancer can be multifactorial. Nevertheless, a number of risk factors have been identified to be promoting the development of breast cancer. Among these are long exposure to endogenous estrogen due to early menarche and late menopause. Early pregnancy, high parity and breastfeeding on the other hand have a long-term protective effect. Further risk factors include, among others, exogenous estrogen, namely oral contraceptives and postmenopausal hormone therapy, high body mass index, old age, alcohol consumption, family history for breast cancer [81, 194, 205], and ionizing radiation [116].

Molecular research of the past decades has revealed carcinogenesis to be a sequential process where a progressive accumulation of mutations and epigenetic changes lead to an abnormal cellular behavior and uncontrolled cell division. Especially changes of tumor suppressor genes and oncogenes are crucial for carcinogenesis [202]. This is most obvious in patients with inherited high penetrant mutations in tumor suppressor genes such as *BRCA1, BRCA2* [184] or *PALB2* [11] as well as *CHEK2* [43] and *RAD51* [39]. However, these inherited genetic factors seem to be responsible for only 5% of all breast cancer cases.

Hormonal risk factors on the other hand seem to be of much more relevance [181]. Unfortunately, neither the mechanisms causing breast cancer nor the ones promoting carcinogenesis are fully understood [202].

1.1.5 Cancer stem cell and clonal evolution models

One striking feature of breast cancer is the marked heterogeneity of histological and molecular characteristics within the tumor. The marked heterogeneity of breast carcinomas is an essential challenge due to resistance and inadequate treatment response following therapeutic regiments [212].

Within the marked heterogeneity of the tumors a distinct cellular hierarchy appears to be present. There are two models explaining this phenomenon.

1.1.5.1 The cancer stem cell model

One possible explanation is the theory of the hierarchical tumor stem cell model [161]. The concept of cancer stem cells is based on the hypothesis that a small subset of cells is able of self-renewal, unlimited, asymmetrical proliferation and generation of the heterogenous cell bulk including tumorigenic and non-tumorigenic cells. Therefore, these cells are responsible for tumor growth as well as disease recurrence [8, 36, 117, 125]. At the base of this model are cancer stem cells that give rise to distinct cellular populations forming the tumor through asymmetrical cell division [148]. Self-renewal of cancer stem cells is represented by the ability of regenerating a heterogenous tumor after transplantation into immunocompromised mice and its continued ability to proliferate even after serial passages [7]. The tissue structure of a tumor resembles normal tissue in the sense that it is organized in a hierarchical pattern. It is hence likely that cancer stem cells develop through mutation of normal stem cells. Normal stem cells are long-lived cells that may accumulate numerous mutations and already poses the ability of self-renewal [161]. Nevertheless, a somatic progenitor cell might acquire mutations and transform into a tumor-initiating cell as well. This tumor-initiating cell then gives rise to a heterogeneous cell bulk. Any proliferative cell in this bulk might acquire a mutation re-instigating the self-renewal capacity and therefore turn it into a cancer stem cell. These cancer stem cells then have the potential to evade therapy and to maintain the tumor and its growth and metastasis [197].

1.1.5.2 The clonal evolution model

In this model the tumor initiation is caused by a series of mutations in a cell which begins to proliferate unchecked and to produce numerous subclones. Genetic and epigenetic aberrations that benefit the cell clone cause the respective cells to expand [155]. This model suggests that every tumor cell has a tumorigenic potential and therefore can develop a new cell clone. Due to differing heritable effects on the fitness of the cells, mutant clones will expand or contract in the tumor due to natural selection and genetic drift [138]. Stochastic, clonal evolution through sequential selection of the mutant subpopulations is thought to cause the hierarchical, cellular heterogeneity found in solid tumors [144].

Clonal evolution, nevertheless, does not explain the fact that evolutionary changes of the cell clones in a tumor do not translate into changes of the major morphology and phenotype of breast cancer lesions. It rather seems that cancer stem or tumor initiating cells determine the underlying phenotype of a tumor and that clonal evolution subsequently is causing the intratumor heterogeneity of breast cancer [83].

1.1.6 Characteristics of breast cancer stem cells

As a result of genomic instability, epigenetic modifications and the influences of the tumor microenvironment on cells arising from the cancer stem cells, the tumor is composed of a heterogenous cluster of cell clones [29]. These three factors also influence the “stemness” of those cell clones and therefore the fate of the tumor and patient survival. In general, cancer stem cells represent a certain minority of cells with self-renewal capacity. However, there are tumors with a large number of cells maintaining a self-renewal capacity. The number of cancer stem cells in a tumor can vary greatly and therefore their impact on patient survival [113]. Accordingly, great effort has been spent to find biomarkers in order to identify these stem cells. After identification of cancer stem cells using surface proteins in acute myelogenous leukemia [118], attempts were made to detect similar stem cells in solid cancers. Al-Hajj et al. were able to verify the existence of such cells in human mammary carcinoma using cell adhesion molecules cluster of differentiation molecule (CD) 44, CD24 and epithelial adhesion molecule (EpCAM). As few as 200 cells with a CD44 positive/CD24 negative/ EpCAM positive marker panel (CD44+/CD24-/EpCAM+) formed tumors after being implanted into a mammary fat pad of a mouse and repeatedly did so after multiple passages in mice [8].

When looking at gene and hormone receptor expression patterns in CD44 positive cells, genes well known from self-renewal pathways in stem cells were upregulated. Equally upregulated were genes involved in cell motility and angiogenesis.

Meanwhile, estrogen receptor was found to be expressed in lower concentrations in CD44 positive breast cancer cells. At the same time CD44 positive tumors were associated with a shorter patient survival, suggesting that CD44 positive cancer cells comprise a mesenchymal, stem-cell like phenotype associated with cell migration and metastasis [177].

As described above, molecular subtypes in breast cancer constitute an additional prognostic factor. Five major molecular subtypes are known with luminal subtypes

being the most prevalent ones, also showing the best prognosis. The basal-like subtype constitutes a heterogeneous group of aggressive, undifferentiated cancers including triple-negative breast carcinomas, with efficient therapeutic options still being scarce [77, 151, 183]. These basal-like carcinomas are associated with a CD44+/CD24- expression pattern. Additionally, these carcinomas showed a trend to a reduced overall and disease-free survival [162]. In luminal subtypes, on the other hand, these CD44+/CD24- subtypes were less common, suggesting an enrichment of cancer stem cells in the more aggressive breast cancer subtypes [92].
An important hallmark of malignancy is the ability to metastasize in distant organs and tissues. CD44+/CD24- cells have been found to be associated with an increased rate of osseous metastases in breast cancer patients [2]. Additionally, CK positive cells in bone marrow of breast cancer patients, representing disseminated tumor cells, have been found to be mostly CD44+/CD24- [16].
As described above, cancer stem cells may be responsible for therapy resistance and relapse in breast cancer patients. In fact, chemotherapy as well as radiation has shown to increase the number of CD44+/CD24- cells in breast carcinomas [40, 123, 154]. Patients with tumors demonstrating such an increase in CD44+/CD24- cells had a significantly reduced disease-free survival, indicating that said cells are chemotherapy resistant and contribute to tumor growth and disease progression [120].
Numerous studies are providing conflicting data. Ahmed et al. investigated the impact of CD44 and CD24 on the prognosis of breast cancer patients in a large cohort. Astonishingly, CD44 was associated with a better prognosis and a more differentiated, luminal tumor type. In fact a CD44 negative/CD24 positive expression pattern was associated with the worst outcome and a dedifferentiated cell type [6]. Similar results are presented by Mylona et al., showing a tendency to a better prognosis for patients with CD44 positive/CD24 negative expression pattern, while tumors with CD44-/CD24+ expression were associated with decreased disease-free and overall survival [142].

1.2 Cell surface markers expressed on breast cancer stem cells

As described above, the main cell surface markers to identify cancer stem cells in breast carcinoma are CD44, CD24 and to a lesser extent EpCAM.

Nevertheless, several additional cancer stem cell markers have been identified in other cancers and subsequently shown to be expressed in breast carcinomas as well.

1.2.1 Cancer stem cell marker CD133 (Prominin 1)

One of those markers is CD133 (Prominin1), a transmembrane glycoprotein, first described to be expressed in hematopoietic stem cells [139, 211]. Initially, CD133 expression was assumed to be restricted to hematopoietic and embryonic stem cells but expression on adult tissue cells and carcinomas was demonstrated later [68]. Subsequently, CD133 has been discovered to be overexpressed in non-small cell lung cancer [90] and was identified as a marker for tumor initiating cells in brain tumors [178] and colon carcinoma [163]. In breast cancer, CD133 was first identified on cells with cancer stem cell properties in mouse mammary tumors [208]. In subsequent studies, CD133 was correlated with lymph node metastases and triple negative breast cancer in humans [26, 129]. In a subgroup analysis of triple negative breast carcinomas, CD133 showed to be significantly correlated with larger tumor size, lymph node metastases, higher UICC stage (staging according to tumor size, lymph node and distant metastases) and reduced disease free and overall survival [214]. Furthermore, in breast cancer patients treated with neoadjuvant chemotherapy, CD133 expression was significantly associated with lymph node metastases, shorter disease free and overall survival [12].

1.2.2 Cancer stem cell marker CD44

As discussed above, CD44 is the most prominent cancer stem cell marker in breast cancer [45]. CD44 was first described as a transmembrane glycoprotein involved in cell-cell- and cell-matrix-adhesion and cell-signaling [94] binding to hyaluronic acid [72]. Although, initially, CD44 was described on hematopoietic cells, the marker is expressed on many cell types including epithelial and mesenchymal cells. Subsequently, CD44 was discovered to be expressed in several carcinomas and their metastases, including breast cancer [174].

CD44 gained its importance as a cancer stem cell marker after the CD44 positive/CD24 negative phenotype had been linked to tumor initiating cells in breast cancer [8]. Multiple studies and publications debate the role of CD44 in breast cancer initiation and progression [8, 177].

1.2.3 Cancer stem cell marker EpCAM

Another potential cancer stem cell marker in breast cancer is EpCAM. This cell surface marker was first discovered in colorectal carcinoma cells [87, 110] and has been shown to be a glycoprotein, expressed on epithelial cancers and in normal tissue [126, 203]. Naturally, among epithelial cancers expressing EpCAM, the mammary carcinoma was present as well [140]. It was found that EpCAM was involved in many other cellular functions next to cell adhesion, such as cell signaling, migration and metastasis [91]. Accordingly, EpCAM expression was shown to be significantly associated with clinical aspects of tumor migration and metastases in the sense of larger tumor size, breast cancer metastasis and increased disease recurrence and mortality [71, 191]. In later studies EpCAM expression was linked to a reduced disease-free and overall survival [168, 169, 188].

1.2.4 Cancer stem cell marker CD166 (ALCAM)

Finally, CD166 (ALCAM) is another cancer stem cell marker expressed on breast cancer cells. CD166 is an immunoglobulin adhesion molecule first discovered on activated leukocytes [24]. Subsequently, CD166 expression was identified in several normal human tissue types as well as in melanoma cells which showed an enhanced metastatic potential [54].

Besides melanoma, CD166 was found to be expressed in colorectal cancer as well, where high CD166 expression was associated with shorter overall survival [201]. In breast cancer, CD166 expression was shown to be correlated with smaller tumor size, fewer lymph node /distant metastases, lower tumor grade and better prognosis on the one hand [50, 100, 106]. On the other hand, subsequent studies demonstrated cytoplasmic CD166 being overexpressed in invasive carcinoma and significantly associated with lymph node metastases and a shorter disease-free survival [28]. The importance of CD166 expression remains somewhat unclear, although high CD166 expression is associated with prolonged overall survival after neoadjuvant chemotherapy [96].

1.3 Synopsis and research goals

The vast heterogeneity of breast cancer reflected in different histopathologic subtypes, varying expression of hormone receptors and intrinsic subtypes challenge the treatment of breast cancer greatly [19]. Cancer stem cells have been described in a variety of carcinomas, including breast cancer [8]. A number of cell surface

proteins identifying cancer stem cells have been found in breast cancer tissue samples and cell lines [45]. The results regarding the association of breast cancer stem cell markers with the prognosis of patients vary in the literature as some studies present contradictory results [61]. A tissue microarray was constructed for this study and four cancer stem cell markers, namely CD133, CD44, EpCAM and CD166, were tested for their association with clinical and histopathological parameters as well as the survival of the patients included. The aim was to identify distinct associations of cancer stem cell markers with clinical aspects and patient survival, indicating possible therapeutic targets to be evaluated in future studies. The analysis of subgroups and the influence of stem cell markers on survival on these patients were of special interest. The distinct research questions for this study were as follows:

Is there an association of

- overall-, five-year- or disease-free survival with cancer stem cell marker expression?
- overall-, five-year- or disease-free survival with cancer stem cell marker expression in subgroups of patients with either large tumors, lymph node or distant metastases, high tumor grading, ER, PR, Her2neu or Ki-67 expression or tumors with specific intrinsic subtypes?
- tumor size, lymph node metastases, tumor grading and distant metastases with cancer stem cell marker expression?
- hormone receptor, Her2neu and Ki-67 expression or the clinico-pathologic surrogate parameters for the intrinsic subtypes with cancer stem cell marker expression?
- stem cell marker expression with normal breast- or breast cancer tissue?

2 Material and Methods

2.1 Patient selection and clinical characteristics of the cancer cohort

For this study, 245 female patients with benign or malignant neoplasms of the breast were selected retrospectively at the University Hospital Schleswig-Holstein, Campus Lübeck. The study was approved by the ethics committee of the University of Lübeck (Ethikvoten Nr. 08-012 und 20-507). The patients selected were treated for breast tumors between the years of 1989 and 1993. Tissue samples were retrieved from the tissue archive of the Institute of Pathology. The corresponding clinical information was obtained as follows: age, date of surgery, tumor location, surgical procedure, histopathology, UICC stage [88], grading [22, 62], disease recurrence and distant metastases. The overall survival was calculated as the interval between the year of surgery and the date of the last follow-up (December 2011) or the date of death. Tissue samples of 207 patients suffering from breast carcinoma were included on the TMA. For 175 (84.5%) of these patients, data were available regarding all clinical parameters assessed in this study. Patients were divided into groups suffering from early (112 patients, 64%) and late stage carcinoma (63 patients, 36%), using the UICC staging [88] (Table 2). Significant differences in tumor size, nodal status and distant metastases are expected, as these factors are used to divide patient groups into early and late stage carcinomas. Additionally, patient age was found to be significantly higher in the late stage carcinoma group ($p=0.012$). While the mean age in the early stage carcinoma was 59.74 years (± 14.38 years), patients in the late stage carcinoma group had a mean age of 65.62 years (± 14.71 years). Besides age, disease recurrence was significantly higher in the late stage carcinoma group ($p=0.001$). 16 patients (25.4%) suffered from disease recurrence in the late stage carcinoma group, while only 8 patients (7.8%) in the early carcinoma group relapsed.

Clinical Parameters	Subgroups	UICC 0-II (early stage) No. of patients (%) 112 (64)	UICC III-IV (late stage) No. of patients (%) 63 (36)	p-Value
Age	Years	59.74 ± 14.38	65.62 ± 14.71	**0.012**
	Range	30 - 93	31 - 90	
Histopathology	DCIS/LIN	5 (4.5%)	0 (0)	0.161
	Invasive ductal carcinoma	92 (82.1)	51 (81)	
	Other carcinoma	15 (13.4)	12 (19)	
Location	Right	52 (48.6)	28 (50)	0.871
	Left	55 (51.4)	28 (50)	
Tumor size	Tis	1 (0.9)	0 (0)	**< 0.001**
	T1	47 (42)	2 (4.1)	
	T2	62 (55.4)	15 (19.5)	
	T3	2 (1.8)	6 (9.8)	
	T4	0 (0)	38 (62.3)	
Nodal status	N0	70 (63.1)	9 (18.4)	**< 0.001**
	N1	41 (36.9)	23 (46.9)	
	N2	0 (0)	17 (34.7)	
Distant metastases	M0	112 (100)	44 (69.8)	**< 0.001**
	M1	0 (0)	19 (30.2)	
Grading	G1	6 (5.6)	0 (0)	0.177
	G2	82 (75.9)	48 (80)	
	G3	20 (18.5)	12 (20)	
Disease recurrence	No recurrence	104 (92.9)	47 (74.6)	**0.001**
	Recurrence	8 (7.1)	16 (25.4)	
Intrinsic classification	Luminal A	45 (47.4)	26 (50)	0.92
	Luminal B Her2 negative	11 (11.6)	6 (11.5)	
	Luminal B Her2 positive	7 (7.4)	3 (5.8)	
	Her2 positive	7 (7.4)	2 (3.8)	
	Triple negative	25 (26.3)	15 (28.8)	
Ki 67 (20% cutoff)	Negative	99 (88.4)	55 (87.3)	0.813
	Positive	13 (11.6)	8 (12.7)	
Estrogen receptor status	Negative	44 (40)	26 (42.6)	0.748
	Positive	66 (60)	35 (57.4)	
Progesterone receptor status	Negative	43 (39.4)	21 (34.4)	0.621
	Positive	66 (60.6)	40 (65.6)	
Her2 neu status	Negative	95 (85.6)	55 (88.7)	0.646
	Positive	16 (14.4)	7 (11.3)	

Table 2 Comparison of clinical parameters in early and late stage carcinoma patients. In some cases, the tissue present in the sample differed from the TNM classification in the original pathology report as described in the following section. The p-Value was calculated with Chi^2 test, Fisher's exact test or non parametric tests (Mann-Whitney U test, Kruskal-Wallis test), as appropriate.

2.2 Identification and retrieval of specimens

Tissue sections that had been stained at the Institute of Pathology at the University of Lübeck with Meyer's Hematoxylin and Eosin (H&E) for the primary diagnosis were retrieved from the archive of the Institute.

One senior pathologist (Prof. Christoph Thorns, M.D.) evaluated and scored all specimens stained with H&E as well as the staining for routine markers, Ki67 and the cancer stem cell markers described hereafter. The sections with H&E staining were reviewed and for each patient the tissue samples with the highest amount of neoplastic or normal tissue were determined and retrieved from the tissue archive of the Institute of Pathology. In case tissue of different types of neoplasms was available in one patient, a representative sample of every tissue type was retrieved from the archive. Additionally, a normal tissue sample was gathered for every patient, if available. The tissue had been fixed in 4% normally buffered formalin and had subsequently been embedded in paraffin.

Slices of 4 µm thickness were cut from the tissue specimens and mounted on glass slides in the Laboratory for Surgical Research. They subsequently were stained with H&E and assessed by the senior pathologist to confirm the primary diagnosis. Samples that did not contain any normal epithelial or neoplastic tissue were not further included into the study. In case the diagnosis in our assessment differed from the entity described in the primary histological diagnosis, the diagnosis made by the pathologist for this study was considered for further analysis. However, the UICC stage and grading described in the primary diagnosis were used for analysis in this study. Tissue samples were available for 245 patients and included in a tissue microarray (TMA). The mean observation time of the patients, calculated as described above, was 11.8 years (Table 3).

			Benign tumors (n = 31)	**DCIS (n = 7)**	**Carcinoma (n = 207)**	**Total (n = 245)**
Age at Diagnosis (years)		Mean	42 ± 17	60 ± 14	62 ± 15	60 ± 16
		Range	18 - 82	41 - 75	30 - 93	18 - 93
Follow up (years)		Mean	18 ± 4	17 ± 5	11 ± 8	12 ± 8
		Range	8 - 21	7 - 21	0 - 23	0 - 23

Table 3 Clinical parameters of the patients included in the TMA.

2.3 Methods

2.3.1 Tissue Microarray construction

The concept of constructing a TMA goes back to the multitumor tissue block developed by Battifora [18]. The first TMA was constructed more than ten years later [111].

This TMA described by Kononen et al. contains up to 1,000 tissue cores retrieved from so called donor tissue blocks. These cores are punched out of the donor blocks using a tissue arrayer and punches with a diameter from 0.6 mm to 2.0 mm. Subsequently these punches are integrated into previously prepared holes in a blank paraffin block. Up to 300 sections of 4 µm thickness can be cut from such a TMA providing a large amount of tissue samples from one block. To determine the area of interest on the tissue samples, the pathologist marked and labeled the neoplastic and normal tissue areas on the HE stained slides that were produced for this study. The paraffin embedded tissue samples of the patients included in this study were marked accordingly. Using a tissue arrayer (Tissue arrayer, Pathology Devices Inc., Westminster, USA) punches with a diameter of 1.5 mm were taken out of these marked areas and inserted into a blank paraffin block. All together, 406 tissue punches were included into 3 TMAs, containing 129, 137 and 140 tissue cores respectively. For one poor quality tissue sample two cores were placed in the TMA to ensure an adequate amount of tissue for analysis in one of the cores. Only one of these cores was used for analysis, hence 405 tissue cores were included in further analysis (Table 4). To enable macroscopic identification of the different TMAs, the layout was varied and tissue cores from human liver were included in distinctive locations on the array.

Histopathology		Samples (% of total)
Main groups (n of samples)	**Subgroups**	
Normal tissue (n = 101)	Normal tissue	101 (24.9)
Benign neoplasia (n = 43)	Usual Ductal Hyperplasia	8 (2)
	Papilloma	2 (0.5)
	Fibroadenoma	33 (8.1)
Phylloides tumor (n = 2)	Phylloidestumor	2 (0.5)
Noninvasive malignant neoplasia (n = 37)	Ductal Carcinoma in Situ	35 (8.6)
	Lobular intraepithelial Neoplasia	1 (0.3)
	Atypical Ductal Hyperplasia	1 (0.3)
Invasive carcinoma (n = 222)	Ductal carcinoma	173 (42.7)
	Lobular carcinoma	9 (2.2)
	Mucinous carcinoma	3 (0.7)
	Medullary carcinoma	2 (0.5)
	Tubular carcinoma	10 (2.5)
	Carcinoma, not further specified	25 (6.2)
	Total	405 (100)

Table 4 Number of tissue cores representing the respective histopathological groups

After completion of the tissue microarrays, the blocks were incubated for 12 hours at 36˚C to improve adhesion of the tissue punches to the paraffin. Afterwards, protruding tissue punches were carefully leveled by gently pressing on a glass slide onto the microarray block. Subsequently, the blocks were incubated at 36˚C for another 30 minutes and finally stored at 4˚C. This protocol, similar to the so called tempering, improves the performance of the tissue microarray during sectioning significantly [89]. In addition to established protocols, an automated rotating microtome (Hyrax M 55, Carl Zeiss, Jena, D) with a cool clamp (Cool Cut UKK, Carl Zeiss, Jena, D) and a water-driven retrieval system (Hyrax STS Section-Transfer-System, Carl Zeiss, Göttingen, D) for the sections were used in this study. These lead to additionally improved performance of the tissue microarray during sectioning. After cutting, the sections were mounted on glass slides, baked over night at 60˚C and thereupon dried at room temperature for 48 hours. Finally, the slides were stored at 4˚C until further processing (Figure 1).

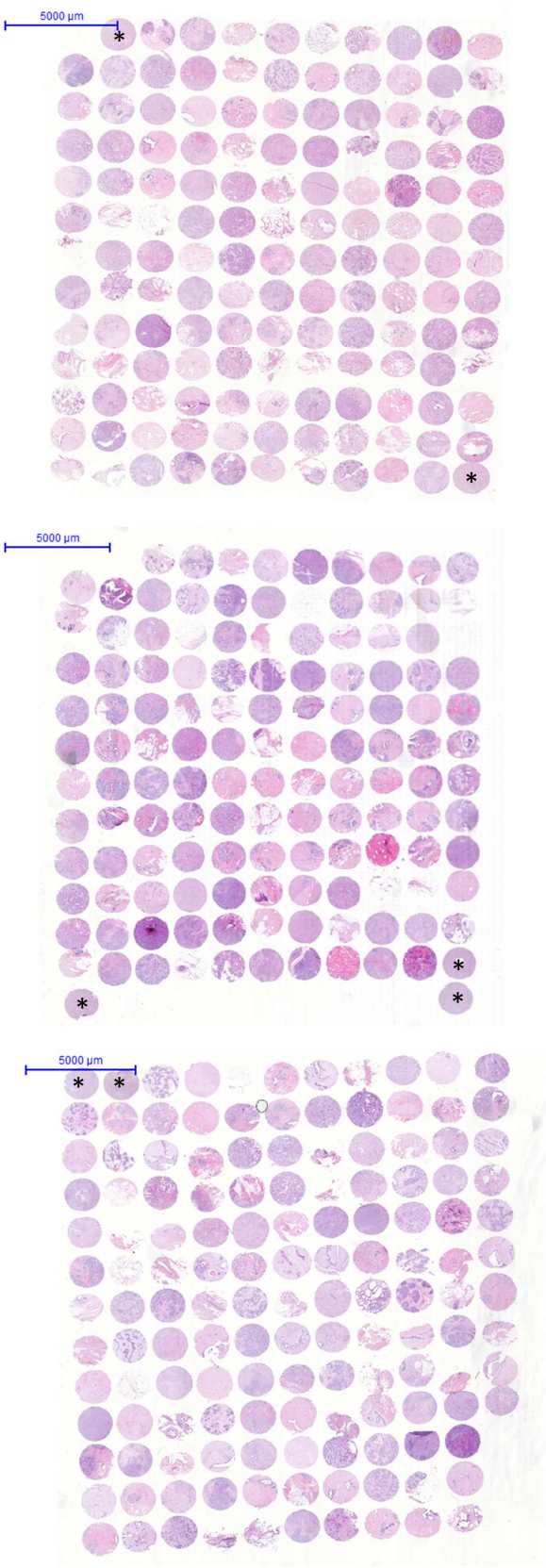


Figure 1 Section of the three TMAs stained with H&E; the cores marked with an asterisk are liver tissue cores included for orientation. The scale measures 5000 µm.

2.3.2 IHC staining and FISH on the tissue microarray

Hormone and Her2neu receptors where not routinely assessed at the time, when the tumor specimens of the patients of this cohort where initially examined at the Institute of Pathology. Hence, most of the tissue samples were not stained for these markers during the primary assessment. In order to be able to compare the cancer stem cell markers examined in this study to nowadays gold standard of pathological diagnosis of breast cancer, the tissue microarray was stained for estrogen (ER) and progesterone receptors (PR) as well as Her2-neu receptor (Her2). As a marker of proliferation, the expression of Ki67 was assessed as well. The staining for these markers was conducted at the Institute of Pathology at the UKSH, Campus Lübeck; ER, PR, Her2 and Ki67 were detected with immunohistochemistry using protocols established in the Institute of Pathology for analysis in clinical routine (Verfahrensanweisungen VA-033, VA-050, VA-015, Institute of Pathology, UKSH, Campus Lübeck).

In order to assess the status of Her2 expression in the tissue samples, a fluorescence in situ hybridization (FISH) with a commercially purchased Her2/CEP17 probe was completed for all cases in addition to the immunohistochemistry staining. Protocols of clinical routine assessment of the Institute of Pathology were used to evaluate the staining.

For the immunohistochemistry staining of cancer stem cell markers, the TMA slides were deparaffinized in Xylene and then rehydrated in a descending Ethanol series. For antigen retrieval, slides were heated in retrieval buffers Table 5 Primary antibodies used in immunohistochemistry staining of cancer stem cell markers.(Table 5) in a microwave oven. To block endogenous peroxidase in the breast tissue, the slides were treated with 3% hydrogen peroxide in methanol for 10 minutes. Subsequently, the slides were incubated with normal serum in PBS to prevent unspecific binding of the secondary antibodies to binding sites in the tissue. Thereafter, the TMAs were incubated overnight with primary antibodies, distinctively diluted in Antibody Diluent, at 4ºC (Table 5, Table 13). All primary antibodies used were monoclonal mouse antibodies. After the primary antibodies were washed off, the slides were incubated with biotinylated secondary antibodies (Table 14).

Primary antibodies	Dilution	Antigen retrieval	Secondary antibodies
CD 133 Miltenyi Biotec Inc. Clone AC133 Monoclonal IgG 1 Mouse	1:10	5 minutes at 900 watts and 14 minutes at 300 watts in citrate buffer (pH 6)	Horse Anti-Mouse Vectastain® Elite ABC Universal Kit
CD 44 BD Biosciences Clone G44-26 Monoclonal IgG 2b Mouse	1:150	5 minutes at 900 watts and 14 minutes at 300 watts in citrate buffer (pH 6)	Horse Anti-Mouse Vectastain® Elite ABC Universal Kit
EpCAM Dako Cytomation A/S Clone Ber-EP4 Monoclonal IgG 1 Mouse	1:50	20 minutes at 900 watts in Antigen Retrieval Solution (Dako Cytomation)	Horse Anti-Mouse Vectastain® Elite ABC Universal Kit
CD 166 Abcam® Clone MOG/07 Monoclonal IgG 2a Mouse	1:100	5 minutes at 900 watts and 14 minutes at 300 watts in citrate buffer (pH 6)	Goat Anti-Mouse Dako Cytomation

Table 5 Primary antibodies used in immunohistochemistry staining of cancer stem cell markers.

Following the secondary antibodies, the slides were incubated with streptavidin-biotin-peroxidase solution. Binding to the biotin on the secondary antibodies, the streptavidin-biotin-peroxidase forms a complex that is attached to the secondary antibodies. As a substrate for the peroxidase, 3-Amino-9-Ethylcarbazole (AEC) or 3-3'-Diamino-benzidine (DAB) were subsequently added onto the slides. In the presence of peroxidase AEC and DAB are oxidized and generate a red or brown precipitate, respectively. Finally, the slides were counterstained with Haematoxylin and covered with Aquatex® and a cover glass (Supplemental Figure 1-4). All TMA slides were analyzed by a senior pathologist who was blinded for all clinical data.

The TMA slides stained with antibodies against ER and PR were analyzed according to the standards of the breast cancer guidelines of the American Society of Clinical Oncology/College of American Pathologists [79]. The immunohistochemistry staining for Her2 was assessed according to the ASCO/CAP guidelines as well [207]. The expression of Ki67 was assessed as the percentage of neoplastic cells that contained stained nuclei. Tumors harboring more than 20% positive cells were rated as positive [58].

The FISH for Her2 was detected with a fluorescence microscope and the appropriate imaging software. The pathologist determined the areas of invasive

carcinoma that were to be imaged for each carcinoma case and subsequently analyzed the Her2 and CEP17 signals for 60 tumor cells in this area. Tumors were determined as Her2 positive when the Her2/CEP17 ratio exceeded 2.2 [207].

The analysis of the immunohistochemistry staining of the cancer stem cell markers was done together with a senior pathologist as described above. The samples were evaluated according to a score established in the Laboratory for Surgical Research at the University of Lübeck. Samples containing less than 1% positive cells received a score of 0, samples with up to 20% positive cells were scored as 1 and samples containing up to 50% and more than 51%, respectively, were scored as 2 and 3 (Figure 2).

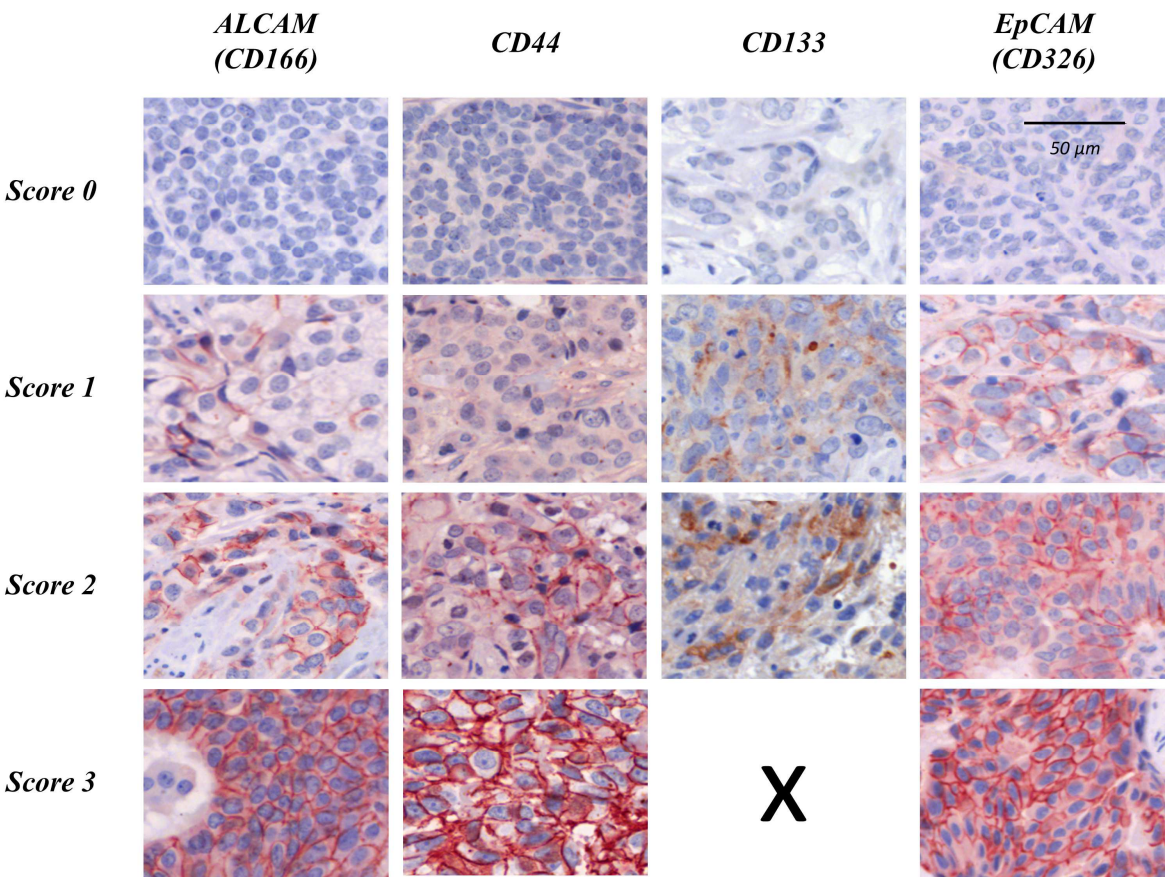


Figure 2 Immunohistochemial staining of mammary TMA sections: The quantity of immune-reactive cells was estimated as a three category score: score 0 (≤1% of total cells), score 1 (2–20%), score 2 (21–50%) and score 3 (51–100%). For the staining of CD133, no sample with high expression was detected (score 3). For statistics, expression of CD133, CD44 and CD166 scored with 0 was assessed as "low" and expression of score 1-3 was summarized as "high". For EpCAM expression, a scoring of 0 and 1 was evaluated as "low" compared to scoring of 2 and 3 as "high". Staining evaluation assessed only epithelial cells. (Magnification 100x); (ALCAM: activated leucocyte adhesion molecule, CD: cluster of differentiation, EpCAM: epithelial cell adhesion molecule)

Marker expression was considered high for CD166, CD44 and CD133 at a score of 1 to 3, for EpCAM expression was considered high with a score of 2 to 3.

Due to partly harsh conditions during IHC staining, numerous tissue cores on the TMA sections were lost. Patients with missing date were excluded from the respective analysis regarding said data.

Therefore, the number of patients differs in each analysis regarding traditional IHC markers, Her2 neu as well as the cancer stem cell markers (Figure 3).

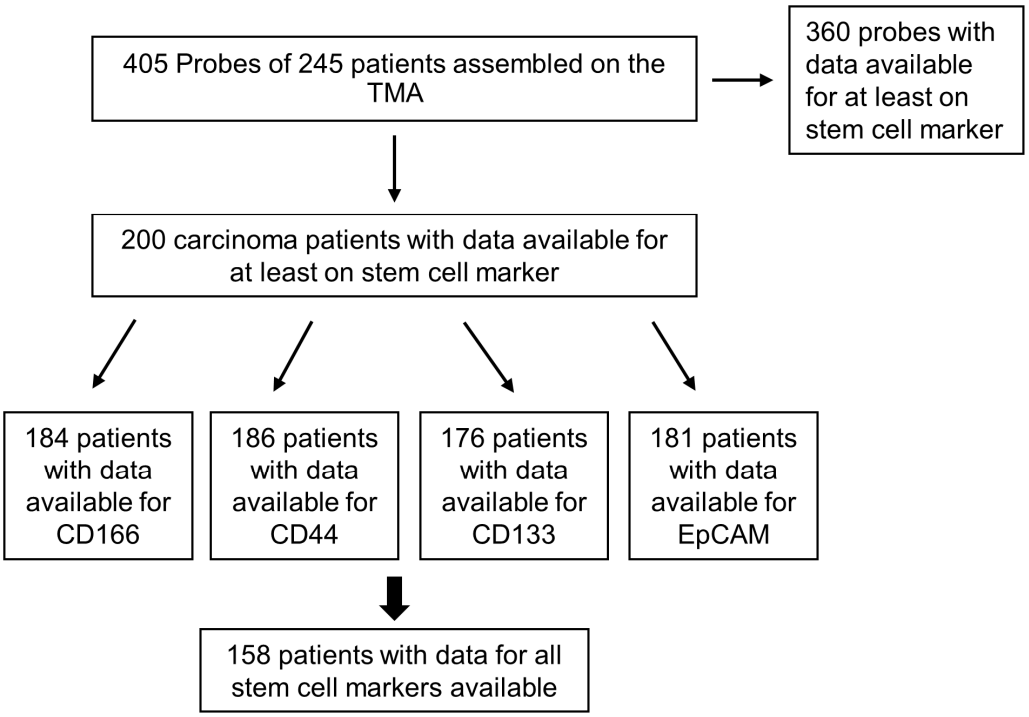


Figure 3 Number of patients included for statistical analysis of the respective cancer stem cell marker. (TMA: tissue microarray, CD: cluster of differentiation molecule, EpCAM: epithelial cell adhesion molecule)

Patients suffering from breast carcinoma comprised 207 patients (84.5%) out of 245 patients whose tissue samples were included on the TMA. For 200 cancer patients (96.6% of all cancer patients included) at least one tissue core stained for a cancer stem cell marker remained available. CD133 was the cancer stem cell marker with the least amount of tissue cores available for analysis with 176 cancer patients (85% of all cancer patients included) being eligible for statistical analysis. Data regarding all four cancer stem cell markers were available for 158 patients (76.3% of all cancer patients included). Hence, statistical analysis for the different cancer stem cell markers involved slightly diverging numbers of patients.

2.3.3 Statistical analysis

Associations between the cancer stem cell marker expression and clinical parameters were statistically evaluated by Chi^2 test or Fisher's exact test, as appropriate. Non parametric tests (Mann-Whitney U test, Kruskal-Wallis test) were used to compare cancer stem cell marker expression levels to continuous variables such as age. Correlations between marker expressions and between all clinical measures mentioned above were assessed by Spearman's rank correlation

coefficient (ρ). Disease-free-survival at five and 22 years of follow-up was calculated using Kaplan-Meier estimator and evaluated by log-rank test for differences due to potential influencing factors. To uncover dependencies between variables and to determine the prognostic value of the stem cell markers upon survival time, simple and multivariable Cox regression analysis was conducted. The model selection procedure was carried out by stepwise backward regression using the likelihood ratio criterion. Clinical parameters were included into the Cox model when the log-rank test yielded an association with survival with p-values less than 0.1. Statistical analyses were performed using SPSS Statistics version 22 (IBM Corporation, Somer, NY) and visualized using additionally GraphPad Prism 4.0 software© (San Diego, CA, USA). P-values of ≤ 0.05 were regarded as statistically significant.

Sandra Freitag-Wolf, Ph.D., of the Institute of Medical Informatics and Statistics at the Christian-Albrechts-University Kiel, supervised statistical calculations for this project.

3 Results

3.1 Cancer stem cell marker expression in normal tissue and benign neoplasia compared to carcinoma tissue

Tissue samples were examined regarding the intensity and distribution of cancer stem cell marker expression as described above. The normal and benign tissue samples were comprised of normal breast tissue, benign neoplasia and phyllodes tumors. The group of carcinoma tissue included samples of DCIS and invasive carcinoma. The overviews of the stained TMA sections are presented in Supplementary Figures 1-4. The samples for analysis of CD133 expression were comprised of 105 samples (34.4%) that contained normal tissue and benign neoplasia and 200 samples (65.6%) contained carcinoma tissue. 62 normal and benign tissue samples (59.1%) stained $CD133^{high}$, while 52 carcinoma samples (26%) displayed a $CD133^{high}$ staining (OR 0.24 (CI 0.15-0.4), $p<0.001$, Figure 4).

In the analysis of CD44 expression, 101 (32.1%) were normal or benign tissue samples, 214 (67.9%) contained malignant tissue. 18 (17.8%) of the normal and benign tissue samples stained $CD44^{high}$ while 62 (29%) of the carcinomas expressed $CD44^{high}$ (OR 1.9 (CI 1.0-3.4), $p=0.038$, Figure 4).

In the analysis of EpCAM expression, 51 (24.8%) carcinoma tissue samples were $EpcAM^{high}$ while 17 (16.3%) of the normal and benign tissues stained $EpCAM^{high}$. There was no statistically significant difference in EpCAM expression between normal and benign tissue and carcinomas (OR 1.7 (CI 0.9-3.1), $p=0.11$, Figure 4).

In the samples analyzed for CD166 expression, 211 samples (67.2%) containing carcinoma tissue of which 104 samples (49.3%) were $CD166^{high}$. 68 (66%) of the normal tissue and benign neoplasia samples were $CD166^{high}$ (OR 0.5 (CI 0.3-0.8), $p=0.006$, Figure 4).

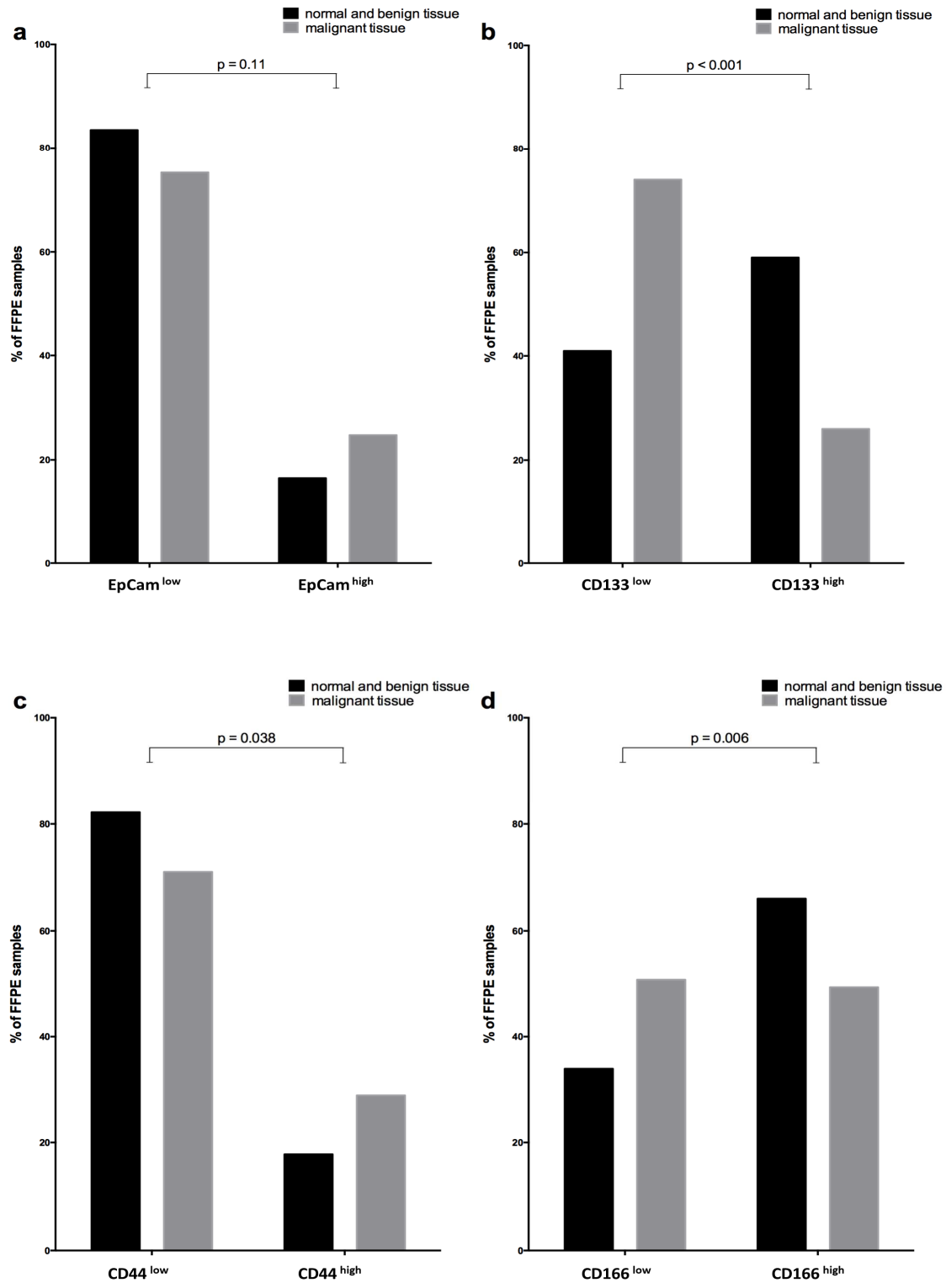


Figure 4 Cancer stem cell marker expression in association with histopathology (normal and benign in black and malignant, DCIS and carcinoma in grey); a) EpCAM, b) CD133, c) CD44, and d) CD166. (FFPE: formalin fixed, paraffin embedded, EpCAM: epithelial cell adhesion molecule, CD: cluster of differentiation molecule)

3.2 Association between cancer stem cell marker expression and clinical parameters

3.2.1 CD133 expression compared to clinical parameters

The associations described in the following paragraph are presented in Figure 5 and Table 6. The high expression of CD133 was significantly associated with non-invasive histopathology. Only 36 samples of invasive carcinoma (21.3%) stained $CD133^{high}$ while 133 invasive carcinomas (78.7%) were $CD133^{low}$. On the other hand, five DCIS samples were $CD133^{high}$ (71.4%), while two (28.6%) were $CD133^{low}$ (OR 0.11 (CI 0.02-0.58), p=0.01). In contrast, $CD133^{high}$ carcinomas were associated with more invasive intrinsic subtypes (p=0.009). Of 40 triple negative carcinomas, 17 carcinomas (42.5%) were $CD133^{high}$, compared to 11 (15.9%) out of 69 luminal A carcinomas. $CD133^{high}$ expression was less common in ten luminal B (Her2-neu positive) (1, 10%) and 23 luminal B (Her2-neu negative) carcinomas (4, 17.4%).

$Ki\text{-}67^{high}$ carcinomas were more often $CD133^{high}$ than $Ki\text{-}67^{low}$ carcinomas: nine (42.9%) out of 21 $Ki67^{high}$ carcinomas expressed high CD133. Only 32 (20.6%) out of 155 $Ki67^{low}$ carcinomas expressed $CD133^{high}$ (OR 2.88 (CI 1.12-7.44), p=0.029). CD133 expression was shown to have an inverse correlation with the ER (OR 0.34 (CI 0.17-0.72), p=0.004). Of the 172 samples included in this analysis, 39 (22.7%) were $CD133^{high}$. 16 ER positive samples (15.2%) were $CD133^{high}$ while 89 (84.8%) ER positive samples were $CD133^{low}$.

The correlation of CD133 expression and PR positivity was equally inverse (OR 0.27 (CI 0.13-0.57), p=0.001). Of 102 PR positive carcinomas 14 (13.7%) were $CD133^{high}$ and 88 (86.3%) were $CD133^{low}$.

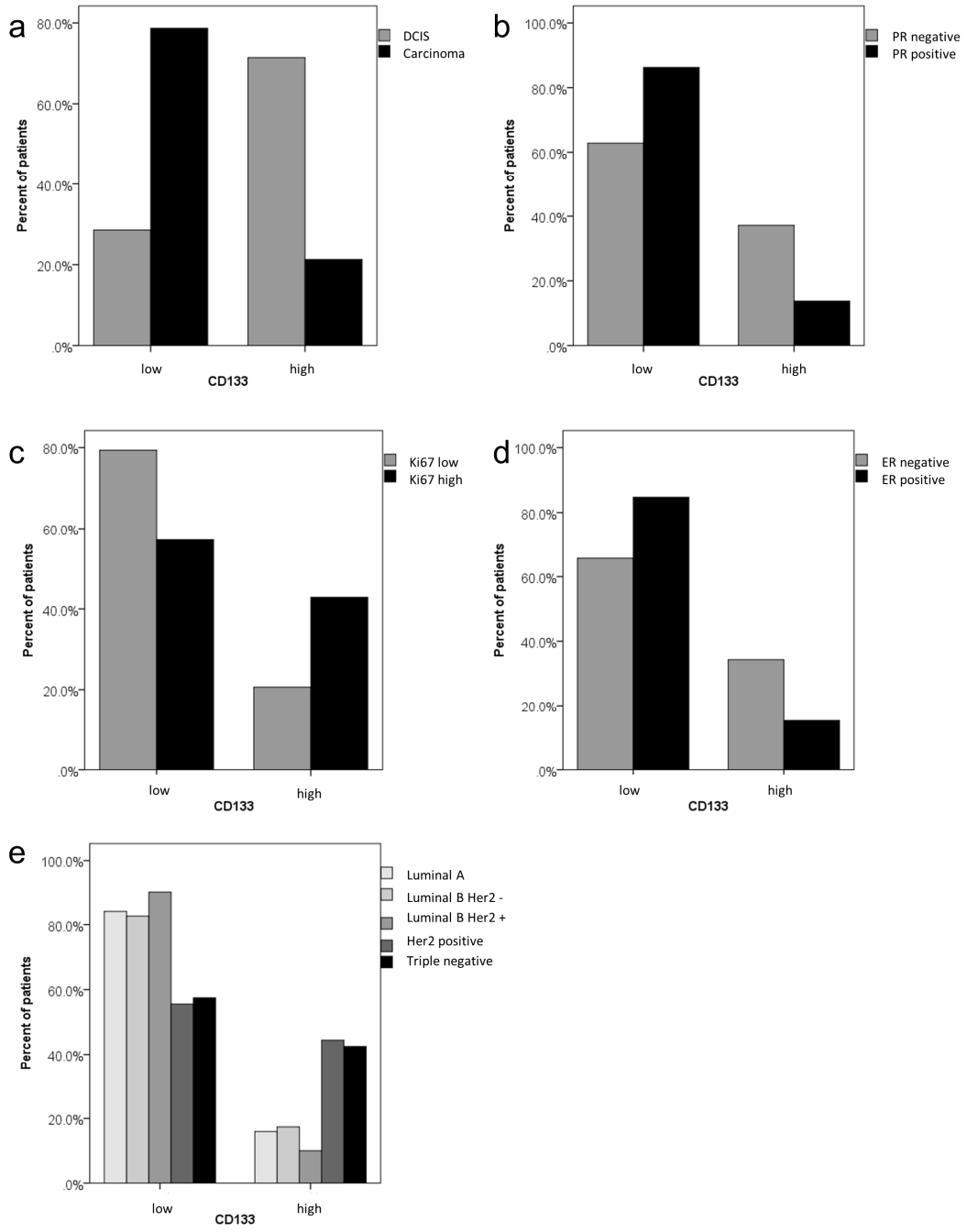


Figure 5 Boxplots of the significant associations of CD133 expression with clinical parameters. a) DCIS were significantly more common with $CD133^{high}$ expression, compared to invasive carcinoma (p = 0.01). b) $CD133^{high}$ expression was significantly associated with PR negative receptor status (p = 0.001). c) $CD133^{high}$ expression was significantly associated with Ki-67 expression (p = 0.029). d) $CD133^{high}$ expression was significantly associated with ER negative receptor status (p = 0.004). e) $CD133^{high}$ expression was significantly associated with Her2 neu positive and triple negative intrinsic subtypes (p = 0.009). (DCIS: ductal carcinoma in situ, PR: progesterone receptor, ER: estrogen receptor, Her2: human epidermal growth receptor 2)

Clinical parameter	Subgroups	CD133^{high} (% of subgroup)	CD133^{low} (% of subgroup)	p-Value*	Odds ratio (95% CI)	p-Value (logistic regression)
Age (years)	**Mean**	58.8 ± 14.76	62.5 ± 15.17	0.172	0.984 (0.961-1.007)	0.172
	Range	32 - 89	30 - 93			
Follow up	**Mean**	11.42 ± 8.4	11.1 ± 7.62	0.856	1.005 (0.958-1.055)	0.825
	Range	0.49 - 21.45	0 - 22.45			
Histo-pathology	**DCIS/LIN**	5 (71.4%)	2 (28.6%)	**0.008**	**0.108 (0.02-0.581)**	**0.01**
	Invasive Carcinoma	36 (21.3%)	133 (78.7%)			
Tumor size	**T < 2cm**	16 (32%)	34 (68%)	0.161	0.55 (0.258-1.172)	0.122
	T > 2cm	22 (20.6%)	85 (79.4%)			
Nodal status	**Negative**	18 (25.4%)	53 (74.6%)	0.557	0.775 (0.355-1.691)	0.522
	Positive	15 (20.8%)	57 (79.2%)			
Distant metastases	**Negative**	37 (23.3%)	122 (76.7%)	1	1.015 (0.312-3.3)	0.981
	Positive	4 (23.5%)	13 (76.5%)			
Grading	**Low grading**	26 (20.3%)	102 (79.7%)	0.236	1.783 (0.753-4.225)	0.189
	High grading	10 (31.2%)	22 (68.8%)			
UICC classification	**Early stage**	28 (26.7%)	77 (73.3%)	0.22	0.656 (0.289-1.487)	0.312
	Late stage	8 (16.7%)	40 (83.3%)			
Disease recurrence	**Negative**	36 (22.8%)	122 (77.2%)	0.769	1.303 (0.435-3.901)	0.636
	Positive	5 (27.8%)	13 (72.2%)			
Intrinsic classification	**Luminal A**	11 (15.9%)	58 (84.1%)	**0.009**		
	Luminal B, Her2 negative	4 (17.4%)	19 (82.6%)			
	Luminal B, Her2 positive	1 (10%)	9 (90%)			
	Her2-neu	4 (44.4%)	5 (55.6%)			
	Triple negative	17 (42.5%)	23 (57.5%)			
Ki67 (20% cutoff)	**Negative**	32 (20.6%)	123 (79.4%)	0.05	**2.883 (1.117-7.437)**	**0.029**
	Positive	9 (42.9%)	12 (57.1%)			
Estrogen receptor	**Negative**	23 (34.3%)	44 (65.7%)	**0.005**	**0.344 (0.165-0.716)**	**0.004**
	Positive	16 (15.2%)	89 (84.8%)			
Progesterone receptor	**Negative**	26 (37.1%)	44 (62.9%)	**< 0.001**	**0.269 (0.128 - 0.566)**	**0.001**
	Positive	14 (13.7%)	88 (86.3%)			
Her2 neu	**Negative**	35 (23.2%)	116 (76.8%)	0.788	1.243 (0.452-3.418)	0.674
	Positive	6 (27.3%)	16 (72.7%)			
CD166	**Low**	20 (24.1%)	63 (75.9%)	0.856	0.921 (0.449-1.886)	0.822
	High	19 (22.6%)	65 (77.4%)			
CD44	**Low**	27 (22.5%)	93 (77.5%)	0.427	1.418 (0.666-3.019)	0.365
	High	14 (29.2%)	34 (70.8%)			
EpCAM	**Low**	24 (19.5%)	99 (80.5%)	0.086	2.139 (0.976-4.687)	0.058
	High	14 (34.1%)	27 (65.9%)			

Table 6 Association of clinical parameters and CD133 expression. Binary logistic regression was calculated for dichotomized parameters. Significant correlations are highlighted in bold. *The p-Value was calculated with Chi^2 test, Fisher's exact test or non parametric tests (Mann-Whitney U test, Kruskal-Wallis test), as appropriate.

3.2.2 CD44 expression compared to clinical parameters

In the analysis of CD44 expression, 56 patients (30.1%) suffered from $CD44^{high}$ carcinomas. The associations described in the following paragraph are presented in Figure 6 and Table 7. $CD44^{high}$ expression was associated with a tumor size of more than 2 cm in this study (OR 2.36 (CI 1.04-5.36), p=0.039). 38 carcinomas (33.6%) larger than 2 cm were $CD44^{high}$, while 9 carcinomas (17.6%) smaller than 2 cm were $CD44^{high}$. In multiple regression analysis, $CD44^{high}$ expression was an independent predictor for tumor size of more than 2 cm (OR 2.46 (CI 1.01 - 6.02), p = 0.048). The only other independent predictor for larger tumor size were lymph node metastases (OR 3.71 (CI 1.76 – 7.82), p=0.001).

Furthermore, $CD44^{high}$ expression was associated with the triple-negative intrinsic subtype (p=0.012). Out of the tissue samples of 42 patients with triple negative carcinomas, 19 tissue samples (45.2%) were $CD44^{high}$. In comparison, no Her2 neu positive, two Luminal B, Her2 neu positive (20%), three Luminal B, Her2 neu negative (13.6%) and 18 Luminal A (24.7%) tissue samples were $CD44^{high}$.

$CD44^{high}$ expression was inversely correlated with ER expression (OR 0.49 (CI 0.25-0.94), p=0.032), with 25 ER positive carcinomas (22.7%) being $CD44^{high}$ and 85 ER positive carcinomas (77.3%) being $CD44^{low}$.

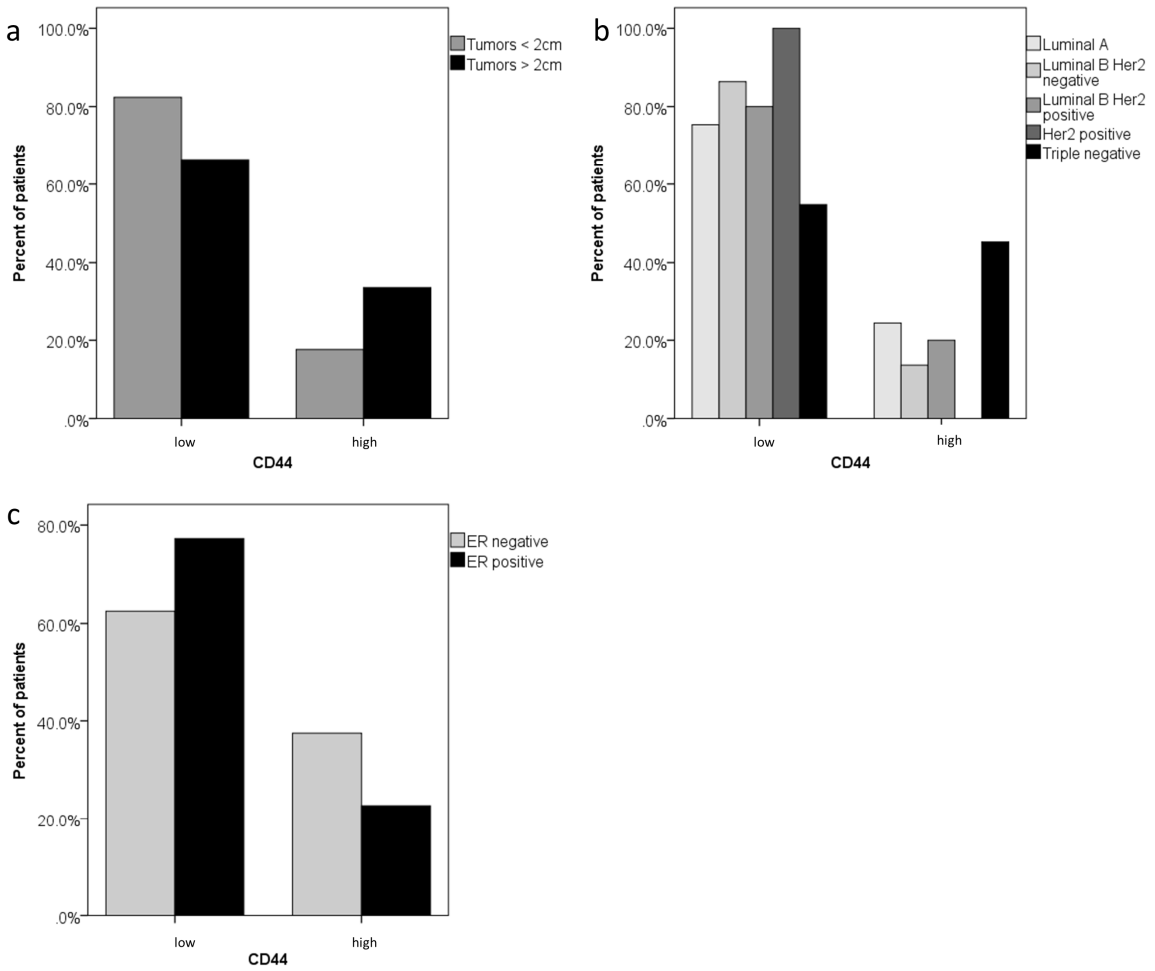


Figure 6 Boxplots of the significant associations of CD44 expression with clinical parameters.

a) CD44 ^{high} expression was significantly higher in Tumors larger than 2 cm ($p = 0.039$). b) CD44 ^{high} expression was significantly higher in triple negative carcinomas ($p = 0.012$). c) ER expression was significantly reduced in CD44 ^{high} carcinomas ($p = 0.032$). (Her2: human epidermal growth receptor 2, ER: estrogen receptor)

Clinical parameter	Subgroups	CD44^{high} (% of subgroup)	CD44^{low} (% of subgroup)	p-Value*	Odds ratio (95% CI)	p-Value (logistic regression)
Age (years)	**Mean**	62.81 ± 17.69	62.3 ± 13.82	0.73	1.002 (0.981-1.024)	0.833
	Range	31 - 93	30 - 90			
Follow up	**Mean**	9.60 ± 7.67	11.57 ± 7.77	0.105	0.967 (0.923-1.013)	0.163
	Range	0 - 20.45	0 - 21.45			
Histo-pathology	**DCIS/LIN**	5 (71.4%)	2 (28.6%)	1	0.996 (0.187-5.3)	0.996
	Invasive Carcinoma	51 (28.5%)	128 (71.5%)			
Location	**Left**	24 (28.6%)	60 (71.4%)	0.865	0.927 (0.437-1.818)	0.826
	Right	23 (27.1%)	62 (72.9%)			
Tumor size	**T < 2cm**	9 (17.6%)	42 (82.4%)	**0.041**	**2.364 (1.043 – 5.363)**	**0.039**
	T > 2cm	38 (33.6%)	75 (66.4%)			
Nodal status	**Negative**	18 (24%)	57 (76%)	0.28	1.552 (0.76-3.17)	0.227
	Positive	25 (32.9%)	51 (67.1%)			
Distant metastases	**Negative**	49 (29.2%)	119 (70.8%)	0.784	0.694 (0.218-2.213)	0.537
	Positive	4 (22.2%)	14 (77.8%)			
Grading	**Low grading**	36 (26.7%)	99 (73.3%)	0.525	1.315 (0.583-2.967)	0.509
	High grading	11 (32.4%)	23 (67.6%)			
UICC classification	**Early stage**	29 (26.1%)	82 (73.9%)	0.348	1.393 (0.692-2.804)	0.353
	Late stage	17 (34%)	33 (66%)			
Disease recurrence	**Negative**	44 (26.8%)	120 (73.2%)	0.208	1.888 (0.754-4.726)	0.175
	Positive	9 (40.9%)	13 (59.1%)			
Intrinsic classification	**Luminal A**	18 (24.7%)	55 (75.3%)	**0.012**		
	Luminal B, Her2 negative	3 (13.6%)	19 (86.4%)			
	Luminal B, Her2 positive	2 (20%)	8 (80%)			
	Her2-neu	0 (0%)	9 (100%)			
	Triple negative	19 (45.2%)	23 (54.8%)			
Ki67 (20% cutoff)	**Negative**	44 (27%)	119 (73%)	0.21	1.872 (0.748-4.687)	0.18
	Positive	9 (40.9%)	13 (59.1%)			
Estrogen receptor	**Negative**	27 (37.5%)	45 (62.5%)	**0.044**	**0.49 (0.255-0.942)**	**0.032**
	Positive	25 (22.7%)	85 (77.3%)			
Progesterone receptor	**Negative**	21 (29.2%)	51 (70.8%)	0.856	0.903 (0.465-1.753)	0.763
	Positive	29 (27.1%)	78 (72.9%)			
Her2 neu	**Negative**	50 (30.9%)	112 (69.1%)	0.088	0.336 (0.095-1.183)	0.089
	Positive	3 (13%)	20 (87%)			
CD166	**Low**	28 (30.8%)	63 (69.2%)	0.41	0.75 (0.389-1.446)	0.39
	High	22 (25%)	66 (75%)			
CD133	**Low**	34 (26.8%)	93 (73.2%)	0.427	1.418 (0.666-3.019)	0.365
	High	14 (34.1%)	27 (65.9%)			
EpCAM	**Low**	33 (25.8%)	95 (74.2%)	0.239	1.599 (0.759-3.369)	0.217
	High	15 (35.7%)	27 (64.3%)			

Table 7 Association of clinical parameters and CD44 expression. Binary logistic regression was calculated for dichotomized parameters. Significant correlations are highlighted in bold. *The p-Value was calculated with Chi^2 test, Fisher's exact test or non parametric tests (Mann-Whitney U test, Kruskal-Wallis test), as appropriate.

3.2.3 Association of EpCAM expression with clinical parameters

In the analysis of EpCAM expression, 43 carcinoma patients (23.8%) suffered from carcinomas with high EpCAM expression. The associations described in the following paragraph are presented in Figure 7 and Table 8. $EpCAM^{high}$ expression was inversely associated with nodal status (OR 0.31 (CI 0.14-0.7), p=0.004). While 11 patients (14.5%) with lymph node metastases had $EpCAM^{high}$ carcinomas, 65 patients (85.5%) with lymph node metastases had $EpCAM^{low}$ carcinomas. Conversely, $EpCAM^{high}$ expression was significantly associated with higher grading and less differentiated carcinomas (OR 3.62 (CI 1.58-8.29), p=0.002). While 14 high grade carcinomas (42.4%) were $EpCAM^{high}$, only 22 low grade carcinomas (16.9%) were $EpCAM^{high}$.

$EpCAM^{high}$ carcinomas were more common in patients with disease recurrence (OR 3.06 (CI 1.17-8), p=0.022). Of 20 patients suffering from disease recurrence during the follow up period, 9 patients (45%) had $EpCAM^{high}$ carcinomas. In contrast, of the 161 relapse free patients, only 34 (21.1%) had $EpCAM^{high}$ carcinomas.

Furthermore, $EpCAM^{high}$ expression was significantly associated with triple-negative carcinomas (p=0.002). While 18 triple-negative carcinomas (45%) were $EpCAM^{high}$, one Her2-neu positive (14.3%), three Luminal B (Her2-neu positive) (30%), seven Luminal B (Her2-neu negative) (31.8%) and nine Luminal A carcinomas (12%) were $EpCAM^{high}$.

Correspondingly, $EpCAM^{high}$ expression was significantly associated with Ki-67 expression (OR 3.85 (CI 1.48-10.02), p=0.006). 10 $Ki\text{-}67^{pos}$ carcinomas (50%) were $EpCAM^{high}$, while 33 $Ki\text{-}67^{neg}$ carcinomas (20.6%) out of 160 $Ki\text{-}67^{neg}$ carcinomas were $EpCAM^{high}$.

On the other hand, $EpCAM^{high}$ was inversely correlated with ER (OR 0.46 (CI 0.22-0.93), p=0.03) and PR (OR 0.29 (CI 0.14-0.59), p=0.001) expression. 20 ER^{pos} carcinomas (17.9%) were $EpCAM^{high}$, while 92 ER^{pos} carcinomas (82.1%) were $EpCAM^{low}$. 16 PR^{pos} carcinomas (15.1%) presented $EpCAM^{high}$, whereas 90 PR^{pos} carcinomas (84.9%) were $EpCAM^{low}$.

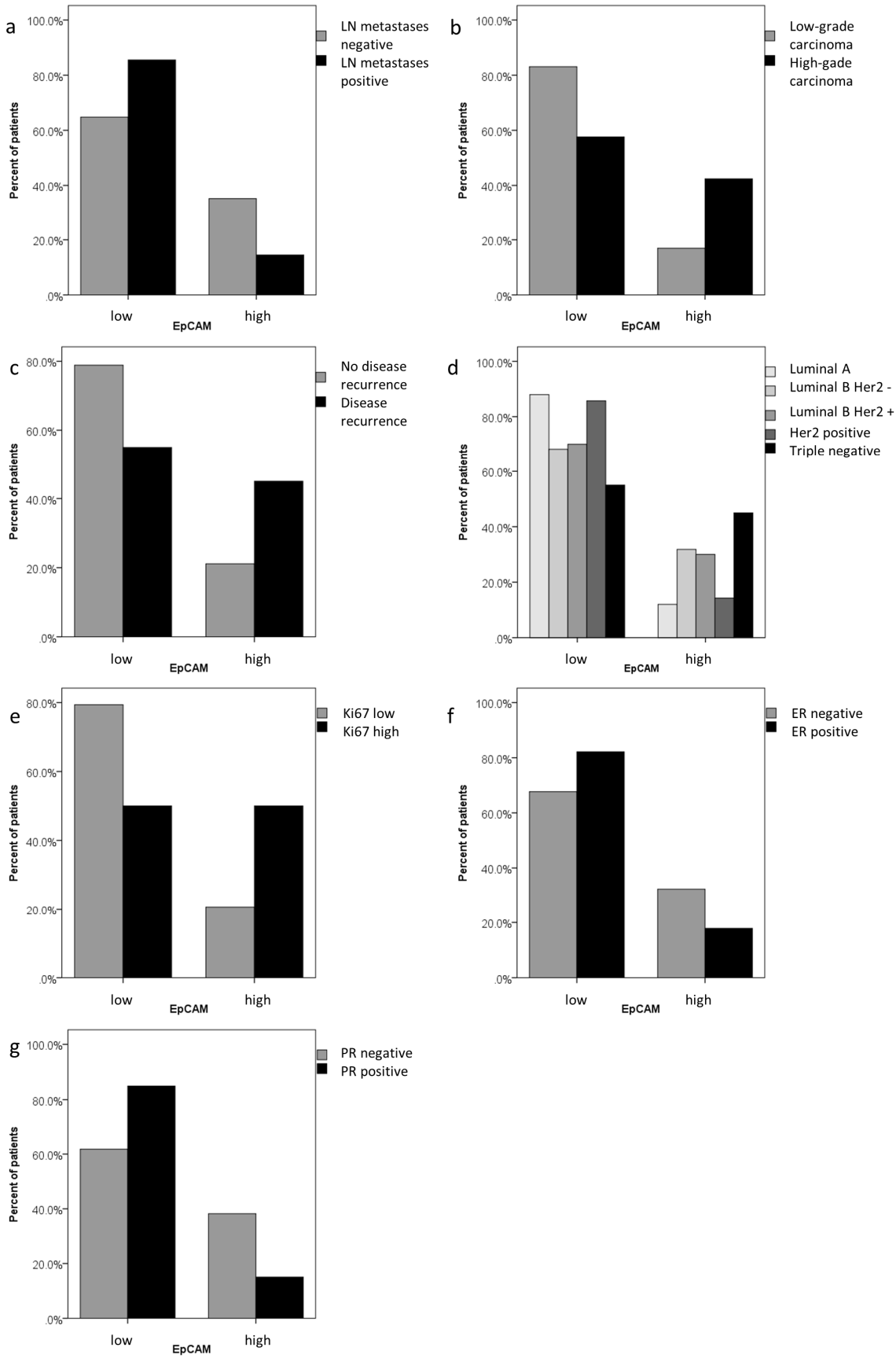

a
100.0%
80.0%
60.0%
40.0%
20.0%
.0%
Percent of patients
low
high
EpCAM
LN metastases negative
LN metastases positive
b
Low-grade carcinoma
High-gade carcinoma
c
No disease recurrence
Disease recurrence
d
Luminal A
Luminal B Her2 -
Luminal B Her2 +
Her2 positive
Triple negative
e
Ki67 low
Ki67 high
f
ER negative
ER positive
g
PR negative
PR positive

Figure 7 (Previous page) Boxplots of the associations of EpCAM expression with clinical parameters. a) $EpCAM^{high}$ expression is higher in carcinomas without lymph node metastases (p = 0.004). b) $EpCAM^{high}$ expression is higher in high-grade carcinomas (p = 0.002). c) $EpCAM^{high}$ expression is higher in patients with disease recurrence (p = 0.022). d) The proportion of triple negative carcinomas was higher in $EpCAM^{high}$ tumors than any other intrinsic subtype (p = 0.002). e) $EpCAM^{high}$ expression was associated with Ki-67 expression (p = 0.006). f) $EpCAM^{high}$ expression was inversely correlated with ER expression (p = 0.03). g) $EpCAM^{high}$ expression was inversely correlated with PR expression (p = 0.001). (LN: lymph node, Her2: human epidermal growth receptor 2, CD: cluster of differentiation molecule, ER: estrogen receptor, PR: progesterone receptor)

Clinical parameter	Subgroups	EpCAM^{high} (% of subgroup)	EpCAM^{low} (% of subgroup)	p-Value*	Odds ratio (95% CI)	p-Value (logistic regression)
Age (years)	**Mean**	59.77 ± 17.31	63.01 ± 14.43	0.247	0.986 (0.964-1.009)	0.221
	Range	30 - 90	31 - 93			
Follow up	**Mean**	10.24 ± 8.31	11.32 ± 7.62	0.495	0.982 (0.935-1.031)	0.468
	Range	0 - 21.45	0 - 22.45			
Histo-pathology	**DCIS/LIN**	2 (28.6%)	5 (71.4%)	0.671	0.771 (0.144-4.122)	0.761
	Invasive Carcinoma	41 (23.6%)	133 (76.4%)			
Tumor size	**T < 2cm**	15 (30%)	35 (70%)	0.232	0.617 (0.289-1.319)	0.213
	T > 2cm	23 (20.9%)	87 (79.1%)			
Nodal status	**Negative**	25 (35.2%)	46 (64.8%)	**0.004**	**0.311 (0.139 - 0.695)**	**0.004**
	Positive	11 (14.5%)	65 (85.5%)			
Distant metastases	**Negative**	38 (23.3%)	125 (76.7%)	0.771	1.265 (0.424-3.776)	0.673
	Positive	5 (27.8%)	13 (72.2%)			
Grading	**Low grading**	22 (16.9%)	108 (83.1%)	**0.004**	**3.617 (1.579 – 8.285)**	**0.002**
	High grading	14 (42.4%)	19 (57.6%)			
UICC classification	**Early stage**	30 (28.8%)	74 (71.2%)	0.076	0.5 (0.223-1.118)	0.091
	Late stage	8 (15.1%)	45 (84.9%)			
Disease recurrence	**Negative**	34 (21.1%)	127 (78.9%)	**0.026**	**3.056 (1.172-7.972)**	**0.022**
	Positive	9 (45%)	11 (55%)			
Intrinsic classification	**Luminal A**	9 (12%)	66 (88%)	**0.002**		
	Luminal B, Her2 negative	7 (31.8%)	15 (68.2%)			
	Luminal B, Her2 positive	3 (30%)	7 (70%)			
	Her2-neu	1 (14.3%)	6 (85.7%)			
	Basal-like	18 (45%)	22 (55%)			
Ki67 (20% cutoff)	**Negative**	33 (20.6%)	127 (79.4%)	**0.009**	**3.848 (1.479-10.016)**	**0.006**
	Positive	10 (50%)	10 (50%)			
Estrogen receptor	**Negative**	21 (32.3%)	44 (67.7%)	**0.041**	**0.455 (0.224-0.926)**	**0.03**
	Positive	20 (17.9%)	92 (82.1%)			
Progesterone receptor	**Negative**	26 (38.2%)	42 (61.8%)	**0.001**	**0.287 (0.139 - 0.591)**	**0.001**
	Positive	16 (15.1%)	90 (84.9%)			
Her2 neu	**Negative**	37 (23.3%)	122 (76.7%)	0.579	1.413 (0.507-3.937)	0.508
	Positive	6 (30%)	14 (70%)			
CD166	**Low**	21 (24.1%)	66 (75.9%)	1	0.952 (0.472-1.920)	0.891
	High	20 (23.3%)	66 (76.7%)			
CD44	**Low**	35 (22.4%)	121 (77.6%)	0.239	1.599 (0.759-3.369)	0.217
	High	7 (50%)	7 (50%)			
CD133	**Low**	37 (23.6%)	120 (76.4%)	0.066	2.139 (0.976-4.687)	0.058
	High	4 (57.1%)	3 (42.9%)			

Table 8 Association of clinical parameters and EpCAM expression. Binary logistic regression was calculated for dichotomized parameters. Significant correlations are highlighted in bold. *The p-Value was calculated with Chi^2 test, Fisher's exact test or non parametric tests (Mann-Whitney U test, Kruskal-Wallis test), as appropriate.

3.2.4 Association of CD166 expression with clinical parameters

The associations described in the following paragraph are presented in Figure 8 and Table 9. The expression of CD166 was significantly associated with lower tumor grading in the examined carcinoma patients (OR 0.37 (CI 0.16-0.82), p=0.015). 70 low grade carcinomas (52.2%) were $CD166^{high}$ while 64 low grade carcinomas (47.8%) were $CD166^{low}$.

$CD166^{high}$ carcinomas were significantly associated with luminal intrinsic subtypes (p=0.034). 37 patients (50%) with luminal A carcinomas, eleven patients (50%) with luminal B (Her2-neu negative) carcinomas and eight patients (80%) with luminal B (Her2-neu positive) carcinomas were $CD166^{high}$. In comparison, four patients with Her2-neu positive carcinomas (44.4%) and 12 patients with triple-negative carcinomas (28.6%) were $CD166^{high}$.

Finally, $CD166^{high}$ expression was significantly associated with PR expression (OR 2 (CI 1.09-3.69), p=0.026). 59 PR^{pos} carcinomas (55.1%) were $CD166^{high}$ while 48 PR^{pos} carcinomas (44.9%) were $CD166^{low}$.

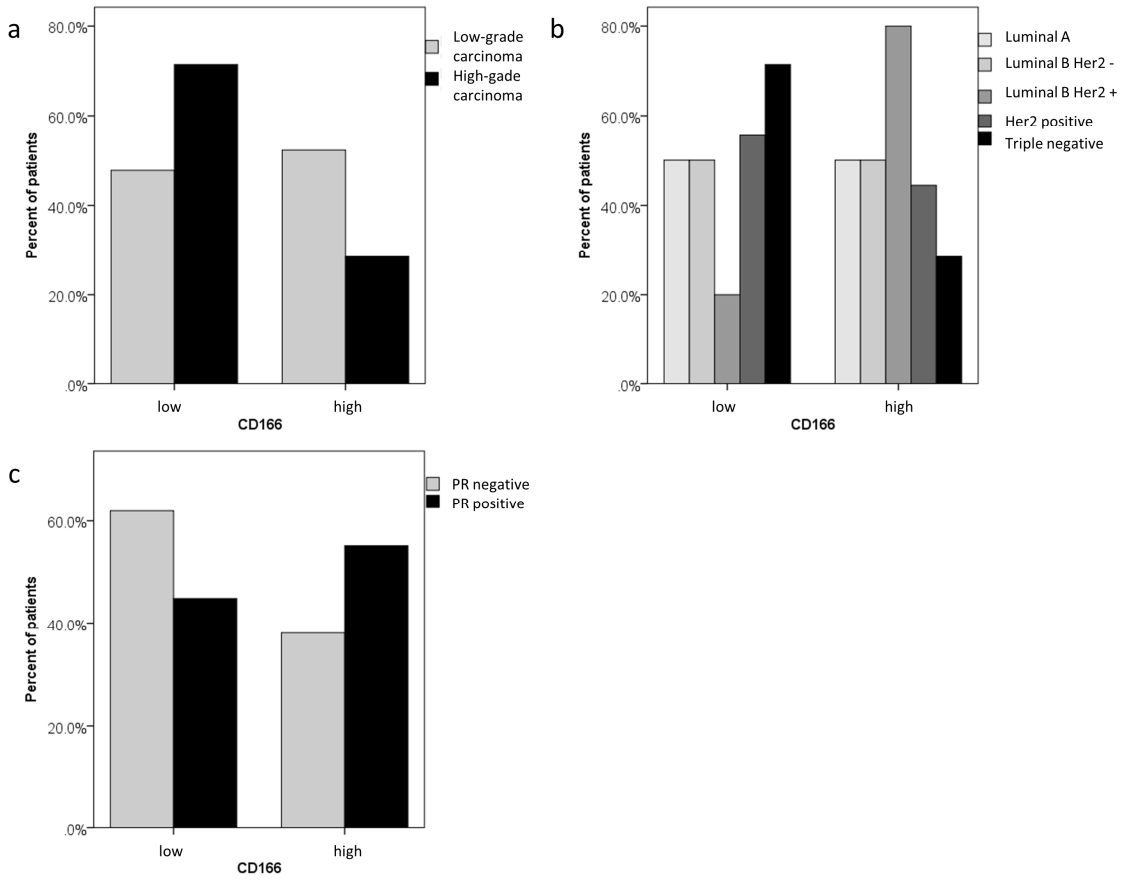


Figure 8 Boxplots of the associations of CD166 expression with clinical parameters. a) CD166^{high} carcinomas were more often low-grade carcinomas (p = 0.015). b) CD166 ^{high} carcinomas were more common among luminal subtypes while triple negative carcinomas were predominantly CD166 ^{low} (p = 0.034). c) The majority of CD166 ^{high} carcinomas were PR positive (p = 0.026). (CD: cluster of differentiation molecule, ER: estrogen receptor, PR: progesterone receptor)

Clinical parameter	Subgroups	CD166^{high} (% of subgroup)	CD166^{low} (% of subgroup)	p-Value*	Odds ratio (95% CI)	p-Value (logistic regression)
Age (years)	**Mean**	61.22 ± 14.94	62.61 ± 15.44	0.518	0.994 (0.975-1.013)	0.535
	Range	31 - 90	30 - 93			
Follow up	**Mean**	12.52 ± 7.56	9.85 ± 7.8	**0.032**	**1.046 (1.003-1.091)**	**0.035**
	Range	0 - 21.45	0 - 21.45			
Histo-pathology	**DCIS/LIN**	5 (83.3%)	1 (16.7%)	0.112	0.183 (0.021-1.596)	0.124
	Invasive Carcinoma	85 (47.8%)	93 (52.2%)			
Location	**Left**	39 (45.3%)	47 (54.7%)	0.354	0.717 (0.39-1.315)	0.282
	Right	44 (53.7%)	38 (46.3%)			
Tumor size	**T < 2cm**	30 (58.8%)	21 (41.2%)	0.064	0.517 (0.264-1.011)	0.054
	T > 2cm	48 (42.5%)	65 (57.5%)			
Nodal status	**Negative**	35 (46.7%)	40 (53.3%)	0.745	0.877 (0.462-1.666)	0.689
	Positive	33 (43.4%)	43 (56.6%)			
Distant metastases	**Negative**	80 (48.2%)	86 (51.8%)	0.624	1.344 (0.505-3.574)	0.554
	Positive	10 (55.6%)	8 (44.4%)			
Grading	**Low grading**	70 (52.2%)	64 (47.8%)	**0.014**	**0.366 (0.163 – 0.820)**	**0.015**
	High grading	10 (28.6%)	25 (71.4%)			
UICC classification	**Early stage**	54 (49.1%)	56 (50.9%)	0.502	0.86 (0.452-1.637)	0.647
	Late stage	22 (43.1%)	29 (56.9%)			
Disease recurrence	**Negative**	80 (49.4%)	82 (50.6%)	0.822	0.854 (0.349-2.088)	0.73
	Positive	10 (45.5%)	12 (54.5%)			
Intrinsic classification	**Luminal A**	37 (50%)	37 (50%)	**0.034**		
	Luminal B, Her2 negative	11 (50%)	11 (50%)			
	Luminal B, Her2 positive	8 (80%)	2 (20%)			
	Her2-neu	4 (44.4%)	5 (55.6%)			
	Triple-negative	12 (28.6%)	30 (71.4%)			
Ki67 (20% cutoff)	**Negative**	82 (50.9%)	79 (49.1%)	0.113	0.45 (0.174-1.161)	0.099
	Positive	7 (31.8%)	15 (68.2%)			
Estrogen receptor	**Negative**	29 (40.8%)	42 (59.2%)	0.130	1.615 (0.884-2.953)	0.119
	Positive	58 (52.7%)	52 (47.3%)			
Progesterone receptor	**Negative**	27 (38%)	44 (62%)	**0.032**	**2.003 (1.086 – 3.694)**	**0.026**
	Positive	59 (55.1%)	48 (44.9%)			
Her2 neu	**Negative**	76 (47.5%)	84 (52.5%)	0.366	1.596 (0.646-3.945)	0.311
	Positive	13 (59.1%)	9 (40.9%)			
CD44	**Low**	66 (51.2%)	63 (48.8%)	0.41	0.75 (0.389-1.446)	0.39
	High	22 (44%)	28 (56%)			
CD133	**Low**	65 (50.8%)	63 (49.2%)	0.856	0.921 (0.449-1.886)	0.822
	High	19 (48.7%)	20 (51.3%)			
EpCAM	**Low**	66 (50%)	66 (50%)	1	0.952 (0.472-1.92)	0.891
	High	20 (48.8%)	21 (51.2%)			

Table 9 Association of clinical parameters and CD166 expression. Binary logistic regression was calculated for dichotomized parameters. Significant correlations are highlighted in bold. *The p-Value was calculated with Chi^2 test, Fisher's exact test or non parametric tests (Mann-Whitney U test, Kruskal-Wallis test), as appropriate.

3.3 Overall, five-year and disease-free survival

Survival data was available for 164 carcinoma patients (79.2%) included in the TMA. Clinical characteristics examined in early- and late-stage carcinomas were examined for their impact on patient survival. Clinical parameters significantly associated with a shorter overall survival were advanced age ($p<0.001$), large tumor size ($p<0.001$), lymph node ($p=0.01$) and distant metastases ($p=0.004$) as well as advanced UICC stage ($p<0.001$) and disease recurrence ($p=0.001$) (Table 10). Reduced five-year survival was significantly associated with large tumor size ($p<0.001$), lymph node metastases ($p=0.012$), high tumor grading ($p=0.029$), advanced UICC stage ($p<0.001$), triple-negative intrinsic subtype ($p=0.032$) and Ki-67 expression ($p=0.001$) while ER- ($p=0.049$) and PR- ($p=0.006$) expression were associated with higher five-year survival rate (Table 11). Disease free survival was reduced in patients suffering from distant metastases ($p<0.001$) and late UICC stage ($p<0.001$) while ER expression was associated with higher disease-free survival ($p=0.007$) (Table 12).

Clinical parameters	Patients	Number of patient deaths	p-Value (Log Rank)	Hazard Ratio (95% CI)	p-Value (Cox Regression)
Age	164	116	**< 0.001**	**2.455 (1.664-3.621)**	**< 0.001**
Histopathology	164	116	0.095	2.563 (0.814-8.071)	0.108
Location	147	103	0.843	0.962 (0.653-1.416)	0.843
Tumor size (</> 2cm)	146	101	**< 0.001**	**2.419 (1.504-3.892)**	**< 0.001**
Nodal status	133	90	**0.01**	**1.72 (1.134-2.609)**	**0.011**
Distant metastases	164	116	**0.004**	**2.174 (1.256-3.762)**	**0.006**
Grading	148	105	0.215	1.352 (0.838-2.181)	0.217
UICC stage (early and late stage)	142	97	**< 0.001**	**2.958 (1.985-4.488)**	**< 0.001**
Disease recurrence	164	116	**0.001**	**2.25 (1.351-3.746)**	**0.002**
Intrinsic classification	139	99	0.316	1.056 (0.938-1.188)	0.369
Ki67 expression	163	115	0.176	1.508 (0.828-2.746)	0.179
Estrogen receptor	159	112	0.631	0.91 (0.618-1.339)	0.631
Progesterone receptor	156	111	0.628	0.91 (0.621-1.334)	0.628
Her2-neu	161	114	0.233	0.696 (0.382-1.267)	0.236
CD166	151	105	0.104	0.728 (0.496-1.069)	0.106
CD44	152	108	0.176	1.329 (0.879-2.01)	0.178
CD133	148	104	0.743	0.927 (0.587-1.463)	0.743
CD326	150	105	0.732	1.083 (0.686-1.708)	0.732

Table 10 Analysis of **overall survival** in association with clinical parameters and cancer stem cell markers.

Clinical parameters	Patients	Number of deaths	p-Value (Log Rank)	Hazard Ratio (95% CI)	p-Value (Cox Regression)
Age	164	52	0.134	1.529 (0.874-2.673)	0.137
Histopathology	164	52	0.094	21.769 (0.091-5194.956)	0.27
Location	147	52	0.755	0.91 (0.504-1.645)	0.755
Tumor size (>/< 2cm)	146	44	**< 0.001**	**6.86 (2.123-22.166)**	**0.001**
Nodal status	133	38	**0.012**	**2.297 (1.175-4.492)**	**0.015**
Distant metastases	164	52	0.1	1.865 (0.877-3.964)	0.105
Grading	148	47	**0.029**	**2.011 (1.061-3.814)**	**0.032**
UICC stage (early and late stage)	143	43	**< 0.001**	**4.042 (2.174-7.515)**	**< 0.001**
Disease recurrence	164	52	0.087	1.855 (0.903-3.811)	0.092
Intrinsic classification	139	45	**0.032**	**1.28 (1.083-1.512)**	**0.004**
Ki67 expression	163	51	**0.001**	**3.077 (1.536-6.163)**	**0.002**
Estrogen receptor	159	50	**0.049**	**0.576 (0.33-1.004)**	0.052
Progesterone receptor	156	48	**0.006**	**0.462 (0.262-0.816)**	**0.008**
Her2-neu	161	51	0.909	1.048 (0.472-2.326)	0.909
CD166	151	48	0.068	0.587 (0.329-1.047)	0.071
CD44	152	50	0.273	1.393 (0.768-2.524)	0.275
CD133	148	49	0.39	1.311 (0.705-2.438)	0.392
CD326	150	47	0.307	1.393 (0.735-2.64)	0.310

Table 11 Analysis of **five-year survival** in association with clinical parameters and cancer stem cell markers.

Clinical parameters	Patients	Number of disease recurrences	p-Value (Log Rank)	Hazard Ratio (95% CI)	p-Value (Cox Regression)
Age	164	24	0.966	0.983 (0.441-2.192)	0.967
Histopathology	164	52	0.866	1.186 (0.16-8.785)	0.868
Location	149	23	0.83	1.092 (0.482-2.476)	0.833
Tumor size (>/< 2cm)	147	24	0.064	2.621 (0.893-7.693)	0.08
Nodal status	135	22	0.235	1.648 (0.703-3.862)	0.25
Distant metastases	164	24	**< 0.001**	**8.954 (3.975-20.167)**	**< 0.001**
Grading	148	23	0.478	1.421 (0.527-3.828)	0.488
UICC stage (early and late stage)	143	24	**< 0.001**	**5.125 (2.172-12.094)**	**< 0.001**
Intrinsic classification	143	18	0.076	**1.321 (1.015-1.72)**	**0.039**
Ki67 expression	163	24	0.121	2.258 (0.769-6.633)	0.139
Estrogen receptor	160	23	**0.007**	**0.34 (0.147-0.785)**	**0.012**
Progesterone receptor	157	24	0.644	0.829 (0.368-1.867)	0.65
Her2-neu	162	24	0.937	0.953 (0.284-3.197)	0.938
CD166	150	22	0.555	0.78 (0.337-1.806)	0.562
CD44	152	22	0.075	2.106 (0.899-4.936)	0.086
CD133	145	18	0.726	1.2 (0.428-3.367)	0.729
CD326	149	20	**0.01**	**2.977 (1.233-7.191)**	**0.015**

Table 12 Analysis of **disease-free** survival in association with clinical parameters and cancer stem cell markers.

3.4 Influence of cancer stem cell markers on overall, five-year and disease-free survival

3.4.1 Overall and five-year survival of patients with CD133 ^{high} carcinomas

There was no influence of CD133 expression on survival, when analyzed in the whole cohort. Nevertheless, in patients with tumors larger than 2 cm, CD133 ^{high} expression was shown to negatively influence overall survival (HR 2.13 (CI 1.14-3-96), p=0.017) (Figure 9 a).

The median survival in CD133^{low} carcinomas was 7.3 years (CI 3.8-10.8) compared to 3.21 years (CI 1.48-4.94) in patients with CD133 ^{high} carcinomas. In line, the five-year survival was equally reduced in patients with CD133 ^{high} tumors (HR 2.503 (CI 1.15-5.43), p=0.02) (Figure 9 b). In multiple regression analysis, CD133 expression was identified as an independent predictor of overall survival in patients suffering from tumors larger than 2 cm (HR 2.397 (CI 1.29-4.47), p=0.006) (Figure 13). Additional negative prognostic factors in this subgroup were age over 58 years at surgery (HR = 2.16 (CI 1.26 - 3.71), p = 0.005) and UICC stage higher than IIb (HR = 2.273 (CI 1.34 - 3.85), p = 0.002). Similarly, CD133^{high} was an independent predictor for five-year survival in patients with tumors larger than 2 cm (HR 2.79 (CI 1.28-6.08), p=0.01). Here, the only other negative prognostic factor was the UICC stage higher than IIB (HR = 2.49 CI (1.23 – 5.08), p = 0.002).

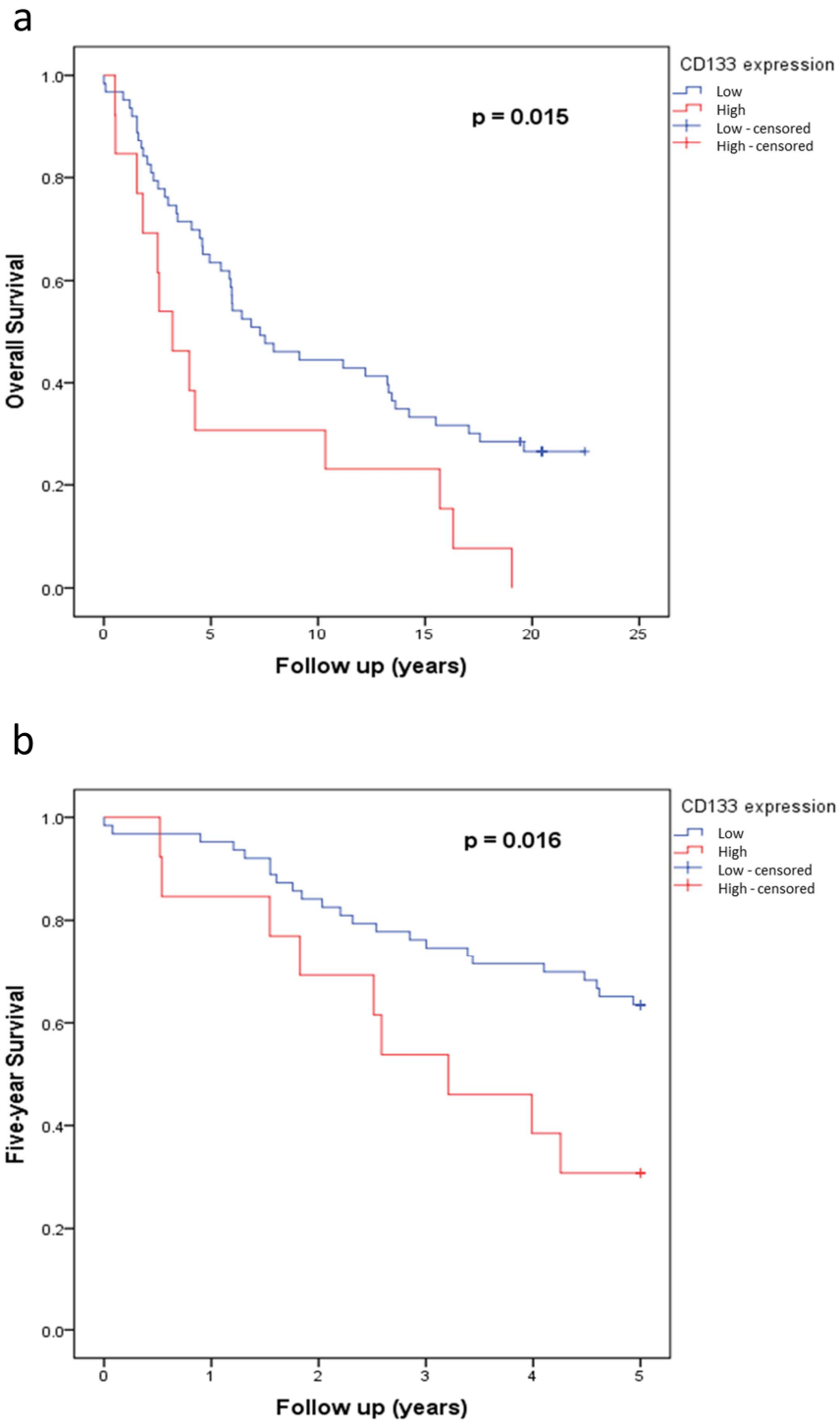


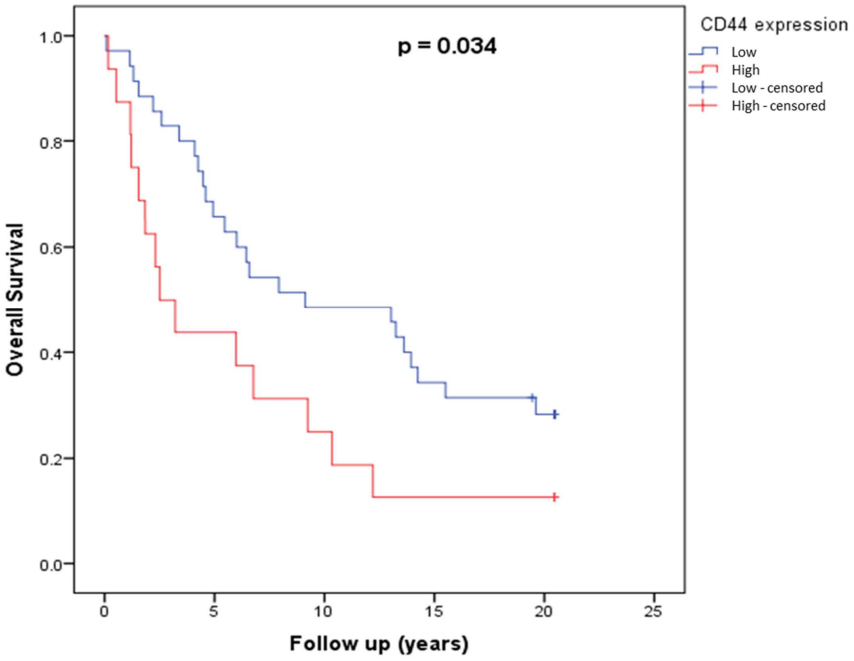
Figure 9 CD133 expression and association to survival. The p-value was calculated with the log-rank test. a) and b) Overall and five-year survival in patients with tumors > 2 cm in association with CD133 expression. CD133 expression is associated with shorter survival. (CD: cluster of differentiation molecule)

3.4.2 Overall, five-year and disease-free survival of patients with CD44^{high} carcinomas

There was no significant association of CD44 on survival in the overall cohort. However, in patients with lymph node metastases, CD44 expression had a significant association with overall survival (HR 2.02 (CI 1.04-3.92), p=0.038) (Figure 10 a). While median survival was 9.13 years (CI 1.25-17.01) in CD44^{low} carcinomas, patients with CD44^{high} carcinomas had a median survival of 2.5 years (CI 0.76-4.27).

In the subgroup of triple-negative carcinomas, CD44^{high} expression was an independent predictor of reduced overall survival (HR 2.224 (CI 1-4.94), p=0.05) (Figure 10 b, Figure 13). The median survival in patients with CD44^{high} carcinomas was 2.32 years (CI 1.25-3.38), while patients with CD44^{low} carcinomas had a median survival of 7.93 years (CI 2.84-13.02). Five-year survival was equally reduced in patients with CD44^{high}, triple-negative carcinomas (HR 3.11 (CI 1.17- 8.3), p=0.023). In the subgroup of patients with ER positive carcinoma, CD44 expression was associated with a reduced disease-free survival in Cox regression analysis (HR 4.99 (CI 1.11-22.34), p=0.036).

a



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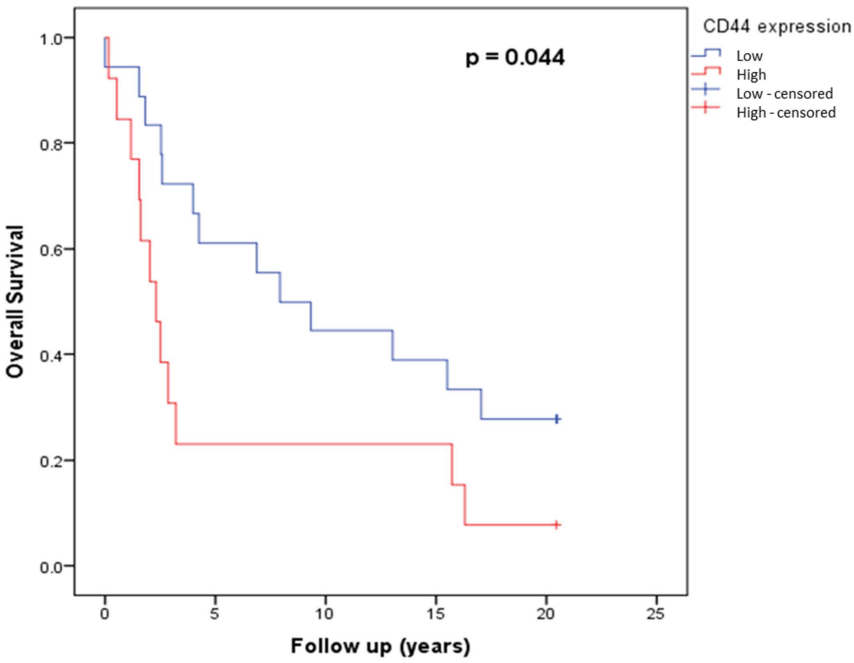


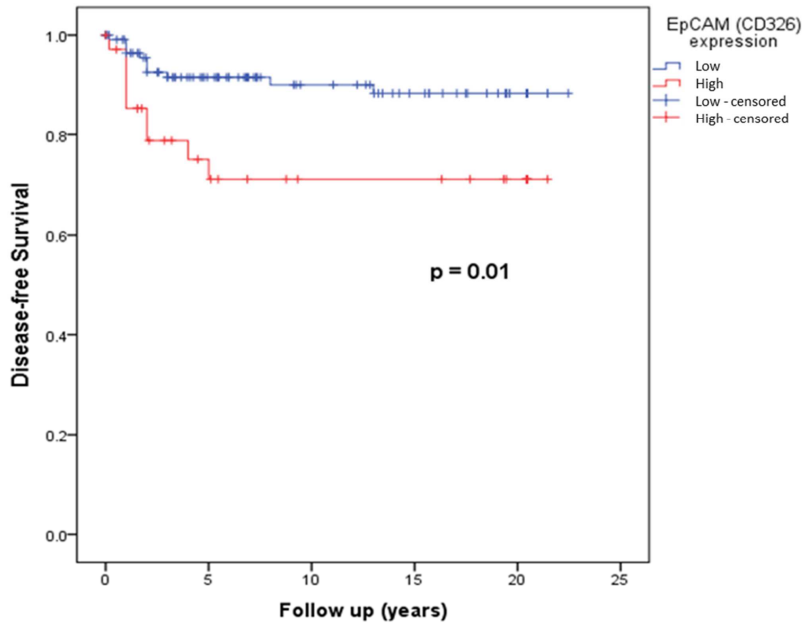
Figure 10 CD44 expression and association to survival. The p-value was calculated with the log-rank test. a) Overall survival in patients with lymph node metastases in association to CD44 expression. CD44 expression is associated with shorter survival. b) Overall survival in patients with triple-negative carcinomas in association with CD44 expression. CD44 expression is associated with shorter survival. (CD: cluster of differentiation molecule)

3.4.3 Disease-free survival of patients with EpCAM^{high} carcinomas

Cox regression analysis showed a reduced disease-free survival for patients with EpCAM^{high} carcinomas in the overall cohort (HR 2.98 (CI 1.23-7.19), p=0.015) (Figure 11 a). The ten-year disease-fee survival probability of patients with EpCAM^{high} breast cancer was 0.71 (CI 0.55-0.87), while EpCAM^{low} patients had a disease-free survival probability of 0.90 (CI 0.84-0.96). In multiple regression analysis, EpCAM proved to be an independent predictor of reduced disease-free survival in patients with breast carcinoma (HR 3.08 (CI 1.27-7.48), p=0.013) (Figure 13). The only additional negative prognostic factor next to high EpCAM expression was distant metastasis (HR = 10.452(CI 4.28 - 25.51), p < 0.001).

In patients with tumors larger than 2 cm, EpCAM expression was associated with disease-free survival in Cox regression analysis (HR 4.08 (CI 1.43-11.7, p=0.009) (Figure 11 b). The ten-year disease-free survival probability for patients with an EpCAM^{high} carcinoma was 0.54 (CI 0.28-0.81), while the ten-year disease-free survival probability in patients with EpCAM^{low} tumors was 0.87 (CI 0.77-0.97). In patients with lymph node metastases, EpCAM expression was associated with a shorter disease-free survival in Cox regression analysis, as well (HR 6.18 (CI 1.58-24.15), p=0.009). The ten-year survival probability was reduced in patients with EpCAM positive carcinomas (0.44 (CI 0.03-0.86)) compared to patients with EpCAM negative carcinomas (0.86 (CI 0.78-1)).

a



b

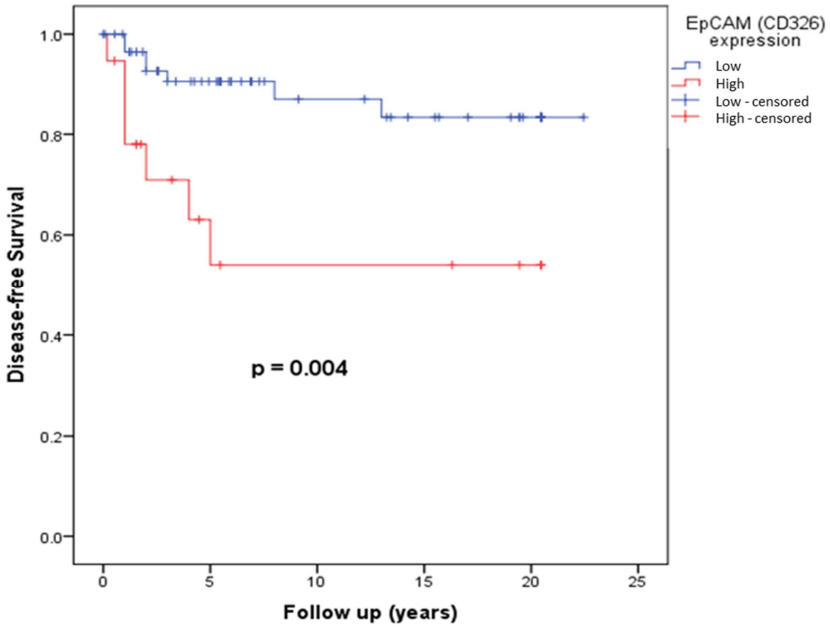


Figure 11 EpCAM expression and association to disease-free survival. The p-value was calculated with the log-rank test. Data of patients who died were censored. a) EpCAM^{high} carcinomas were associated with reduced disease-free survival. b) EpCAM expression in patients with large tumors was association with reduced disease-free survival. (CD: cluster of differentiation molecule, EpCAM: epithelial cell adhesion molecule)

3.4.4 Overall survival of patients with CD166^{high} carcinomas

In the group of ER positive carcinoma patients, those patients with CD166 positive carcinomas had a longer overall survival in Cox regression analysis (HR 0.56 (CI 0.32-0.98), p=0.04) (Figure 12 a). The median survival in patients with CD166^{high} carcinomas was 16.36 years (CI 8.5-24.22) while patients with CD166^{low} carcinomas had a median survival of 6.58 years (CI 2.21-10.95). In patients with ER positive carcinomas, the expression of CD166 was an independent predictor of longer survival in multiple regression analysis (HR 0.48 (CI 0.26-0.89), p=0.02) (Figure 13). One further independent but negative prognostic factors was UICC stage higher than IIb (HR = 3.922 (CI 2.03-7.59), p = 0.001).

In patients with PR positive carcinomas, the expression of CD166 was significantly associated with prolonged overall survival as well (HR 0.52 (CI 0.29-0.92), p=0.025) (Figure 12 b). The median survival in patients with CD166^{high} carcinomas was 15.69 years (CI 10.31-21.06) while median survival in CD166^{low} carcinomas was 6.58 years (CI 2.68-10.48). In patients with PR positive carcinomas, CD166 expression was an independent positive predictor of survival in multiple regression analysis (HR 0.495 (CI 2.7-0.92). p=0.027) (Figure 13). Other independent negative prognostic factors in this analysis were age over 58 years (HR = 3.512 (CI 1.82 – 6.76), p = 0.001) and carcinomas larger than 2 cm (HR = 2.568 (CI 1.14 – 5.77), p = 0.022).

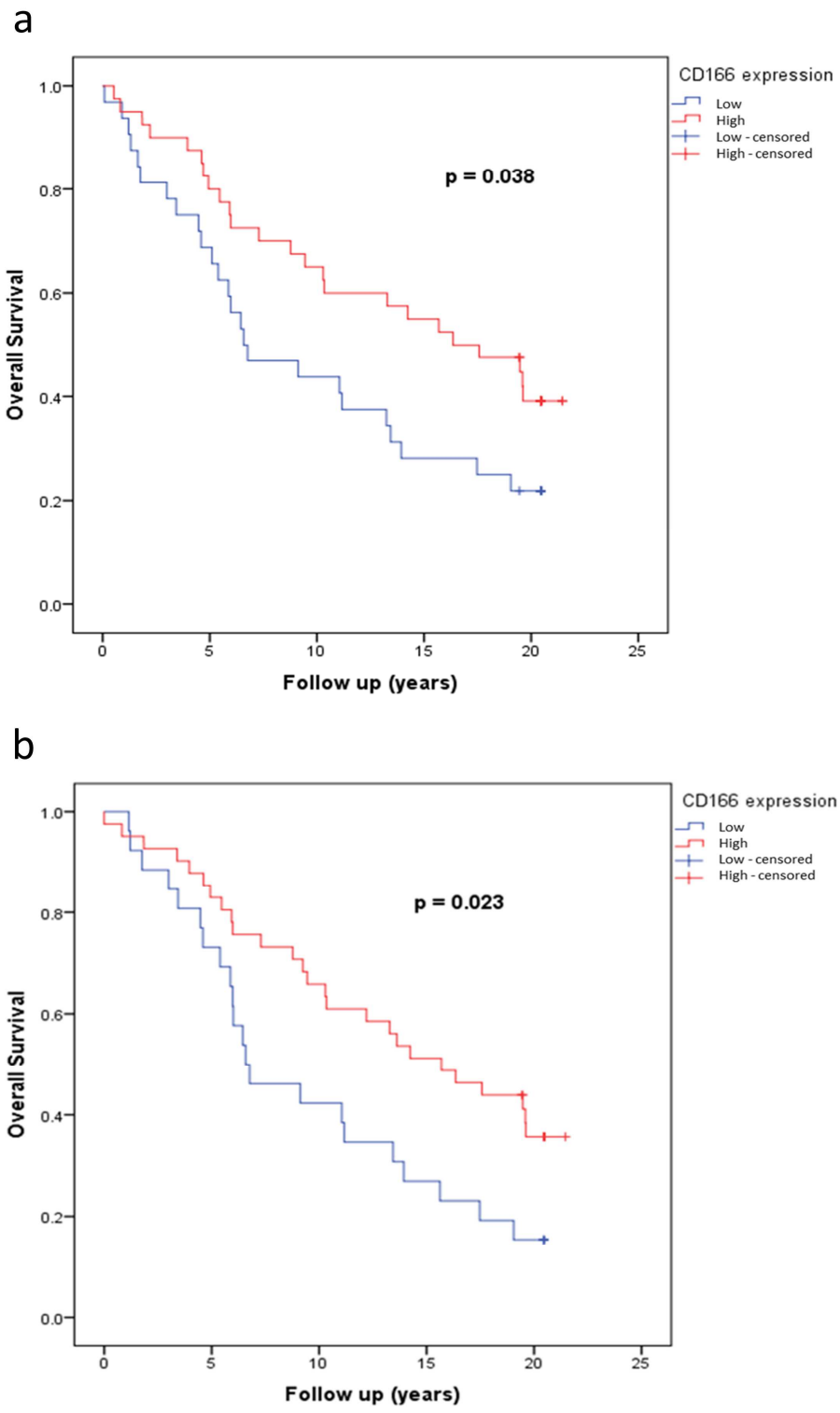


Figure 12 CD166 expression and association to survival. The p-value was calculated with the log-rank test. a) Expression of CD166^{high} was associated with prolonged survival in patients with ER positive carcinomas. b) CD166^{high} expression in patients with PR positive carcinomas was associated with longer overall survival. (CD: cluster of differentiation molecule)

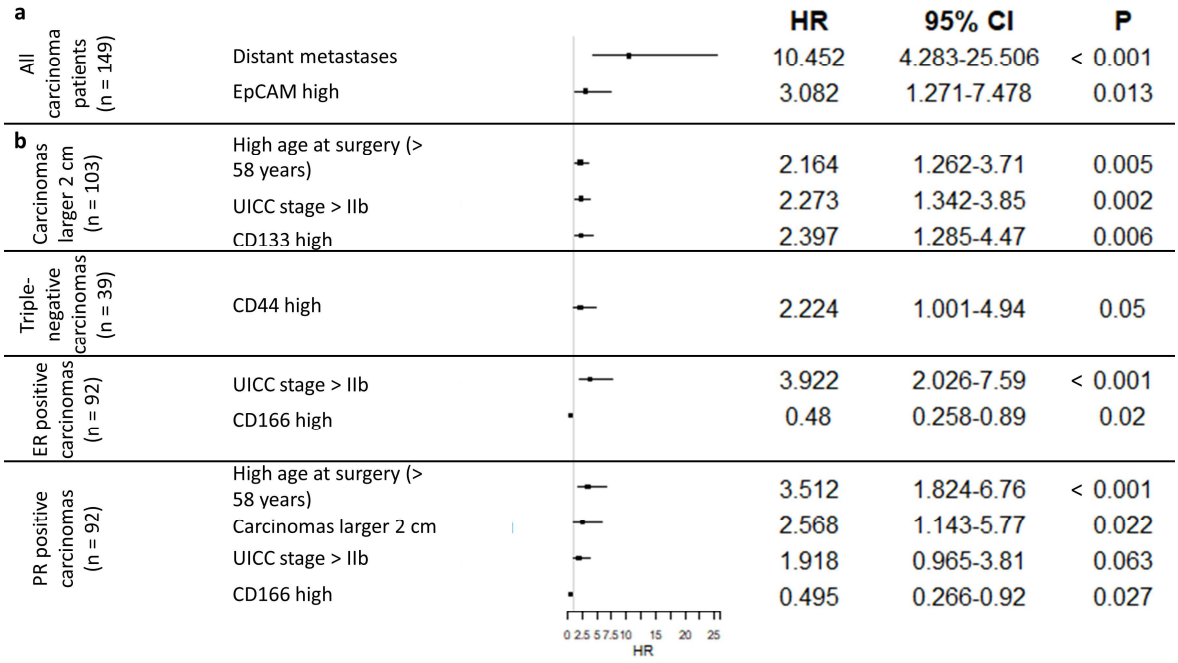


Figure 13 Multiple Cox regression analysis in the overall cohort and four subgroups where CSC marker expression was found to be an independent predictor of overall survival. a) EpCAM was associated with a reduced disease-free survival in the overall cohort. b) CD133 and CD44 were associated with reduced overall survival, CD166 was associated with a prolonged overall survival in the respective subgroups. (HR: hazard ratio, CI: confidence interval, EpCAM: Epithelial cell adhesion molecule, UICC: Union for International Cancer Control; CD: cluster of differentiation molecule, ER: estrogen receptor, PR: progesterone receptor)

4 Discussion

4.1 Overview of experimental results

The aim of this study was to further elucidate the association of cancer stem cell markers with clinical parameters and survival of breast cancer patients. All cancer stem cell markers were significantly associated with several clinical parameters and either overall, five-year or disease-free survival in the cohort as a whole or in various subgroups. These associations underline the importance of cancer stem cells not only as possible drivers of carcinogenesis. Moreover, cancer stem cell markers have a significant role for the determination of patients' individual prognosis and as possible therapeutic targets. Nevertheless, cancer stem cells and their respective markers have been shown to influence tumor biology and the clinical course in variable ways due to their plasticity, depending on the different tumor types and the tumor microenvironment [113]. Accordingly, varying results have been presented in the literature describing the impact of cancer stem cells on carcinoma prognosis. In this study, cancer stem cell markers were further evaluated in a well characterized cohort of 254 breast cancer patients with a minimum follow-up of 18 years for their clinical applicability. Here, only statistically significant associations of cancer stem cell expression with clinical parameters or survival are discussed.

4.2 Comparison of cancer stem cell marker expression to clinical parameters and survival

4.2.1 CD133 expression vs clinical parameters and survival

4.2.1.1 CD133 in normal tissue/benign neoplasia vs carcinoma

$CD133^{high}$ expression was associated with normal and benign tissue samples rather than malignant tissue (OR 0.24, (CI 0.15-0.4), $p<0.001$). While 26% of carcinoma tissue samples showed a positive CD133 expression, 59% of normal and benign tissue samples were $CD133^{high}$. Other studies, however, have shown a reduced CD133 expression in tumor-adjacent normal tissue [80]. When CD133 was first discovered by immunohistochemistry it was found to be situated on hematopoietic stem cells and leukemic cells. While protein expression detected with immunohistochemistry was limited to normal tissue in the bone marrow and leukemic cells, its mRNA was detected in a variety of normal tissues [139].

CD133 expression has also been described in normal mouse mammary epithelia [59, 180]. Atkinson et al. looked at the expression of a stem cell marker panel including CD133 in normal tissue adjacent to human breast cancer. Hereby, the expression of the stem cell markers was found to be significantly elevated in tissue adjacent to triple-negative carcinomas. The authors hypothesized that these normal cells could either represent cancer stem cells as tumor initiating cells leading to breast cancer or a cell population of the microenvironment necessary for the development of breast cancer [13]. Finally, Martin et al. also found increased expression of stem cell markers in tumor adjacent normal breast tissue, including CD133 expression in a large cohort of invasive breast cancer samples [135]. This is in line with the results of this work, which shows an increased expression of CD133 in tumor adjacent tissue suggesting that normal cells expressing stem cell markers might play an important role in tumor development in breast tissue.

4.2.1.2 CD133 in carcinoma tissue vs clinical parameters

When comparing the expression of CD133 in invasive carcinoma and DCIS, the latter were significantly more often $CD133^{high}$ (21.3% of invasive carcinomas, 71,4% of DCIS). In one other study the expression of CD133 was assessed in DCIS associated with invasive carcinomas and was found to be positive in only 14% of DCIS [42]. While the DCIS tissues in our study were from different patients than the carcinoma samples, Currie et al. examined DCIS samples associated with an invasive carcinoma by immunohistochemistry. While both comparisons have their advantages, the DCIS samples in our study - not accompanied by invasive carcinoma - might potentially reflect overall cell populations with an increased malignant potential. This could be the reason why the DCIS samples in our cohort show a much higher level of CD133 expression, interestingly accompanied by an equally high level of CD44 expression (71.4% of the DCIS samples). However, due to the small number of DCIS samples in our study these results have to be interpreted with caution and further research is warranted to elucidate the role of cancer stem cell markers in early stages of breast cancer. Furthermore, even though CD133 expression was assessed by immunohistochemistry in both studies, its evaluation is depending on several factors such as protocols and antibodies applied, age and storage of samples or the experience of the evaluator.

When examining the relation of CD133 expression and intrinsic subtypes in invasive cancers, 42.5% of triple-negative carcinomas were found to be $CD133^{high}$, while only

15.9% of Luminal A carcinomas were CD133 ^{high} (p=0.009). In contrast, some studies associate triple-negative breast carcinomas with low CD133 expression (20% and 22% respectively) [38, 55]. On the other hand, Liu et al. presented a high CD133 expression in 71% of triple-negative breast cancers [129]. They postulated CD133 expression to be associated with an increased self-renewal capacity of tumor cells and increased vascular mimicry, both signs of malignant potential and cancer stem cell characteristics. In addition, Liu et al. found CD133 expression to be associated with lymph node metastases and high tumor grade. In a similar study, Zhao et al. detected CD133 expression in 43.3% of triple negative breast cancers which was associated with shorter overall- and disease-free survival [214]. One explanation for these diverging results is the use of different antibodies. While Collina and Di Bonito et al. used monoclonal antibodies, Zhao et al. used polyclonal antibodies to detect CD133 expression. Nevertheless, we detected a high CD133 expression in triple-negative breast cancer using the same monoclonal antibody as the first two studies described. The reason that CD133 is not associated with overall- and disease-free survival in our study might be due to a smaller samples size and patients with less advanced disease compared to the study by Zaho et al. Overall, CD133 expression seems to be associated with triple-negative breast cancer, and hence a clinically more aggressive phenotype [30, 159, 167].

Furthermore, CD133 expression was positively associated with Ki-67 expression (OR 2.88, (CI 1.12-7.44), p=0.029) and negatively associated with ER and PR expression (OR 0.34, (CI 0.17-0.72), p=0.004 and OR 0.27, (CI 0.13-0.57), p=0.001, respectively). Also Di Bonito et al. observed a significant association of CD133 with Ki-67 expression in twelve breast cancer patients [56]. Ki-67 has been described as a marker for increased cellular proliferation in breast cancer [73, 74, 171] and its expression is associated with a reduced disease-free and overall survival [51, 97, 153].

Results regarding ER, PR and CD133 expression in this study and the association of CD133 expression with triple-negative breast cancer and hence ER and PR negative cancers, has been discussed above. In normal breast tissue, ER and PR was found to be mainly expressed in mature luminal cells [124]. Interestingly, normal mouse mammary epithelia was described to mainly express CD133 on ER and PR positive, luminal cells forming a hormone sensing compartment with low colony forming, and hence, low stem cell activity [180].

On the other hand, when examining breast cancer cells and cell lines, CD133 is associated with a low ER expression and loss of ER expression, respectively. When culturing those cells in mammospheres to select for cells with cancer stem cell properties, this phenomenon was maintained [44, 189]. This effect of increased expression of CD133 and loss of ER in breast cancer cells was also demonstrated after neoadjuvant hormonal therapy, probably as cause of metastatic relapse after hormonal therapy [165]. Apart from ER negativity being an indicator for a stem-cell like phenotype in cancer cells [134], it also limits the therapeutic options and therefore the prognosis [60, 119, 147]. As described above, CD133 expression was negatively associated with ER and PR expression and further confirms the previous findings [42, 105]. Overall, this work suggests an association of CD133 expression with a hormone negative, aggressive breast cancer phenotype with cancer stem cell characteristics.

4.2.1.3 CD133 in carcinoma tissue vs survival in patients with advanced tumors

CD133 expression was shown to negatively influence survival in patients with tumors larger than 2 cm (HR 2.13 (CI 1.14-3-96), p=0.017). The five-year survival was equally reduced in patients with $CD133^{high}$ tumors (HR 2.5 (CI 1.15-5.43), p=0.02). Most importantly, CD133 expression was identified as an independent predictor of overall survival (HR 2.4 (CI 1.29-4.47), p=0.006) and five-year survival (HR 2.79 (CI 1.28-6.08), p=0.01) in patients with large tumors in multiple regression analysis. Although, there was no significant association of CD133 expression and the size of a tumor, the results presented above distinguish CD133 as a prognostic marker in advanced carcinomas. Liu et al. described a correlation of CD133 with larger tumors in breast cancer patients [128]. However, the cohort of 74 patients analyzed by Liu et al. was smaller yet representing a larger group of patients with large tumors. The first study associating high CD133 expression with reduced overall- and disease-free survival was published by Zhao et al. [213]. Kim et al. were able to show that CD133 expression was associated with larger tumors as well as overall- and disease-free survival in breast cancer patients [105].

Finally, Zhao et al. also demonstrated the association of CD133 expression and tumor size as well as overall- and disease-free survival in patients suffering from triple negative breast cancer [214].

However, none of the studies mentioned above, illustrated a connection of CD133 expression and a reduced overall- and five-year survival in patients suffering from large tumors as outlined in our study. Interestingly, two phase I trials in hepatocellular, pancreatic and colorectal carcinomas [199] and glioblastomas (NCT02049489) target CD133. Our results indicate high CD133 expression to be a potential therapeutic target also for breast cancer patients.

4.2.2 CD44 vs clinical parameters and survival

4.2.2.1 CD44 in normal tissue and benign neoplasia vs carcinoma

The expression of CD44 was significantly more common in DCIS and invasive breast carcinomas compared to normal tissue and benign tumors (OR 1.9 (CI 1.0-3.4), p=0.038). While 17.8% of normal and benign tumor tissue showed high CD44 expression, 29 % of carcinoma samples were high for CD44. Expression of CD44 in normal breast tissue has been described early on [47], with myoepithelial cells being positive for CD44 while the luminal epithelial cells did not express CD44. In samples of atypical epithelial proliferation, DCIS and breast carcinomas, CD44 continued to be present, although the prevalence of splice variants changed. Similar results were presented by Bankfalvi et al. [17] with CD44 splice variant expression being associated with carcinoma grading and steroid receptor expression. Later studies associated the number of cells expressing CD44 in normal tissue and breast carcinomas with a majority of normal cells being $CD44^{high}$. Afify et al. found up to 100% of normal cells, DCIS and invasive carcinoma to express CD44 [4], while Da Cruz Paula et al. detected CD44 expression in non-malignant cells in 81% and in DCIS and invasive carcinoma in 95% and 81%, respectively [46]. The studies had included only a small number of samples or did not present any clinical data, hence rendering the interpretation of the results difficult. Nevertheless, further studies also present high expression rates of CD44 in carcinoma tissue [14, 101], however no comparison with CD44 expression in normal tissue was presented.

4.2.2.2 CD44 in carcinoma tissue vs clinical parameters

When comparing the frequency of CD44 expression in breast cancer patients to the clinical characteristics, $CD44^{high}$ expression was significantly associated with a tumor size of 2 cm or larger (OR 2.36, CI 1.04 – 5.36, p=0.039). The size of breast carcinomas is a parameter significantly associated with survival and part of the

UICC classification [10, 33, 67, 112]. The association of CD44 expression in breast cancer has been evaluated with conflicting results: a large number of studies did not find an association of CD44 expression or any splice variants with tumor size [35, 53, 69, 85, 101, 141, 164, 172, 193]. Several of these studies were conducted on smaller patient cohorts and with varying techniques and antibodies. Nevertheless, a larger study performed by Joensuu et al. [101] did show an association of CD44 expression with lymph node metastases, high histopathological grade, higher mitotic counts and ER negativity. After the study by Al-Hajj et al. [8] the focus shifted from identifying the relevant CD44 isoform expression to evaluating the impact of CD44+/CD24- expression patterns on prognosis and tumor biology. Despite convincing evidence from in vitro experiments, the association of CD44+/CD24- expression patterns to clinical parameters such as tumor size remained to be difficult to prove [14, 52, 57, 98, 103, 142, 146, 152, 192, 195]. Abraham et al. did not find CD44+/CD24- cell containing tumors to be associated with tumor size or survival and argued that CD44+/CD24- cells could be a subset of tumorigenic cells as Al-Hajj et al. did find a subgroup with CD24+ cells to have tumorigenic potential [2]. Honeth et al. made a similar discovery describing CD44+/CD24- cells to be enriched in basal like tumors while other breast cancer subtypes did not express CD44+/CD24- cells at all, suggesting that tumorigenic cells might have a CD44+/CD24- expression pattern without this pattern being exclusive for breast cancer stem cells [92]. On the other hand, several studies have indeed shown a relation between CD44 expression and clinical parameters such as tumor size. While our study shows a higher frequency of $CD44^{high}$ tumors to be larger than 2 cm, Friedrichs et al. associated CD44 expression to tumors smaller than 5 cm [70]. Other groups did show an association of larger tumors with a higher frequency of CD44 or CD44 isoform expression [20, 108, 133, 145].

As described above, breast cancer can be divided into molecular subtypes which are associated to survival and clinical aggressiveness of the tumor [31, 151, 156, 157]. For the evaluation of such molecular subtypes, defined markers have been published [77]. The group of basal-like tumors is of special interest due to reduced survival and clinically aggressive phenotype [115, 170]. 80% of basal-like tumors are triple-negative carcinomas. Therefore, the triple-negativity is considered to be the clinicopathological substitute for the basal-like molecular subtype when molecular assessment of tumors is not available [77].

When comparing the CD44 expression in our cohort to the substitute subtype definition, $CD44^{high}$ expression was significantly associated with triple-negativity and inversely correlated to the luminal A subtype (p=0.012). Rabinovich et al. presented that CD44+/CD24- tumors were associated with the ErbB2 overexpressing subtype while no association with the basal-like subtype was found [158]. Reasons for the diverging results found in our study and the work of Rabinovich et al. might be the smaller sample size and the different staining methods used. On the other hand, a number of studies have shown a similar association of CD44 expression and triple-negative carcinomas or cell lines as was found in our study [6, 15, 34, 66, 75, 76, 92, 95, 109, 149, 162, 176]. These many confirmatory studies as well as our results strongly suggest an association of $CD44^{high}$ tumors with the triple-negative or basal-like subtype and hence a clinically aggressive phenotype.

As the association of $CD44^{high}$ tumors with the triple-negative subtype suggests, $CD44^{high}$ carcinomas where inversely correlated to ER expression in our study (OR 0.49 (CI 0.25-0.94), p=0.032). Only a few studies with conflicting results exist, associating $CD44^{high}$ expression with ER positivity [57, 70]. In both studies, tumor samples were examined for the CD44 standard isoform and CD44 variant exon expression, while ours and most other studies investigated CD44 expression overall. These studies show an inverse association of CD44 expression and positive ER status [69, 101, 104, 193].

A metanalysis by Xu et al. including 23 studies demonstrated the association of $CD44^{high}$ expression to negative ER expression in line with our results [209]. This also suggests that CD44 is associated with less differentiated cell types. Hebbard et al. linked CD44 to cell differentiation [84] and Wei et al. demonstrated a reduction of CD44 positive cells with increased ERα expression [200], thus linking CD44 expression with less differentiated cells and stem cell characteristics.

4.2.2.3 CD44 expression and survival in breast cancer patients with lymph-node positive carcinoma, triple-negative and ER positive carcinoma

Since CD44 expression has been linked to less differentiated tumors in our study, we then examined the association of $CD44^{high}$ expression and survival in this cohort. CD44 expression had a significant association with overall survival in patients with lymph-node metastases (HR 2.02 (CI 1.04-3.92), p=0.038). Adamzcyk et al. investigated this association as well and did not find any significant association of CD44 expression with survival in patients with lymph-node metastases.

When looking at CD44+/CD24- cells, however, this phenotype associated with improved outcome [3]. Dan et al. described a significantly longer survival in patients with lymph-node metastases and $CD44^{high}$ tumors. While the study comprised an extensive follow-up period, the number of patients with lymph-node metastases (n = 29) was rather small, potentially impacting on the results [48]. An extensive study by Joensuu et al., on the other hand, demonstrated CD44 expression to be associated with a significantly shorter survival [101]. Thus, these two studies by Joensuu et al. and ours, both with larger patient cohorts and extensive follow-up periods, strongly suggest that CD44 expression is associated with worse survival in patients with lymph-node metastases.

When investigating patients with triple-negative carcinomas and the impact of CD44 expression on survival in this subgroup, we found a reduced overall (HR 2.22 (CI 1-4.94), p=0.05) and five-year survival (HR 3.11 (CI 1.17- 8.3), p=0.023). $CD44^{high}$ expression even proved to be an independent predictor of reduced overall survival (HR 2.22 (CI 1-4.94), p=0.05) in triple-negative carcinomas. When comparing the expression of CD44 and CD24 to overall survival, Giatromanolaki et al., on the other hand, described an association of the CD44+/CD24- as well as CD44-/CD24- phenotype with a reduced overall survival in triple negative carcinomas [76]. Nevertheless, due to the very small sample size of the CD44-/CD24- tumor sample, these results must be interpreted with caution. Wang et al. reported a reduced overall survival in CD44+/CD24-, triple-negative carcinomas [198] and Collina et al. presented results demonstrating an association of CD44 expression and disease-free survival [38]. These results support our finding, that $CD44^{high}$, triple-negative breast cancer is associated with a reduced five-year and overall survival.

When comparing survival of patients suffering from $CD44^{high}$ tumors to patients with $CD44^{low}$ disease in the subgroup of ER positive carcinomas, CD44 expression was significantly associated with a reduced disease-free survival of these patients (HR 4.99 (CI 1.11-22.34), p=0.036). Horimoto et al. on the other hand found tumors with a CD44+/CD24- subtype to be associated with a prolonged disease-fee survival in ER positive cancers while ER negative patients experienced shorter overall survival [93]. The discrepancy of these results and our study might be due to small samples sizes in our and Horimoto's cohort. Nevertheless, the follow up period in our study is remarkedly longer. Apart from these two studies, no other investigation found an association of CD44 expression, ER positivity and survival.

Two studies showed increased and reduced overall survival in ER negative tumors, respectively [104, 215].

The vast heterogeneity of the results regarding the association of CD44 and clinical parameters as well as survival is striking. Technical reasons might be attributable to this, such as differing antibodies used, varying staining technics and clinically distinct and variable patient cohorts. Nevertheless, the biological variability of CD44 expression [130] due to possible changes of CD44 expression during epithelial-mesenchymal transition [134, 177] or varying functions depending on epigenetic variations and splice variants [132, 137, 182] might play additional important roles in obscuring its influence on breast cancer outcome. Nonetheless, we were able to demonstrate an association of $CD44^{high}$ expression with less differentiated, clinically aggressive tumors and a reduced survival in subpopulations with triple-negative and lymph node positive cancers.

4.2.3 High-level EpCAM expression vs clinical parameters and survival

4.2.3.1 High-level EpCAM expression in carcinoma tissue vs clinical parameters

When comparing high-level EpCAM expression to the presence of lymph node metastases in breast cancer patients, EpCAM expression was inversely associated with nodal metastases (OR 0.31 (CI 0.14-0.7), p=0.004). Today there is only limited evidence for the involvement of EpCAM expression and its association to lymph node metastases. Two studies revealed a significant association between $EpCAM^{high}$ expression and lymph-node metastases [188, 191].

Gastl et al. on the other hand did not find an association of EpCAM expression and lymph node involvement [71]. When considering the available data on EpCAM function and its role in supporting cell-cell adhesion [127], a dual function of EpCAM depending on the microenvironment and epigenetic changes has to be considered [150, 196]. Hence, despite the overall negative impact of high-level EpCAM expression on outcome in our cohort, a negative association of EpCAM expression and local lymph node involvement is shown here.

Conversely, high-level EpCAM expression was significantly associated with higher grading and less differentiated carcinomas (OR 3.62 (CI 1.58-8.29), p=0.002). This is in line with several other studies [1, 5, 71, 168, 188].

As the association of EpCAM expression and high grade suggests, high-level EpCAM expression was inversely associated with ER (OR 0.45 (CI 0.22-0.93), p=0.03) and PR (OR 0.29 (CI 0.14-0.59), p=0.001) expression. Schmidt et al. found the same association between EpCAM expression and ER negativity [168]. When examining the relation of EpCAM expression and Ki-67 positive tumors, EpCAM was significantly associated with Ki-67 expression (OR 3.84 (CI 1.48-10.02), p=0.006). The high expression of Ki-67 has been conclusively linked to an adverse outcome in breast cancer in multiple retrospective evaluations of randomized trials and meta-analyses [51, 153, 210]. As a marker for cell proliferation, Ki-67 is associated with clinically aggressive and less differentiated breast cancer.

The findings described above support the hypothesis that high-level EpCAM expression is associated with hormone receptor negative carcinomas. In this study, EpCAM expression was significantly associated with triple-negative carcinomas (p=0.002) while high-level EpCAM expression was barely present in luminal A breast cancer. A previous study by Soysal et al. came to the same conclusion when investigating a large cohort of breast cancer patients [185]. These results lead to the conclusion that high-level EpCAM expression is indeed associated with high-proliferative breast cancers. It is thus in line that patients with high-level EpCAM expressing carcinomas present more frequently with disease recurrence (OR 3.06 (CI 1.17-8), p=0.022); which has been described by Tandon et al. as well [191].

4.2.3.2 High-level EpCAM expression and disease-free survival in breast cancer patients

High-level EpCAM expression was significantly associated with disease-free survival in our cohort (HR 2.98 (CI 1.23-7.19), p=0.015). In multiple regression analysis, EpCAM proved to be an independent predictor of reduced disease-free survival (HR 3.08 (CI 1.27-7.48), p=0.013). Several other studies have described an association of high-level EpCAM expression with disease-free [187] and overall survival [71, 168, 169, 186, 188, 191]. Thus, EpCAM expression seems to have a very strong association with disease-free survival and most likely exerts its impact on overall survival through evoking disease recurrence in breast cancer patients. Our cohort might be too small to detect this impact on overall survival. Nevertheless, when examining subgroups for the impact of high-level EpCAM expression on survival, in carcinoma patients with tumors larger than 2 cm and high-level EpCAM

expression, disease-free survival was significantly reduced (HR 4.08 (CI 1.43-11.7, p=0.009).

In patients with carcinomas with lymph node metastases, EpCAM expression was significantly associated with a shorter disease-free survival as well (HR 6.18 (CI 1.58-24.15), p=0.009). Spizzo et al. found high-level EpCAM expression to be associated with reduced overall survival in the subgroup of breast cancer patients with positive lymph nodes [188]. Due to the much larger cohort in their study, Spizzo et al. were able to find an association with overall survival. Nevertheless, since one of the most important determinants of survival in breast cancer patients is disease recurrence, one can assume that our results are in line with the study of Spizzo et al..

Interestingly, EpCAM expression was associated with a reduced number of lymph node metastases. When considering the negative impact of EpCAM on the survival in node positive patients, this finding supports the notion of EpCAM having a dual role in cancer progression and metastases as discussed above. EpCAM's clinical importance is further underlined by the fact that treating malignant ascites by targeting EpCAM (Catumaxomab) has been approved for clinical use [173].

4.2.4 CD166 expression vs clinical parameters and survival

4.2.4.1 CD166 expression in normal tissue and benign neoplasia vs carcinoma

When examining the expression of CD166 on normal breast tissue and benign neoplasia compared to DCIS and carcinoma, CD166 expression was found to be primarily positive in normal and benign tissue. Of the carcinoma samples 104 (49.3%) were $CD166^{high}$ (OR 0.5 (CI 0.3-0.8), p=0.005). On the other hand, 68 (66%) of the normal tissue and benign neoplasia samples were found to be $CD166^{high}$. Only two studies show contradictory results [28, 204]: while Wiiger et al. only examined four normal tissue specimens, Burkhardt et al. studied a larger cohort. Nevertheless, Burkhardt et al. do not present any statistics whether the difference of CD166 expression was indeed statistically significant between normal and cancer tissue in their cohort.

On the other hand and in line with our results, two studies have shown a significantly increased CD166 expression in normal breast tissue in comparison with breast cancer cells [50, 106].

These results together with our data suggest a loss of CD166 expression in breast carcinomas as cells become less differentiated and obtain a more malignant phenotype.

4.2.4.2 CD166 expression in carcinoma tissue vs clinical parameters

We found CD166 expression to be associated with lower tumor grading in our cohort (OR 0.37 (CI 0.16-0.82), p=0.015). Only a few publications describe the association of CD166 expression and tumor grading. Burkhardt et al. showed cytoplasmic staining for CD166 to be significantly associated with high grade tumors. When examining membranous staining, there were no significant associations with clinical characteristics [28].

King et al. on the other hand have demonstrated a significant association of CD166 expression to low grade tumors [106]. Burkhardt et al. argue that the high concentration of the Anti-CD166 antibody in the study by King et al. may have resulted in overstaining. Here, we present a study using the same antibody as the authors mentioned above, using the same dilution as Burkhardt et al.. Nevertheless, we can confirm the findings by King et al. showing a significant association of CD166 expression to less malignant and higher differentiated breast cancer cells.

$CD166^{high}$ carcinomas were associated with luminal intrinsic subtype while triple-negative carcinomas showed less CD166 expression (p=0.034). Tan et al. found CD166 expression to be significantly lower in triple-negative compared to non-triple-negative breast carcinomas [190]. Nevertheless, Ferragut et al. observed increased tumor growth of triple-negative cell lines in a xenograft, when silenced for CD166 expression [65]. These results have not been confirmed in clinical studies, though. Since CD166 expression seems to be associated to low grade tumors, it seems rational that luminal type carcinomas, that are known to be less aggressive and more differentiated [23, 63, 86], exhibit a higher CD166 expression.

Finally, CD166 expression was associated with PR expression (OR 2 (CI 1.09-3.69), p=0.026). Two earlier studies have shown a positive relation between CD166 expression and the positivity for hormone receptors in breast cancer [100, 190]. As mentioned above, breast cancers with luminal subtype and hormone receptor expression have a more differentiated phenotype and a better prognosis due to their tumor biology and the possibility of endocrine treatment [37]. Overall, these results associate CD166 expression to better differentiated, less proliferative and less malignant disease in breast cancer patients.

4.2.4.3 CD166 expression and overall survival in hormone receptor positive breast cancer

In the subgroup of patients with ER positive carcinomas, the expression of CD166 was an independent predictor of prolonged overall survival in multiple regression analysis (HR 0.48 (CI 0.26-0.89), p=0.02). In patients with PR positive carcinomas, CD166 expression was an independent positive predictor of survival in multiple regression analysis as well (HR 0.5 (CI 2.7-0.92). p=0.027).

As Darvishi et al. report in their review, the results regarding the association of CD166 and survival are inconclusive [49]. Our study reports on an association of shortened survival in hormone receptor positive breast cancer with CD166 expression. Burkhardt et al. have shown a significant association between high CD166 expression and reduced overall and disease-free survival in their cohort of breast cancer patients. In multivariate analysis, only cytoplasmic CD166 expression was significantly associated with reduced disease-free survival [28]. King et al. on the other hand described an increased time to event, defined as diagnosis of distant metastases, disease recurrence or death, in their cohort of breast cancer patients [106]. Burandt et al. examined CD166 expression in a cohort of 2,100 breast cancer patients [27]. Here, loss of CD166 was associated with increased malignancy and reduced survival. Despite conflicting reports in the literature, CD166 recruitment to the epithelium and epithelial CD166 expression seem to reduce cell migration in cancer cells due to cell clustering [107, 136]. The correlation of cytoplasmic CD166 expression with worse prognosis shown by Burkhard et al. might thus reflect the loss of epithelial CD166 expression. Hence, expression of CD166 on breast cancer cells appears to be a favorable prognostic factor.

5 Summary

Breast cancer is the most common cancer in women. Surgery in conjunction with chemo- and radiotherapy remain the main therapeutic options. One major obstacle in the treatment of breast cancer has been and still is the therapy resistance of a small minority of cancer cells, capable of eliciting disease recurrence. These cells have been identified as tumor initiating or cancer stem cells. These cancer stem cells are understood as the basis for tumor heterogeneity, proliferation, metastasis formation and therapy resistance. The identification of cancer stem cells has become possible by using cell surface markers, expressed by cells capable of tumor initiation and proliferation in mouse models, even after multiple passages. Despite major efforts, it has been difficult to connect these cancer stem cell markers to the clinical course of breast cancer patients including prognosis or therapeutic options.
This study was undertaken to further elucidate the association of four stem cell markers with clinical parameters and survival in 245 patients. Using a tissue microarray for immunohistochemical analysis, 405 normal, benign and carcinomatous tissue specimens were stained and analyzed for the expression of CD133, CD44, EpCAM and CD166.

The expression of the cancer stem cell marker CD133 was significantly higher in normal and benign tissues compared to carcinomas. In triple-negative breast cancer, however, CD133 expression was more frequent compared to other intrinsic subtypes. Accordingly, CD133 was associated with a high Ki-67 and low ER and PR expression, overall suggesting an association with an undifferentiated and highly proliferative phenotype. CD133 expression was also shown to be an independent predictor for a reduced five-year- and overall-survival in patients suffering from breast tumors larger than two cm. Overall, CD133 expression showed to be associated with a cancer stem cell like phenotype with reduced overall und five-year survival.

CD44 expression was significantly more frequent in DCIS and carcinoma samples as opposed to normal or benign tissue. Furthermore, CD44 expression was associated with larger tumor size, triple-negativity and accordingly, negative estrogen receptor status. Thus, CD44 expression is a marker for less differentiated, clinically more aggressive breast cancers. This is reflected in the survival analysis showing CD44 expression to be a predictor for (i) reduced survival in lymph-node positive and triple-negative breast carcinomas as well as (ii) reduced disease-free

survival in ER positive carcinomas. While the impact of CD44 expression on breast cancer is still a matter of debate, we and others have been able to show a striking association of CD44 expression in breast cancer with an aggressive tumor biology and a reduced overall and disease-free survival.

EpCAM expression was present in patients without lymph-node metastases. Conversely, breast carcinomas with high grading, hormone receptor negativity, triple-negative subtype and high Ki-67 expression were associated with EpCAM as well. While the inverse association of EpCAM with lymph-node metastases suggests a more inhibitory role towards invasiveness and carcinoma progression, EpCAM expression was associated with disease recurrence and an independent predictor of reduced disease-free survival. These results suggest a dual role of EpCAM expression in breast cancer, potentially depending on the microenvironment and epigenetic changes.

CD166 expression is the only marker in our panel associated with low grade tumors, luminal subtypes and positive hormone receptor expression. Most remarkable is the positive association of CD166 expression with a prolonged survival in patients with hormone positive breast carcinomas. This phenomenon has been reported previously and suggests a protective role of CD166 expression regarding carcinoma cell invasion and metastases.

As a novel finding we have shown that CD133 is an independent predictor of poor survival in breast cancers larger than two cm. Additionally, our results demonstrate the importance of EpCAM, CD133, and CD44 as potential markers of poor and CD166 as a potential marker of good prognosis in breast cancer. These markers should be further evaluated for their prognostic means in prospective trials. Regarding the impact of cancer stem cell marker expression on effectiveness of neoadjuvant chemotherapy and the therapy of triple-negative carcinomas further prospective clinical trials are warranted as well. Using CD133, CD44 and EpCAM expression to guide treatment decisions in personalized therapy or even as targets for specific pathway inhibitors is a possibility to improve the outcome of breast cancer patients.

6 Zusammenfassung

Mammakarzinome sind die häufigsten Karzinome und die häufigste karzinomassoziierte Todesursache bei Frauen weltweit. Trotz großer Anstrengungen in der präklinischen und klinischen Forschung haben viele Brustkrebspatienten nach wie vor eine sehr eingeschränkte Prognose. Die Diagnose wird in der Regel durch eine Mammographie oder Sonografie gestellt und durch eine Biopsie und anschließende histologische Untersuchung bestätigt. Die operative Resektion, zusammen mit Chemotherapie und Bestrahlung, stellen die wesentlichen therapeutischen Maßnahmen dar. Die genauen Mechanismen der Entstehung des Mammakarzinoms sind unklar, allerdings gibt es eine Reihe an Risikofaktoren, wie etwa exogene Östrogene oder Adipositas. Eine wesentliche Hürde in der Behandlung des Brustkarzinoms ist die ausgeprägte Heterogenität der Zellen innerhalb eines Tumors und der Tumore untereinander. Als eine Ursache für diese Heterogenität gelten Krebsstammzellen. Diese Krebsstammzellen scheinen die Basis der Proliferation, der Metastasierung und der Therapieresistenz zu sein. Sie sind in der Lage, sich asymmetrisch zu teilen und eine große Zahl an heterogenen Zellklonen hervorzubringen. Die Identifizierung von Krebsstammzellen wurde durch die Nutzung von Zelloberflächenmarkern möglich. Trotz großer Anstrengungen ist es bisher nur zum Teil gelungen diese Krebsstammzellmarker mit dem klinischen Verlauf und der Prognose von Brustkrebspatientinnen zu verknüpfen. CD44 wurde, ebenso wie CD133, bei Mammakarzinomen im Zusammenhang mit einer schlechteren Prognose beschrieben. EpCAM wurde eine Schlüsselrolle in Bezug auf die Zellmigration und Metastasierung zugeschrieben während für die CD166 Expression in der aktuellen Literatur eher eine Assoziation mit einer verbesserten Prognose beschrieben ist. Viele Untersuchungen brachten allerdings widersprüchliche Ergebnisse zu Tage. Daher war das Ziel dieser Studie, die Expression dieser vier Krebsstammzellmarker (CD133, CD44, EpCAM und CD166) und ihren Einfluss auf die Prognose von Brustkrebspatientinnen zu untersuchen.

Für diese Studie wurden die Daten von 245 Patientinnen mit benignen und malignen Neoplasien der Brust erhoben und Gewebeproben aller Patientinnen auf einem „tissue microarry“ (TMA) für die immunhistochemische Analyse zusammengefasst. Um die Überlebenszeiten berechnen zu können wurden die Todeszeitpunkte der verstorbenen Patientinnen bei den jeweiligen Standesämtern erhoben.

Schließlich konnten 207 Patientinnen mit einem Brustkarzinom, sieben Patientinnen mit einem DCIS und 31 Patientinnen mit einer benignen Neoplasie in die Analyse eingeschlossen werden. Zur Evaluation der Gewebeprobe und zur Auswahl einer repräsentativen Region für die Entnahme einer Gewebestanze zum Einschluss in den TMA, wurden Schnitte der Gewebeproben erstellt, mit Haematoxylin und Eosin gefärbt und gemeinsam mit einem erfahrenen Pathologen analysiert. Dann wurden Gewebstanzen mit 1,5 mm Durchmesser aus den in Paraffin eingebetteten Gewebeproben entnommen und in den TMA integriert. Nach der Fertigstellung der TMAs wurden Schnitte erstellt und zur immunhistochemischen Färbung vorbereitet. 405 normale, benigne und maligne Gewebeproben wurde immunhistochemisch gefärbt und in Bezug auf die Expression von CD133, CD44, EpCAM und CD166 hin analysiert. Für die Färbungen wurden monoklonale Primärantikörper eingesetzt, anschließend wurden die Gewebeproben mit biotinylierten Sekundärantikörpern inkubiert. Die Färbung erfolgte mittels eines Streptavidin-Biotin-Peroxidase-Komplexes, als Substrate der Peroxidase wurden 3-Amino-9-Ethylcarbazole (AEC) oder 3-3'-Diamino-benzidine (DAB) eingesetzt. Neben den immunhistochemischen Färbungen für CD133, CD44, EpCAM und CD166 wurden auch immunhistochemische Färbungen für Östrogen- und Progesteronrezeptoren sowie Her2neu und Ki-67 durchgeführt. Die Analyse der Her2neu Expression wurde durch eine Fluoreszenz-in-situ-Hybridisierung vervollständigt. Die Analyse der Krebsstammzellmarker-Expression sowie der Hormonrezeptor-, Her2neu- und Ki-67 Expression erfolgte erneut gemeinsam mit einem erfahrenen Pathologen. Die statistische Analyse beinhaltete die Untersuchung der Assoziation von Krebsstammzellmarker-Expression mit den klinischen Parametern mit Chi^2 oder Fisher's Exact Test. Nicht parametrische Tests (Kruskal-Wallis- und Mann-Whitney-U Test) wurde für die Untersuchung der kontinuierlichen Variablen eingesetzt. Die Überlebenszeitanalysen wurden mittels Kaplan-Meier-Test und Cox Regressionsanalysen durchgeführt.

Die Expression des Krebsstammzellmarkers CD133 war in normalem und benignem Gewebe signifikant höher als in Karzinomen. In trippel-negativen Karzinomen hingegen war CD133 häufiger exprimiert als in anderen intrinsischen Subtypen. Dazu passend war die CD133 Expression mit der hohen Expression von Ki-67 und einer niedrigen Östrogen- und Progesteronrezeptor Expression assoziiert, was eine Assoziation mit einem gering differenzierten und hoch

proliferativen Phänotyp nahelegt. Es konnte auch gezeigt werden, dass die CD133 Expression einen unabhängigen Prädiktor für ein reduziertes Fünfjahres- und Gesamtüberleben bei Patientinnen mit Brusttumoren größer als zwei cm darstellte. Die CD44 Expression war in DCIS und Karzinomgewebe deutlich häufiger als in normalem und benignem Gewebe. Zudem war die CD44 Expression assoziiert mit großen Tumoren, dreifach negativen Karzinomen und dazu passend mit einem negativen Östrogenrezeptor-Status. Daher kann CD44 als ein Marker angesehen werden, der mit weniger differenzierten, klinisch aggressiveren Brustkarzinomen assoziiert ist. Dies zeigt sich auch in der Überlebensanalyse, in der die CD44 Expression mit (i) reduziertem Überleben bei Patientinnen mit Lymphknotenmetastasen und dreifach-negativen Karzinomen, sowie (ii) reduziertem erkrankungsfreien Überleben bei Patientinnen mit Östrogenrezeptor positiven Karzinomen asoziiert ist.

Die EpCAM Expression war assoziiert mit Tumoren bei Patientinnen ohne Lymphknotenmetastasen. Im Gegensatz dazu war die EpCAM Expression allerdings auch mit Tumoren mit hoher Graduierung, negativem Hormonrezeptor-Status, trippel-negativem intrinsischem Subtyp und Tumoren mit einer Ki-67 Expression assoziiert. Während die negative Assoziation mit Metastasierung und Tumorprogression eine inhibitorische Rolle von EpCAM in diesen Qualitäten nahe legt, zeigte sich allerdings auch eine Assoziation von EpCAM Expression mit Erkrankungsrezidiven sowie einem reduzierten erkrankungsfreien Überleben.

CD166 ist der einzige Stammzellmarker, dessen Expression mit einer niedrigen Tumorgraduierung, dem luminalen intrinsischen Subtyp und einer positiven Hormonrezeptor-Expression einhergeht. Besonders bemerkenswert ist die Assoziation der CD166 Expression mit einem verlängerten Überleben bei Patientinnen mit einem Hormonrezeptor positiven Tumor.

Wie oben beschrieben, zeigte sich CD133 assoziiert mit einem den Krebsstammzellen ähnlichen Phänotyp und einem reduzierten Fünfjahres- und Gesamtüberleben. In dieser Arbeit zeigen wir zum ersten Mal, dass CD133 ein unabhängiger Prädiktor für ein reduziertes Überleben bei Patientinnen mit Mammakarzinomen ist, deren Tumoren größer als zwei cm sind. Zusätzlich zeigen unsere Ergebnisse die Bedeutung der Expression von EpCAM, CD133 und CD44 als Marker für eine schlechte und CD166 als Marker für eine verbesserte Prognose von Brustkrebspatientinnen.

Während der Einfluss der CD44 Expression auf das Mammakarzinom nach wie vor debattiert wird, konnten wir in dieser Studie eine deutliche Assoziation der CD44 Expression mit einer aggressiven Tumorbiologie und reduziertem erkrankungsfreien und Gesamtüberleben zeigen. Diese Daten entsprechen den Ergebnissen, die in der aktuellen Literatur bereits beschrieben sind. Die Assoziation der EpCAM Expression mit Karzinomen ohne Lymphknotenmetastasen auf der einen Seite und reduziertem erkrankungsfreien Überleben auf der anderen Seite legen nahe, dass EpCAM eine duale Rolle in der Proliferation und Metastasierung von Mammakarzinomen spielt. Diese duale Rolle ist Möglicherweise abhängig von der Mikroumgebung der Zellen und epigenetischen Veränderungen. Die Assoziation der Expression von CD166 mit einer verbesserten Prognose wurde bereits in der Vergangenheit beobachtet und lässt einen protektiven Effekt der CD166 Expression in Bezug auf Invasivität und Metastasierung vermuten.

Die hier untersuchten Marker sollten in prospektiven Studien weiter auf ihre potentielle prognostische Bedeutung hin untersucht werden. Um eine Korrelation der Expression von Krebsstammzellmarkern und der Effektivität von neoadjuvanter Chemotherapie sowie der Therapie tripple-negativer Karzinome zu untersuchen empfehlen sich ebenfalls weitere prospektive, klinische Studien. Die Nutzung der Expression von EpCAM, CD133 und CD44, um Behandlungsentscheidungen in der personalisierten Therapie zu treffen oder gar als Ziele für spezifische Signalweginhibitoren stellt eine Möglichkeit dar, die Prognose von Brustkrebspatientinnen zu verbessern.

7 References

1. Abd El-Maqsoud, N.M. and Abd El-Rehim, D.M.: *Clinicopathologic implications of EpCAM and Sox2 expression in breast cancer.* Clin Breast Cancer **14**: e1-9 (2014)
2. Abraham, B.K., Fritz, P., McClellan, M., Hauptvogel, P., Athelogou, M., and Brauch, H.: *Prevalence of CD44+/CD24-/low cells in breast cancer may not be associated with clinical outcome but may favor distant metastasis.* Clin Cancer Res **11**: 1154-9 (2005)
3. Adamczyk, A., Niemiec, J.A., Ambicka, A., Mucha-Malecka, A., Mitus, J., and Rys, J.: *CD44/CD24 as potential prognostic markers in node-positive invasive ductal breast cancer patients treated with adjuvant chemotherapy.* J Mol Histol **45**: 35-45 (2014)
4. Afify, A., McNiel, M.A., Braggin, J., Bailey, H., and Paulino, A.F.: *Expression of CD44s, CD44v6, and hyaluronan across the spectrum of normal-hyperplasia-carcinoma in breast.* Appl Immunohistochem Mol Morphol **16**: 121-7 (2008)
5. Agboola, A.J., Paish, E.C., Rakha, E.A., Powe, D.G., Macmillan, R.D., Ellis, I.O., and Green, A.R.: *EpCAM expression is an indicator of recurrence in basal-like breast cancer.* Breast Cancer Res Treat **133**: 575-82 (2012)
6. Ahmed, M.A., Aleskandarany, M.A., Rakha, E.A., Moustafa, R.Z., Benhasouna, A., Nolan, C., Green, A.R., Ilyas, M., and Ellis, I.O.: *A CD44(-)/CD24(+) phenotype is a poor prognostic marker in early invasive breast cancer.* Breast Cancer Res Treat **133**: 979-95 (2012)
7. Al-Hajj, M., Becker, M.W., Wicha, M., Weissman, I., and Clarke, M.F.: *Therapeutic implications of cancer stem cells.* Curr Opin Genet Dev **14**: 43-7 (2004)
8. Al-Hajj, M., Wicha, M.S., Benito-Hernandez, A., Morrison, S.J., and Clarke, M.F.: *Prospective identification of tumorigenic breast cancer cells.* Proc Natl Acad Sci U S A **100**: 3983-8 (2003)
9. Allemani, C., Weir, H.K., Carreira, H., Harewood, R., Spika, D., Wang, X.S., Bannon, F., Ahn, J.V., Johnson, C.J., Bonaventure, A., Marcos-Gragera, R., Stiller, C., Azevedo, E.S.G., Chen, W.Q., Ogunbiyi, O.J., Rachet, B., Soeberg, M.J., You, H., Matsuda, T., Bielska-Lasota, M., Storm, H., Tucker, T.C., and Coleman, M.P.: *Global surveillance of cancer survival 1995-2009: analysis of individual data for 25 676 887 patients from 279 population-based registries in 67 countries (CONCORD-2).* Lancet **385**: 977-1010 (2014)
10. Amin, M.B., Greene, F.L., Edge, S.B., Compton, C.C., Gershenwald, J.E., Brookland, R.K., Meyer, L., Gress, D.M., Byrd, D.R., and Winchester, D.P.: *The Eighth Edition AJCC Cancer Staging Manual: Continuing to build a bridge from a population-based to a more "personalized" approach to cancer staging.* CA Cancer J Clin **67**: 93-99 (2017)

11. Antoniou, A.C., Casadei, S., Heikkinen, T., Barrowdale, D., Pylkas, K., Roberts, J., Lee, A., Subramanian, D., De Leeneer, K., Fostira, F., Tomiak, E., Neuhausen, S.L., Teo, Z.L., Khan, S., Aittomaki, K., Moilanen, J.S., Turnbull, C., Seal, S., Mannermaa, A., Kallioniemi, A., Lindeman, G.J., Buys, S.S., Andrulis, I.L., Radice, P., Tondini, C., Manoukian, S., Toland, A.E., Miron, P., Weitzel, J.N., Domchek, S.M., Poppe, B., Claes, K.B., Yannoukakos, D., Concannon, P., Bernstein, J.L., James, P.A., Easton, D.F., Goldgar, D.E., Hopper, J.L., Rahman, N., Peterlongo, P., Nevanlinna, H., King, M.C., Couch, F.J., Southey, M.C., Winqvist, R., Foulkes, W.D., and Tischkowitz, M.: *Breast-cancer risk in families with mutations in PALB2.* N Engl J Med **371**: 497-506 (2014)
12. Aomatsu, N., Yashiro, M., Kashiwagi, S., Takashima, T., Ishikawa, T., Ohsawa, M., Wakasa, K., and Hirakawa, K.: *CD133 is a useful surrogate marker for predicting chemosensitivity to neoadjuvant chemotherapy in breast cancer.* PLoS One **7**: e45865 (2012)
13. Atkinson, R.L., Yang, W.T., Rosen, D.G., Landis, M.D., Wong, H., Lewis, M.T., Creighton, C.J., Sexton, K.R., Hilsenbeck, S.G., Sahin, A.A., Brewster, A.M., Woodward, W.A., and Chang, J.C.: *Cancer stem cell markers are enriched in normal tissue adjacent to triple negative breast cancer and inversely correlated with DNA repair deficiency.* Breast Cancer Res **15**: R77 (2013)
14. Auvinen, P., Tammi, R., Kosma, V.M., Sironen, R., Soini, Y., Mannermaa, A., Tumelius, R., Uljas, E., and Tammi, M.: *Increased hyaluronan content and stromal cell CD44 associate with HER2 positivity and poor prognosis in human breast cancer.* Int J Cancer **132**: 531-9 (2013)
15. Badve, S. and Nakshatri, H.: *Breast-cancer stem cells-beyond semantics.* Lancet Oncol **13**: e43-8 (2012)
16. Balic, M., Lin, H., Young, L., Hawes, D., Giuliano, A., McNamara, G., Datar, R.H., and Cote, R.J.: *Most early disseminated cancer cells detected in bone marrow of breast cancer patients have a putative breast cancer stem cell phenotype.* Clin Cancer Res **12**: 5615-21 (2006)
17. Bankfalvi, A., Terpe, H.J., Breukelmann, D., Bier, B., Rempe, D., Pschadka, G., Krech, R., and Bocker, W.: *Gains and losses of CD44 expression during breast carcinogenesis and tumour progression.* Histopathology **33**: 107-16 (1998)
18. Battifora, H.: *The multitumor (sausage) tissue block: novel method for immunohistochemical antibody testing.* Lab Invest **55**: 244-8 (1986)
19. Beca, F. and Polyak, K.: *Intratumor Heterogeneity in Breast Cancer.* Adv Exp Med Biol **882**: 169-89 (2016)
20. Bhatavdekar, J.M., Patel, D.D., Shah, N.G., Vora, H.H., Suthar, T.P., Chikhlikar, P.R., Ghosh, N., and Trivedi, T.I.: *Prognostic significance of immunohistochemically localized biomarkers in stage II and stage III breast cancer: a multivariate analysis.* Ann Surg Oncol **7**: 305-11 (2000)
21. Blok, E.J., Bastiaannet, E., van den Hout, W.B., Liefers, G.J., Smit, V., Kroep, J.R., and van de Velde, C.J.H.: *Systematic review of the clinical and economic value of gene expression profiles for invasive early breast cancer available in Europe.* Cancer Treat Rev **62**: 74-90 (2018)
22. Bloom, H.J. and Richardson, W.W.: *Histological grading and prognosis in breast cancer; a study of 1409 cases of which 359 have been followed for 15 years.* Br J Cancer **11**: 359-77 (1957)

23. Blows, F.M., Driver, K.E., Schmidt, M.K., Broeks, A., van Leeuwen, F.E., Wesseling, J., Cheang, M.C., Gelmon, K., Nielsen, T.O., Blomqvist, C., Heikkila, P., Heikkinen, T., Nevanlinna, H., Akslen, L.A., Begin, L.R., Foulkes, W.D., Couch, F.J., Wang, X., Cafourek, V., Olson, J.E., Baglietto, L., Giles, G.G., Severi, G., McLean, C.A., Southey, M.C., Rakha, E., Green, A.R., Ellis, I.O., Sherman, M.E., Lissowska, J., Anderson, W.F., Cox, A., Cross, S.S., Reed, M.W., Provenzano, E., Dawson, S.J., Dunning, A.M., Humphreys, M., Easton, D.F., Garcia-Closas, M., Caldas, C., Pharoah, P.D., and Huntsman, D.: *Subtyping of breast cancer by immunohistochemistry to investigate a relationship between subtype and short and long term survival: a collaborative analysis of data for 10,159 cases from 12 studies.* PLoS Med **7**: e1000279 (2010)
24. Bowen, M.A., Patel, D.D., Li, X., Modrell, B., Malacko, A.R., Wang, W.C., Marquardt, H., Neubauer, M., Pesando, J.M., Francke, U., and et al.: *Cloning, mapping, and characterization of activated leukocyte-cell adhesion molecule (ALCAM), a CD6 ligand.* J Exp Med **181**: 2213-20 (1995)
25. Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R.L., Torre, L.A., and Jemal, A.: *Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries.* CA Cancer J Clin **68**: 394-424 (2018)
26. Brugnoli, F., Grassilli, S., Piazzi, M., Palomba, M., Nika, E., Bavelloni, A., Capitani, S., and Bertagnolo, V.: *In triple negative breast tumor cells, PLC-beta2 promotes the conversion of CD133high to CD133low phenotype and reduces the CD133-related invasiveness.* Mol Cancer **12**: 165 (2013)
27. Burandt, E., Bari Noubar, T., Lebeau, A., Minner, S., Burdelski, C., Janicke, F., Muller, V., Terracciano, L., Simon, R., Sauter, G., Wilczak, W., and Lebok, P.: *Loss of ALCAM expression is linked to adverse phenotype and poor prognosis in breast cancer: a TMA-based immunohistochemical study on 2,197 breast cancer patients.* Oncol Rep **32**: 2628-34 (2014)
28. Burkhardt, M., Mayordomo, E., Winzer, K.J., Fritzsche, F., Gansukh, T., Pahl, S., Weichert, W., Denkert, C., Guski, H., Dietel, M., and Kristiansen, G.: *Cytoplasmic overexpression of ALCAM is prognostic of disease progression in breast cancer.* J Clin Pathol **59**: 403-9 (2006)
29. Burrell, R.A., McGranahan, N., Bartek, J., and Swanton, C.: *The causes and consequences of genetic heterogeneity in cancer evolution.* Nature **501**: 338-45 (2013)
30. Burstein, H.J.: *Patients with triple negative breast cancer: is there an optimal adjuvant treatment?* Breast **22 Suppl 2**: S147-8 (2013)
31. Cancer Genome Atlas Network: *Comprehensive molecular portraits of human breast tumours.* Nature **490**: 61-70 (2012)
32. Cardoso, F., Costa, A., Norton, L., Senkus, E., Aapro, M., Andre, F., Barrios, C.H., Bergh, J., Biganzoli, L., Blackwell, K.L., Cardoso, M.J., Cufer, T., El Saghir, N., Fallowfield, L., Fenech, D., Francis, P., Gelmon, K., Giordano, S.H., Gligorov, J., Goldhirsch, A., Harbeck, N., Houssami, N., Hudis, C., Kaufman, B., Krop, I., Kyriakides, S., Lin, U.N., Mayer, M., Merjaver, S.D., Nordstrom, E.B., Pagani, O., Partridge, A., Penault-Llorca, F., Piccart, M.J., Rugo, H., Sledge, G., Thomssen, C., Van't Veer, L., Vorobiof, D., Vrieling, C., West, N., Xu, B., and Winer, E.: *ESO-ESMO 2nd international consensus guidelines for advanced breast cancer (ABC2)dagger.* Ann Oncol **25**: 1871-88 (2014)

33. Carter, C.L., Allen, C., and Henson, D.E.: *Relation of tumor size, lymph node status, and survival in 24,740 breast cancer cases.* Cancer **63**: 181-7 (1989)
34. Charafe-Jauffret, E., Ginestier, C., Monville, F., Finetti, P., Adelaide, J., Cervera, N., Fekairi, S., Xerri, L., Jacquemier, J., Birnbaum, D., and Bertucci, F.: *Gene expression profiling of breast cell lines identifies potential new basal markers.* Oncogene **25**: 2273-84 (2006)
35. Charpin, C., Garcia, S., Bouvier, C., Devictor, B., Andrac, L., Choux, R., Lavaut, M.N., and Allasia, C.: *Automated and quantitative immunocytochemical assays of CD44v6 in breast carcinomas.* Hum Pathol **28**: 289-96 (1997)
36. Clarke, M.F., Dick, J.E., Dirks, P.B., Eaves, C.J., Jamieson, C.H., Jones, D.L., Visvader, J., Weissman, I.L., and Wahl, G.M.: *Cancer stem cells--perspectives on current status and future directions: AACR Workshop on cancer stem cells.* Cancer Res **66**: 9339-44 (2006)
37. Coates, A.S., Winer, E.P., Goldhirsch, A., Gelber, R.D., Gnant, M., Piccart-Gebhart, M., Thurlimann, B., Senn, H.J., and Panel, M.: *Tailoring therapies--improving the management of early breast cancer: St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2015.* Ann Oncol **26**: 1533-46 (2015)
38. Collina, F., Di Bonito, M., Li Bergolis, V., De Laurentiis, M., Vitagliano, C., Cerrone, M., Nuzzo, F., Cantile, M., and Botti, G.: *Prognostic Value of Cancer Stem Cells Markers in Triple-Negative Breast Cancer.* Biomed Res Int **2015**: 158682 (2015)
39. Couch, F.J., Shimelis, H., Hu, C., Hart, S.N., Polley, E.C., Na, J., Hallberg, E., Moore, R., Thomas, A., Lilyquist, J., Feng, B., McFarland, R., Pesaran, T., Huether, R., LaDuca, H., Chao, E.C., Goldgar, D.E., and Dolinsky, J.S.: *Associations Between Cancer Predisposition Testing Panel Genes and Breast Cancer.* JAMA Oncol **3**: 1190-1196 (2017)
40. Creighton, C.J., Li, X., Landis, M., Dixon, J.M., Neumeister, V.M., Sjolund, A., Rimm, D.L., Wong, H., Rodriguez, A., Herschkowitz, J.I., Fan, C., Zhang, X., He, X., Pavlick, A., Gutierrez, M.C., Renshaw, L., Larionov, A.A., Faratian, D., Hilsenbeck, S.G., Perou, C.M., Lewis, M.T., Rosen, J.M., and Chang, J.C.: *Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features.* Proc Natl Acad Sci U S A **106**: 13820-5 (2009)
41. Cserni, G., Chmielik, E., Cserni, B., and Tot, T.: *The new TNM-based staging of breast cancer.* Virchows Arch **472**: 697-703 (2018)
42. Currie, M.J., Beardsley, B.E., Harris, G.C., Gunningham, S.P., Dachs, G.U., Dijkstra, B., Morrin, H.R., Wells, J.E., and Robinson, B.A.: *Immunohistochemical analysis of cancer stem cell markers in invasive breast carcinoma and associated ductal carcinoma in situ: relationships with markers of tumor hypoxia and microvascularity.* Hum Pathol **44**: 402-11 (2013)
43. Cybulski, C., Wokolorczyk, D., Jakubowska, A., Huzarski, T., Byrski, T., Gronwald, J., Masojc, B., Deebniak, T., Gorski, B., Blecharz, P., Narod, S.A., and Lubinski, J.: *Risk of breast cancer in women with a CHEK2 mutation with and without a family history of breast cancer.* J Clin Oncol **29**: 3747-52 (2011)

44. D'Anello, L., Sansone, P., Storci, G., Mitrugno, V., D'Uva, G., Chieco, P., and Bonafe, M.: *Epigenetic control of the basal-like gene expression profile via Interleukin-6 in breast cancer cells.* Mol Cancer **9**: 300 (2010)
45. Da Cruz Paula, A. and Lopes, C.: *Implications of Different Cancer Stem Cell Phenotypes in Breast Cancer.* Anticancer Res **37**: 2173-2183 (2017)
46. Da Cruz Paula, A., Marques, O., Rosa, A.M., De Fatima Faria, M., Rema, A., and Lopes, C.: *Co-expression of stem cell markers ALDH1 and CD44 in non-malignant and neoplastic lesions of the breast.* Anticancer Res **34**: 1427-34 (2014)
47. Dall, P., Heider, K.H., Sinn, H.P., Skroch-Angel, P., Adolf, G., Kaufmann, M., Herrlich, P., and Ponta, H.: *Comparison of immunohistochemistry and RT-PCR for detection of CD44v-expression, a new prognostic factor in human breast cancer.* Int J Cancer **60**: 471-7 (1995)
48. Dan, T., Hewitt, S.M., Ohri, N., Ly, D., Soule, B.P., Smith, S.L., Matsuda, K., Council, C., Shankavaram, U., Lippman, M.E., Mitchell, J.B., Camphausen, K., and Simone, N.L.: *CD44 is prognostic for overall survival in the NCI randomized trial on breast conservation with 25 year follow-up.* Breast Cancer Res Treat **143**: 11-8 (2014)
49. Darvishi, B., Boroumandieh, S., Majidzadeh, A.K., Salehi, M., Jafari, F., and Farahmand, L.: *The role of activated leukocyte cell adhesion molecule (ALCAM) in cancer progression, invasion, metastasis and recurrence: A novel cancer stem cell marker and tumor-specific prognostic marker.* Exp Mol Pathol **115**: 104443 (2020)
50. Davies, S.R., Dent, C., Watkins, G., King, J.A., Mokbel, K., and Jiang, W.G.: *Expression of the cell to cell adhesion molecule, ALCAM, in breast cancer patients and the potential link with skeletal metastasis.* Oncol Rep **19**: 555-61 (2008)
51. de Azambuja, E., Cardoso, F., de Castro, G., Jr., Colozza, M., Mano, M.S., Durbecq, V., Sotiriou, C., Larsimont, D., Piccart-Gebhart, M.J., and Paesmans, M.: *Ki-67 as prognostic marker in early breast cancer: a meta-analysis of published studies involving 12,155 patients.* Br J Cancer **96**: 1504-13 (2007)
52. de Beca, F.F., Caetano, P., Gerhard, R., Alvarenga, C.A., Gomes, M., Paredes, J., and Schmitt, F.: *Cancer stem cells markers CD44, CD24 and ALDH1 in breast cancer special histological types.* J Clin Pathol **66**: 187-91 (2013)
53. de la Torre, M., Heldin, P., and Bergh, J.: *Expression of the CD44 glycoprotein (lymphocyte-homing receptor) in untreated human breast cancer and its relationship to prognostic markers.* Anticancer Res **15**: 2791-5 (1995)
54. Degen, W.G., van Kempen, L.C., Gijzen, E.G., van Groningen, J.J., van Kooyk, Y., Bloemers, H.P., and Swart, G.W.: *MEMD, a new cell adhesion molecule in metastasizing human melanoma cell lines, is identical to ALCAM (activated leukocyte cell adhesion molecule).* Am J Pathol **152**: 805-13 (1998)
55. Di Bonito, M., Cantile, M., Collina, F., Scognamiglio, G., Cerrone, M., La Mantia, E., Barbato, A., Liguori, G., and Botti, G.: *Overexpression of Cell Cycle Progression Inhibitor Geminin is Associated with Tumor Stem-Like Phenotype of Triple-Negative Breast Cancer.* J Breast Cancer **15**: 162-71 (2012)

56. Di Bonito, M., Collina, F., Cantile, M., Camerlingo, R., Cerrone, M., Marra, L., Liguori, G., Pirozzi, G., and Botti, G.: *Aberrant Expression of Cancer Stem Cells Marker Prominin-1 in Low-Grade Tubulolobular Breast Carcinoma: A Correlative Study between qRT-PCR, Flow-Cytometric and Immunohistochemistry Analysis [corrected].* J Breast Cancer **15**: 15-23 (2012)
57. Diaz, L.K., Zhou, X., Wright, E.T., Cristofanilli, M., Smith, T., Yang, Y., Sneige, N., Sahin, A., and Gilcrease, M.Z.: *CD44 expression is associated with increased survival in node-negative invasive breast carcinoma.* Clin Cancer Res **11**: 3309-14 (2005)
58. Dowsett, M., Nielsen, T.O., A'Hern, R., Bartlett, J., Coombes, R.C., Cuzick, J., Ellis, M., Henry, N.L., Hugh, J.C., Lively, T., McShane, L., Paik, S., Penault-Llorca, F., Prudkin, L., Regan, M., Salter, J., Sotiriou, C., Smith, I.E., Viale, G., Zujewski, J.A., and Hayes, D.F.: *Assessment of Ki67 in breast cancer: recommendations from the International Ki67 in Breast Cancer working group.* J Natl Cancer Inst **103**: 1656-64 (2011)
59. Drobysheva, D., Smith, B.A., McDowell, M., Guillen, K.P., Ekiz, H.A., and Welm, B.E.: *Transformation of enriched mammary cell populations with polyomavirus middle T antigen influences tumor subtype and metastatic potential.* Breast Cancer Res **17**: 132 (2015)
60. Early Breast Cancer Trialists' Collaborative Group: *Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials.* Lancet **365**: 1687-717 (2005)
61. Economopoulou, P., Kaklamani, V.G., and Siziopikou, K.: *The role of cancer stem cells in breast cancer initiation and progression: potential cancer stem cell-directed therapies.* Oncologist **17**: 1394-401 (2012)
62. Elston, C.W. and Ellis, I.O.: *Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up.* Histopathology **19**: 403-10 (1991)
63. Engstrom, M.J., Opdahl, S., Hagen, A.I., Romundstad, P.R., Akslen, L.A., Haugen, O.A., Vatten, L.J., and Bofin, A.M.: *Molecular subtypes, histopathological grade and survival in a historic cohort of breast cancer patients.* Breast Cancer Res Treat **140**: 463-73 (2013)
64. Ferlay, J., Colombet, M., Soerjomataram, I., Mathers, C., Parkin, D.M., Pineros, M., Znaor, A., and Bray, F.: *Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods.* Int J Cancer **144**: 1941-1953 (2019)
65. Ferragut, F., Cagnoni, A.J., Colombo, L.L., Sanchez Terrero, C., Wolfenstein-Todel, C., Troncoso, M.F., Vanzulli, S.I., Rabinovich, G.A., Marino, K.V., and Elola, M.T.: *Dual knockdown of Galectin-8 and its glycosylated ligand, the activated leukocyte cell adhesion molecule (ALCAM/CD166), synergistically delays in vivo breast cancer growth.* Biochim Biophys Acta Mol Cell Res **1866**: 1338-1352 (2019)
66. Fillmore, C.M. and Kuperwasser, C.: *Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy.* Breast Cancer Res **10**: R25 (2008)
67. Fisher, B., Slack, N.H., and Bross, I.D.: *Cancer of the breast: size of neoplasm and prognosis.* Cancer **24**: 1071-80 (1969)

68. Florek, M., Haase, M., Marzesco, A.M., Freund, D., Ehninger, G., Huttner, W.B., and Corbeil, D.: *Prominin-1/CD133, a neural and hematopoietic stem cell marker, is expressed in adult human differentiated cells and certain types of kidney cancer.* Cell Tissue Res **319**: 15-26 (2005)
69. Foekens, J.A., Dall, P., Klijn, J.G., Skroch-Angel, P., Claassen, C.J., Look, M.P., Ponta, H., Van Putten, W.L., Herrlich, P., and Henzen-Logmans, S.C.: *Prognostic value of CD44 variant expression in primary breast cancer.* Int J Cancer **84**: 209-15 (1999)
70. Friedrichs, K., Franke, F., Lisboa, B.W., Kugler, G., Gille, I., Terpe, H.J., Holzel, F., Maass, H., and Gunthert, U.: *CD44 isoforms correlate with cellular differentiation but not with prognosis in human breast cancer.* Cancer Res **55**: 5424-33 (1995)
71. Gastl, G., Spizzo, G., Obrist, P., Dunser, M., and Mikuz, G.: *Ep-CAM overexpression in breast cancer as a predictor of survival.* Lancet **356**: 1981-2 (2000)
72. Gee, K., Kryworuchko, M., and Kumar, A.: *Recent advances in the regulation of CD44 expression and its role in inflammation and autoimmune diseases.* Arch Immunol Ther Exp (Warsz) **52**: 13-26 (2004)
73. Gerdes, J., Lemke, H., Baisch, H., Wacker, H.H., Schwab, U., and Stein, H.: *Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67.* J Immunol **133**: 1710-5 (1984)
74. Gerdes, J., Schwab, U., Lemke, H., and Stein, H.: *Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation.* Int J Cancer **31**: 13-20 (1983)
75. Gerhard, R., Ricardo, S., Albergaria, A., Gomes, M., Silva, A.R., Logullo, A.F., Cameselle-Teijeiro, J.F., Paredes, J., and Schmitt, F.: *Immunohistochemical features of claudin-low intrinsic subtype in metaplastic breast carcinomas.* Breast **21**: 354-60 (2012)
76. Giatromanolaki, A., Sivridis, E., Fiska, A., and Koukourakis, M.I.: *The CD44+/CD24- phenotype relates to 'triple-negative' state and unfavorable prognosis in breast cancer patients.* Med Oncol **28**: 745-52 (2011)
77. Goldhirsch, A., Winer, E.P., Coates, A.S., Gelber, R.D., Piccart-Gebhart, M., Thurlimann, B., and Senn, H.J.: *Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013.* Ann Oncol **24**: 2206-23 (2013)
78. Gray, E., Marti, J., Brewster, D.H., Wyatt, J.C., Hall, P.S., and Group, S.A.: *Independent validation of the PREDICT breast cancer prognosis prediction tool in 45,789 patients using Scottish Cancer Registry data.* Br J Cancer **119**: 808-814 (2018)
79. Hammond, M.E., Hayes, D.F., Dowsett, M., Allred, D.C., Hagerty, K.L., Badve, S., Fitzgibbons, P.L., Francis, G., Goldstein, N.S., Hayes, M., Hicks, D.G., Lester, S., Love, R., Mangu, P.B., McShane, L., Miller, K., Osborne, C.K., Paik, S., Perlmutter, J., Rhodes, A., Sasano, H., Schwartz, J.N., Sweep, F.C., Taube, S., Torlakovic, E.E., Valenstein, P., Viale, G., Visscher, D., Wheeler, T., Williams, R.B., Wittliff, J.L., and Wolff, A.C.: *American Society of Clinical Oncology/College Of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer.* J Clin Oncol **28**: 2784-95 (2010)

80. Han, Z., Chen, Z., Zheng, R., Cheng, Z., Gong, X., and Wang, D.: *Clinicopathological significance of CD133 and CD44 expression in infiltrating ductal carcinoma and their relationship to angiogenesis.* World J Surg Oncol **13**: 56 (2015)
81. Hankinson, S., Tamimi, R.M., and Hunter, D.J.: *Breast Cancer*. In: Adami, H.-O., Hunter, D.J., and Trichopoulos, D. (Ed.): Textbook of cancer epidemiology. 403-445, Oxford University Press, Oxford ; New York, (2008)
82. Harris, L.N., Ismaila, N., McShane, L.M., Andre, F., Collyar, D.E., Gonzalez-Angulo, A.M., Hammond, E.H., Kuderer, N.M., Liu, M.C., Mennel, R.G., Van Poznak, C., Bast, R.C., Hayes, D.F., and American Society of Clinical, O.: *Use of Biomarkers to Guide Decisions on Adjuvant Systemic Therapy for Women With Early-Stage Invasive Breast Cancer: American Society of Clinical Oncology Clinical Practice Guideline.* J Clin Oncol **34**: 1134-50 (2016)
83. Haynes, B., Sarma, A., Nangia-Makker, P., and Shekhar, M.P.: *Breast cancer complexity: implications of intratumoral heterogeneity in clinical management.* Cancer Metastasis Rev **36**: 547-555 (2017)
84. Hebbard, L., Steffen, A., Zawadzki, V., Fieber, C., Howells, N., Moll, J., Ponta, H., Hofmann, M., and Sleeman, J.: *CD44 expression and regulation during mammary gland development and function.* J Cell Sci **113 (Pt 14)**: 2619-30 (2000)
85. Hefler, L., Tempfer, C., Haeusler, G., Kucera, E., Mayerhofer, K., Zeillinger, R., Reinthaller, A., and Kainz, C.: *Cytosol concentrations of CD44 isoforms in breast cancer tissue.* Int J Cancer **79**: 541-5 (1998)
86. Hennigs, A., Riedel, F., Gondos, A., Sinn, P., Schirmacher, P., Marme, F., Jager, D., Kauczor, H.U., Stieber, A., Lindel, K., Debus, J., Golatta, M., Schutz, F., Sohn, C., Heil, J., and Schneeweiss, A.: *Prognosis of breast cancer molecular subtypes in routine clinical care: A large prospective cohort study.* BMC Cancer **16**: 734 (2016)
87. Herlyn, M., Steplewski, Z., Herlyn, D., and Koprowski, H.: *Colorectal carcinoma-specific antigen: detection by means of monoclonal antibodies.* Proc Natl Acad Sci U S A **76**: 1438-42 (1979)
88. Hermanek, P., Sobin, L.H., and International Union against Cancer.: *TNM classification of malignant tumours*. Springer-Verlag, Berlin ; London, (1987)
89. Hewitt, S.M.: *Design, construction, and use of tissue microarrays.* Methods Mol Biol **264**: 61-72 (2004)
90. Hilbe, W., Dirnhofer, S., Oberwasserlechner, F., Schmid, T., Gunsilius, E., Hilbe, G., Woll, E., and Kahler, C.M.: *CD133 positive endothelial progenitor cells contribute to the tumour vasculature in non-small cell lung cancer.* J Clin Pathol **57**: 965-9 (2004)
91. Hiraga, T., Ito, S., and Nakamura, H.: *EpCAM expression in breast cancer cells is associated with enhanced bone metastasis formation.* Int J Cancer **138**: 1698-708 (2016)
92. Honeth, G., Bendahl, P.O., Ringner, M., Saal, L.H., Gruvberger-Saal, S.K., Lovgren, K., Grabau, D., Ferno, M., Borg, A., and Hegardt, C.: *The CD44+/CD24- phenotype is enriched in basal-like breast tumors.* Breast Cancer Res **10**: R53 (2008)
93. Horimoto, Y., Arakawa, A., Sasahara, N., Tanabe, M., Sai, S., Himuro, T., and Saito, M.: *Combination of Cancer Stem Cell Markers CD44 and CD24 Is Superior to ALDH1 as a Prognostic Indicator in Breast Cancer Patients with Distant Metastases.* PLoS One **11**: e0165253 (2016)

94. Iczkowski, K.A.: *Cell adhesion molecule CD44: its functional roles in prostate cancer.* Am J Transl Res **3**: 1-7 (2010)
95. Idowu, M.O., Kmieciak, M., Dumur, C., Burton, R.S., Grimes, M.M., Powers, C.N., and Manjili, M.H.: *CD44(+)/CD24(-/low) cancer stem/progenitor cells are more abundant in triple-negative invasive breast carcinoma phenotype and are associated with poor outcome.* Hum Pathol **43**: 364-73 (2012)
96. Ihnen, M., Muller, V., Wirtz, R.M., Schroder, C., Krenkel, S., Witzel, I., Lisboa, B.W., Janicke, F., and Milde-Langosch, K.: *Predictive impact of activated leukocyte cell adhesion molecule (ALCAM/CD166) in breast cancer.* Breast Cancer Res Treat **112**: 419-27 (2008)
97. Inwald, E.C., Klinkhammer-Schalke, M., Hofstadter, F., Zeman, F., Koller, M., Gerstenhauer, M., and Ortmann, O.: *Ki-67 is a prognostic parameter in breast cancer patients: results of a large population-based cohort of a cancer registry.* Breast Cancer Res Treat **139**: 539-52 (2013)
98. Jang, M.H., Kang, H.J., Jang, K.S., Paik, S.S., and Kim, W.S.: *Clinicopathological analysis of CD44 and CD24 expression in invasive breast cancer.* Oncol Lett **12**: 2728-2733 (2016)
99. Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E., and Forman, D.: *Global cancer statistics.* CA Cancer J Clin **61**: 69-90 (2011)
100. Jezierska, A., Olszewski, W.P., Pietruszkiewicz, J., Olszewski, W., Matysiak, W., and Motyl, T.: *Activated Leukocyte Cell Adhesion Molecule (ALCAM) is associated with suppression of breast cancer cells invasion.* Med Sci Monit **12**: BR245-56 (2006)
101. Joensuu, H., Klemi, P.J., Toikkanen, S., and Jalkanen, S.: *Glycoprotein CD44 expression and its association with survival in breast cancer.* Am J Pathol **143**: 867-74 (1993)
102. Kaatsch, P., Spix, C., Katalinic, A., Hentschel, S., Luttmann, S., Waldeyer-Sauerland, M., Waldmann, A., Christ, M., Folkerts, J., Hansmann, J., Klein, S., Kranzhöfer, K., Kunz, B., Manegold, K., Penzkofer, A., Treml, K., Vollmer, G., Weg-Remers, S., Barnes, B., Buttmann-Schweiger, N., Dahm, S., Fiebig, J., Franke, M., Gurung-Schönfeld, I., Haberland, J., Kraywinkel, K., and Wienecke, A.: *Krebs in Deutschland 2015/2016*. Ed.: Robert Koch-Institut and Gesellschaft der epidemiologischen Krebsregister in Deutschland e.V., Vol. **12**, 1-161, (2019)
103. Kapucuoglu, N., Bozkurt, K.K., Baspinar, S., Kocer, M., Eroglu, H.E., Akdeniz, R., and Akcil, M.: *The clinicopathological and prognostic significance of CD24, CD44, CD133, ALDH1 expressions in invasive ductal carcinoma of the breast: CD44/CD24 expression in breast cancer.* Pathol Res Pract **211**: 740-7 (2015)
104. Kim, H.J., Kim, M.J., Ahn, S.H., Son, B.H., Kim, S.B., Ahn, J.H., Noh, W.C., and Gong, G.: *Different prognostic significance of CD24 and CD44 expression in breast cancer according to hormone receptor status.* Breast **20**: 78-85 (2011)
105. Kim, S.J., Kim, Y.S., Jang, E.D., Seo, K.J., and Kim, J.S.: *Prognostic Impact and Clinicopathological Correlation of CD133 and ALDH1 Expression in Invasive Breast Cancer.* J Breast Cancer **18**: 347-55 (2015)
106. King, J.A., Ofori-Acquah, S.F., Stevens, T., Al-Mehdi, A.B., Fodstad, O., and Jiang, W.G.: *Activated leukocyte cell adhesion molecule in breast cancer: prognostic indicator.* Breast Cancer Res **6**: R478-87 (2004)

107. King, J.A., Tan, F., Mbeunkui, F., Chambers, Z., Cantrell, S., Chen, H., Alvarez, D., Shevde, L.A., and Ofori-Acquah, S.F.: *Mechanisms of transcriptional regulation and prognostic significance of activated leukocyte cell adhesion molecule in cancer.* Mol Cancer **9**: 266 (2010)
108. Kinoshita, J., Haga, S., Shimizu, T., Imamura, H., Watanabe, O., and Kajiwara, T.: *The expression of variant exon v7-v8 CD44 antigen in relation to lymphatic metastasis of human breast cancer.* Breast Cancer Res Treat **53**: 177-83 (1999)
109. Klingbeil, P., Natrajan, R., Everitt, G., Vatcheva, R., Marchio, C., Palacios, J., Buerger, H., Reis-Filho, J.S., and Isacke, C.M.: *CD44 is overexpressed in basal-like breast cancers but is not a driver of 11p13 amplification.* Breast Cancer Res Treat **120**: 95-109 (2010)
110. Kohler, G. and Milstein, C.: *Continuous cultures of fused cells secreting antibody of predefined specificity.* Nature **256**: 495-7 (1975)
111. Kononen, J., Bubendorf, L., Kallioniemi, A., Barlund, M., Schraml, P., Leighton, S., Torhorst, J., Mihatsch, M.J., Sauter, G., and Kallioniemi, O.-P.: *Tissue microarrays for high-throughput molecular profiling of tumor specimens.* Nature Medicine **4**: 844-847 (1998)
112. Koscielny, S., Tubiana, M., Le, M.G., Valleron, A.J., Mouriesse, H., Contesso, G., and Sarrazin, D.: *Breast cancer: relationship between the size of the primary tumour and the probability of metastatic dissemination.* Br J Cancer **49**: 709-15 (1984)
113. Kreso, A. and Dick, J.E.: *Evolution of the cancer stem cell model.* Cell Stem Cell **14**: 275-91 (2014)
114. Krop, I., Ismaila, N., Andre, F., Bast, R.C., Barlow, W., Collyar, D.E., Hammond, M.E., Kuderer, N.M., Liu, M.C., Mennel, R.G., Van Poznak, C., Wolff, A.C., and Stearns, V.: *Use of Biomarkers to Guide Decisions on Adjuvant Systemic Therapy for Women With Early-Stage Invasive Breast Cancer: American Society of Clinical Oncology Clinical Practice Guideline Focused Update.* J Clin Oncol **35**: 2838-2847 (2017)
115. Kumar, P. and Aggarwal, R.: *An overview of triple-negative breast cancer.* Arch Gynecol Obstet **293**: 247-69 (2016)
116. Land, C.E.: *Studies of cancer and radiation dose among atomic bomb survivors. The example of breast cancer.* JAMA **274**: 402-7 (1995)
117. Lang, F., Wojcik, B., and Rieger, M.A.: *Stem Cell Hierarchy and Clonal Evolution in Acute Lymphoblastic Leukemia.* Stem Cells Int **2015**: 137164 (2015)
118. Lapidot, T., Sirard, C., Vormoor, J., Murdoch, B., Hoang, T., Caceres-Cortes, J., Minden, M., Paterson, B., Caligiuri, M.A., and Dick, J.E.: *A cell initiating human acute myeloid leukaemia after transplantation into SCID mice.* Nature **367**: 645-8 (1994)
119. Leary, A.F., Drury, S., Detre, S., Pancholi, S., Lykkesfeldt, A.E., Martin, L.A., Dowsett, M., and Johnston, S.R.: *Lapatinib restores hormone sensitivity with differential effects on estrogen receptor signaling in cell models of human epidermal growth factor receptor 2-negative breast cancer with acquired endocrine resistance.* Clin Cancer Res **16**: 1486-97 (2010)
120. Lee, H.E., Kim, J.H., Kim, Y.J., Choi, S.Y., Kim, S.W., Kang, E., Chung, I.Y., Kim, I.A., Kim, E.J., Choi, Y., Ryu, H.S., and Park, S.Y.: *An increase in cancer stem cell population after primary systemic therapy is a poor prognostic factor in breast cancer.* Br J Cancer **104**: 1730-8 (2011)

121. Leong, A.S. and Zhuang, Z.: *The changing role of pathology in breast cancer diagnosis and treatment.* Pathobiology **78**: 99-114 (2011)
122. Lester, S.C.: *The Breast*. In: Robbins, S.L., Kumar, V., and Cotran, R.S. (Ed.): Robbins and Cotran pathologic basis of disease. 1066-1094, Saunders/Elsevier, Philadelphia, (2010)
123. Li, X., Lewis, M.T., Huang, J., Gutierrez, C., Osborne, C.K., Wu, M.F., Hilsenbeck, S.G., Pavlick, A., Zhang, X., Chamness, G.C., Wong, H., Rosen, J., and Chang, J.C.: *Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy.* J Natl Cancer Inst **100**: 672-9 (2008)
124. Lim, E., Vaillant, F., Wu, D., Forrest, N.C., Pal, B., Hart, A.H., Asselin-Labat, M.L., Gyorki, D.E., Ward, T., Partanen, A., Feleppa, F., Huschtscha, L.I., Thorne, H.J., kConFab, Fox, S.B., Yan, M., French, J.D., Brown, M.A., Smyth, G.K., Visvader, J.E., and Lindeman, G.J.: *Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers.* Nat Med **15**: 907-13 (2009)
125. Lin, C.Y., Barry-Holson, K.Q., and Allison, K.H.: *Breast cancer stem cells: are we ready to go from bench to bedside?* Histopathology **68**: 119-37 (2016)
126. Linnenbach, A.J., Seng, B.A., Wu, S., Robbins, S., Scollon, M., Pyrc, J.J., Druck, T., and Huebner, K.: *Retroposition in a family of carcinoma-associated antigen genes.* Mol Cell Biol **13**: 1507-15 (1993)
127. Litvinov, S.V., Velders, M.P., Bakker, H.A., Fleuren, G.J., and Warnaar, S.O.: *Ep-CAM: a human epithelial antigen is a homophilic cell-cell adhesion molecule.* J Cell Biol **125**: 437-46 (1994)
128. Liu, Q., Li, J.G., Zheng, X.Y., Jin, F., and Dong, H.T.: *Expression of CD133, PAX2, ESA, and GPR30 in invasive ductal breast carcinomas.* Chin Med J (Engl) **122**: 2763-9 (2009)
129. Liu, T.J., Sun, B.C., Zhao, X.L., Zhao, X.M., Sun, T., Gu, Q., Yao, Z., Dong, X.Y., Zhao, N., and Liu, N.: *CD133+ cells with cancer stem cell characteristics associates with vasculogenic mimicry in triple-negative breast cancer.* Oncogene **32**: 544-53 (2013)
130. Louderbough, J.M. and Schroeder, J.A.: *Understanding the dual nature of CD44 in breast cancer progression.* Mol Cancer Res **9**: 1573-86 (2011)
131. Luo, M., Clouthier, S.G., Deol, Y., Liu, S., Nagrath, S., Azizi, E., and Wicha, M.S.: *Breast cancer stem cells: current advances and clinical implications.* Methods Mol Biol **1293**: 1-49 (2015)
132. Lyzak, J.S., Yaremko, M.L., Recant, W., Baunoch, D.A., and Joseph, L.: *Role of CD44 in nonpalpable T1a and T1b breast cancer.* Hum Pathol **28**: 772-8 (1997)
133. Ma, W., Deng, Y., and Zhou, L.: *The prognostic value of adhesion molecule CD44v6 in women with primary breast carcinoma: a clinicopathologic study.* Clin Oncol (R Coll Radiol) **17**: 258-63 (2005)
134. Mani, S.A., Guo, W., Liao, M.J., Eaton, E.N., Ayyanan, A., Zhou, A.Y., Brooks, M., Reinhard, F., Zhang, C.C., Shipitsin, M., Campbell, L.L., Polyak, K., Brisken, C., Yang, J., and Weinberg, R.A.: *The epithelial-mesenchymal transition generates cells with properties of stem cells.* Cell **133**: 704-15 (2008)
135. Martin, T.A. and Jiang, W.G.: *Evaluation of the expression of stem cell markers in human breast cancer reveals a correlation with clinical progression and metastatic disease in ductal carcinoma.* Oncol Rep **31**: 262-72 (2014)

136. Masedunskas, A., King, J.A., Tan, F., Cochran, R., Stevens, T., Sviridov, D., and Ofori-Acquah, S.F.: *Activated leukocyte cell adhesion molecule is a component of the endothelial junction involved in transendothelial monocyte migration.* FEBS Lett **580**: 2637-45 (2006)
137. Matsuki, H., Yonezawa, K., Obata, K., Iwata, K., Nakamura, H., Okada, Y., and Seiki, M.: *Monoclonal antibodies with defined recognition sequences in the stem region of CD44: detection of differential glycosylation of CD44 between tumor and stromal cells in tissue.* Cancer Res **63**: 8278-83 (2003)
138. Merlo, L.M., Pepper, J.W., Reid, B.J., and Maley, C.C.: *Cancer as an evolutionary and ecological process.* Nat Rev Cancer **6**: 924-35 (2006)
139. Miraglia, S., Godfrey, W., Yin, A.H., Atkins, K., Warnke, R., Holden, J.T., Bray, R.A., Waller, E.K., and Buck, D.W.: *A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning.* Blood **90**: 5013-21 (1997)
140. Momburg, F., Moldenhauer, G., Hammerling, G.J., and Moller, P.: *Immunohistochemical study of the expression of a Mr 34,000 human epithelium-specific surface glycoprotein in normal and malignant tissues.* Cancer Res **47**: 2883-91 (1987)
141. Morris, S.F., O'Hanlon, D.M., McLaughlin, R., McHale, T., Connolly, G.E., and Given, H.F.: *The prognostic significance of CD44s and CD44v6 expression in stage two breast carcinoma: an immunohistochemical study.* Eur J Surg Oncol **27**: 527-31 (2001)
142. Mylona, E., Giannopoulou, I., Fasomytakis, E., Nomikos, A., Magkou, C., Bakarakos, P., and Nakopoulou, L.: *The clinicopathologic and prognostic significance of CD44+/CD24(-/low) and CD44-/CD24+ tumor cells in invasive breast carcinomas.* Hum Pathol **39**: 1096-102 (2008)
143. Norum, J.H., Andersen, K., and Sorlie, T.: *Lessons learned from the intrinsic subtypes of breast cancer in the quest for precision therapy.* Br J Surg **101**: 925-38 (2014)
144. Nowell, P.C.: *The clonal evolution of tumor cell populations.* Science **194**: 23-8 (1976)
145. Nunez, M.I., Arias, J.I., Del Rio, M.C., Martinez, M.I., Alba, A., Allende, M.T., and Ruibal, A.: *Cell surface CD44v5 levels correlate with progesterone receptors and a tumor size > 2 cm in infiltrating ductal carcinomas of the breast.* Int J Biol Markers **11**: 220-2 (1996)
146. Olsson, E., Honeth, G., Bendahl, P.O., Saal, L.H., Gruvberger-Saal, S., Ringner, M., Vallon-Christersson, J., Jonsson, G., Holm, K., Lovgren, K., Ferno, M., Grabau, D., Borg, A., and Hegardt, C.: *CD44 isoforms are heterogeneously expressed in breast cancer and correlate with tumor subtypes and cancer stem cell markers.* BMC Cancer **11**: 418 (2011)
147. Osborne, C.K., Wakeling, A., and Nicholson, R.I.: *Fulvestrant: an oestrogen receptor antagonist with a novel mechanism of action.* Br J Cancer **90 Suppl 1**: S2-6 (2004)
148. Pannuti, A., Foreman, K., Rizzo, P., Osipo, C., Golde, T., Osborne, B., and Miele, L.: *Targeting Notch to target cancer stem cells.* Clin Cancer Res **16**: 3141-52 (2010)
149. Park, S.Y., Lee, H.E., Li, H., Shipitsin, M., Gelman, R., and Polyak, K.: *Heterogeneity for stem cell-related markers according to tumor subtype and histologic stage in breast cancer.* Clin Cancer Res **16**: 876-87 (2010)

150. Patriarca, C., Macchi, R.M., Marschner, A.K., and Mellstedt, H.: *Epithelial cell adhesion molecule expression (CD326) in cancer: a short review.* Cancer Treat Rev **38**: 68-75 (2012)
151. Perou, C.M., Sorlie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Rees, C.A., Pollack, J.R., Ross, D.T., Johnsen, H., Akslen, L.A., Fluge, O., Pergamenschikov, A., Williams, C., Zhu, S.X., Lonning, P.E., Borresen-Dale, A.L., Brown, P.O., and Botstein, D.: *Molecular portraits of human breast tumours.* Nature **406**: 747-52 (2000)
152. Perrone, G., Gaeta, L.M., Zagami, M., Nasorri, F., Coppola, R., Borzomati, D., Bartolozzi, F., Altomare, V., Trodella, L., Tonini, G., Santini, D., Cavani, A., and Muda, A.O.: *In situ identification of CD44+/CD24- cancer cells in primary human breast carcinomas.* PLoS One **7**: e43110 (2012)
153. Petrelli, F., Viale, G., Cabiddu, M., and Barni, S.: *Prognostic value of different cut-off levels of Ki-67 in breast cancer: a systematic review and meta-analysis of 64,196 patients.* Breast Cancer Res Treat **153**: 477-91 (2015)
154. Phillips, T.M., McBride, W.H., and Pajonk, F.: *The response of CD24(-/low)/CD44+ breast cancer-initiating cells to radiation.* J Natl Cancer Inst **98**: 1777-85 (2006)
155. Polyak, K.: *Breast cancer: origins and evolution.* J Clin Invest **117**: 3155-63 (2007)
156. Prat, A., Parker, J.S., Fan, C., and Perou, C.M.: *PAM50 assay and the three-gene model for identifying the major and clinically relevant molecular subtypes of breast cancer.* Breast Cancer Res Treat **135**: 301-6 (2012)
157. Prat, A. and Perou, C.M.: *Deconstructing the molecular portraits of breast cancer.* Mol Oncol **5**: 5-23 (2011)
158. Rabinovich, I., Sebastiao, A.P.M., Lima, R.S., Urban, C.A., Junior, E.S., Anselmi, K.F., Elifio-Esposito, S., De Noronha, L., and Moreno-Amaral, A.N.: *Cancer stem cell markers ALDH1 and CD44+/CD24- phenotype and their prognosis impact in invasive ductal carcinoma.* Eur J Histochem **62** (2018)
159. Rakha, E.A., El-Sayed, M.E., Reis-Filho, J., and Ellis, I.O.: *Patho-biological aspects of basal-like breast cancer.* Breast Cancer Res Treat **113**: 411-22 (2009)
160. Redig, A.J. and McAllister, S.S.: *Breast cancer as a systemic disease: a view of metastasis.* J Intern Med **274**: 113-26 (2013)
161. Reya, T., Morrison, S.J., Clarke, M.F., and Weissman, I.L.: *Stem cells, cancer, and cancer stem cells.* Nature **414**: 105-11 (2001)
162. Ricardo, S., Vieira, A.F., Gerhard, R., Leitao, D., Pinto, R., Cameselle-Teijeiro, J.F., Milanezi, F., Schmitt, F., and Paredes, J.: *Breast cancer stem cell markers CD44, CD24 and ALDH1: expression distribution within intrinsic molecular subtype.* J Clin Pathol **64**: 937-46 (2011)
163. Ricci-Vitiani, L., Lombardi, D.G., Pilozzi, E., Biffoni, M., Todaro, M., Peschle, C., and De Maria, R.: *Identification and expansion of human colon-cancer-initiating cells.* Nature **445**: 111-5 (2007)
164. Sanchez Lockhart, M., Hajos, S.E., Basilio, F.M., Mongini, C., and Alvarez, E.: *Splice variant expression of CD44 in patients with breast and ovarian cancer.* Oncol Rep **8**: 145-51 (2001)
165. Sansone, P., Ceccarelli, C., Berishaj, M., Chang, Q., Rajasekhar, V.K., Perna, F., Bowman, R.L., Vidone, M., Daly, L., Nnoli, J., Santini, D., Taffurelli, M., Shih, N.N., Feldman, M., Mao, J.J., Colameco, C., Chen, J.,

DeMichele, A., Fabbri, N., Healey, J.H., Cricca, M., Gasparre, G., Lyden, D., Bonafe, M., and Bromberg, J.: *Self-renewal of CD133(hi) cells by IL6/Notch3 signalling regulates endocrine resistance in metastatic breast cancer.* Nat Commun **7**: 10442 (2016)
166. Sawaki, M., Shien, T., and Iwata, H.: *TNM classification of malignant tumors (Breast Cancer Study Group).* Jpn J Clin Oncol **49**: 228-231 (2019)
167. Schmadeka, R., Harmon, B.E., and Singh, M.: *Triple-negative breast carcinoma: current and emerging concepts.* Am J Clin Pathol **141**: 462-77 (2014)
168. Schmidt, M., Hasenclever, D., Schaeffer, M., Boehm, D., Cotarelo, C., Steiner, E., Lebrecht, A., Siggelkow, W., Weikel, W., Schiffer-Petry, I., Gebhard, S., Pilch, H., Gehrmann, M., Lehr, H.A., Koelbl, H., Hengstler, J.G., and Schuler, M.: *Prognostic effect of epithelial cell adhesion molecule overexpression in untreated node-negative breast cancer.* Clin Cancer Res **14**: 5849-55 (2008)
169. Schmidt, M., Petry, I.B., Bohm, D., Lebrecht, A., von Torne, C., Gebhard, S., Gerhold-Ay, A., Cotarelo, C., Battista, M., Schormann, W., Freis, E., Selinski, S., Ickstadt, K., Rahnenfuhrer, J., Sebastian, M., Schuler, M., Koelbl, H., Gehrmann, M., and Hengstler, J.G.: *Ep-CAM RNA expression predicts metastasis-free survival in three cohorts of untreated node-negative breast cancer.* Breast Cancer Res Treat **125**: 637-46 (2011)
170. Schneider, B.P., Winer, E.P., Foulkes, W.D., Garber, J., Perou, C.M., Richardson, A., Sledge, G.W., and Carey, L.A.: *Triple-negative breast cancer: risk factors to potential targets.* Clin Cancer Res **14**: 8010-8 (2008)
171. Scholzen, T. and Gerdes, J.: *The Ki-67 protein: from the known and the unknown.* J Cell Physiol **182**: 311-22 (2000)
172. Schumacher, U., Horny, H.P., Horst, H.A., Herrlich, P., and Kaiserling, E.: *A CD44 variant exon 6 epitope as a prognostic indicator in breast cancer.* Eur J Surg Oncol **22**: 259-61 (1996)
173. Seimetz, D., Lindhofer, H., and Bokemeyer, C.: *Development and approval of the trifunctional antibody catumaxomab (anti-EpCAM x anti-CD3) as a targeted cancer immunotherapy.* Cancer Treat Rev **36**: 458-67 (2010)
174. Senbanjo, L.T. and Chellaiah, M.A.: *CD44: A Multifunctional Cell Surface Adhesion Receptor Is a Regulator of Progression and Metastasis of Cancer Cells.* Front Cell Dev Biol **5**: 18 (2017)
175. Senkus, E., Kyriakides, S., Penault-Llorca, F., Poortmans, P., Thompson, A., Zackrisson, S., and Cardoso, F.: *Primary breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up.* Ann Oncol **24 Suppl 6**: vi7-23 (2013)
176. Sheridan, C., Kishimoto, H., Fuchs, R.K., Mehrotra, S., Bhat-Nakshatri, P., Turner, C.H., Goulet, R., Jr., Badve, S., and Nakshatri, H.: *CD44+/CD24- breast cancer cells exhibit enhanced invasive properties: an early step necessary for metastasis.* Breast Cancer Res **8**: R59 (2006)
177. Shipitsin, M., Campbell, L.L., Argani, P., Weremowicz, S., Bloushtain-Qimron, N., Yao, J., Nikolskaya, T., Serebryiskaya, T., Beroukhim, R., Hu, M., Halushka, M.K., Sukumar, S., Parker, L.M., Anderson, K.S., Harris, L.N., Garber, J.E., Richardson, A.L., Schnitt, S.J., Nikolsky, Y., Gelman, R.S., and Polyak, K.: *Molecular definition of breast tumor heterogeneity.* Cancer Cell **11**: 259-73 (2007)

178. Singh, S.K., Hawkins, C., Clarke, I.D., Squire, J.A., Bayani, J., Hide, T., Henkelman, R.M., Cusimano, M.D., and Dirks, P.B.: *Identification of human brain tumour initiating cells.* Nature **432**: 396-401 (2004)
179. Skibinski, A. and Kuperwasser, C.: *The origin of breast tumor heterogeneity.* Oncogene **34**: 5309-16 (2015)
180. Sleeman, K.E., Kendrick, H., Robertson, D., Isacke, C.M., Ashworth, A., and Smalley, M.J.: *Dissociation of estrogen receptor expression and in vivo stem cell activity in the mammary gland.* J Cell Biol **176**: 19-26 (2007)
181. Smith, G.H.: *Stem cells, hormones, and mammary cancer.* Adv Exp Med Biol **617**: 69-78 (2008)
182. Sneath, R.J. and Mangham, D.C.: *The normal structure and function of CD44 and its role in neoplasia.* Mol Pathol **51**: 191-200 (1998)
183. Sorlie, T., Perou, C.M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Thorsen, T., Quist, H., Matese, J.C., Brown, P.O., Botstein, D., Lonning, P.E., and Borresen-Dale, A.L.: *Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications.* Proc Natl Acad Sci U S A **98**: 10869-74 (2001)
184. Sowter, H.M. and Ashworth, A.: *BRCA1 and BRCA2 as ovarian cancer susceptibility genes.* Carcinogenesis **26**: 1651-6 (2005)
185. Soysal, S.D., Muenst, S., Barbie, T., Fleming, T., Gao, F., Spizzo, G., Oertli, D., Viehl, C.T., Obermann, E.C., and Gillanders, W.E.: *EpCAM expression varies significantly and is differentially associated with prognosis in the luminal B HER2(+), basal-like, and HER2 intrinsic subtypes of breast cancer.* Br J Cancer **108**: 1480-7 (2013)
186. Spizzo, G., Gastl, G., Wolf, D., Gunsilius, E., Steurer, M., Fong, D., Amberger, A., Margreiter, R., and Obrist, P.: *Correlation of COX-2 and Ep-CAM overexpression in human invasive breast cancer and its impact on survival.* Br J Cancer **88**: 574-8 (2003)
187. Spizzo, G., Obrist, P., Ensinger, C., Theurl, I., Dunser, M., Ramoni, A., Gunsilius, E., Eibl, G., Mikuz, G., and Gastl, G.: *Prognostic significance of Ep-CAM AND Her-2/neu overexpression in invasive breast cancer.* Int J Cancer **98**: 883-8 (2002)
188. Spizzo, G., Went, P., Dirnhofer, S., Obrist, P., Simon, R., Spichtin, H., Maurer, R., Metzger, U., von Castelberg, B., Bart, R., Stopatschinskaya, S., Kochli, O.R., Haas, P., Mross, F., Zuber, M., Dietrich, H., Bischoff, S., Mirlacher, M., Sauter, G., and Gastl, G.: *High Ep-CAM expression is associated with poor prognosis in node-positive breast cancer.* Breast Cancer Res Treat **86**: 207-13 (2004)
189. Storci, G., Sansone, P., Trere, D., Tavolari, S., Taffurelli, M., Ceccarelli, C., Guarnieri, T., Paterini, P., Pariali, M., Montanaro, L., Santini, D., Chieco, P., and Bonafe, M.: *The basal-like breast carcinoma phenotype is regulated by SLUG gene expression.* J Pathol **214**: 25-37 (2008)
190. Tan, F., Mosunjac, M., Adams, A.L., Adade, B., Taye, O., Hu, Y., Rizzo, M., and Ofori-Acquah, S.F.: *Enhanced down-regulation of ALCAM/CD166 in African-American Breast Cancer.* BMC Cancer **14**: 715 (2014)
191. Tandon, A.K., Clark, G.M., Chamness, G.C., and McGuire, W.L.: *Association of the 323/A3 surface glycoprotein with tumor characteristics and behavior in human breast cancer.* Cancer Res **50**: 3317-21 (1990)

192. Tiezzi, D.G., Valejo, F.A., Marana, H.R., Carrara, H.H., Benevides, L., Antonio, H.M., Sicchieri, R.D., Milanezi, C.M., Silva, J.S., and de Andrade, J.M.: *CD44+/CD24- cells and lymph node metastasis in stage I and II invasive ductal carcinoma of the breast.* Med Oncol **29**: 1479-85 (2012)
193. Tokue, Y., Matsumura, Y., Katsumata, N., Watanabe, T., Tarin, D., and Kakizoe, T.: *CD44 variant isoform expression and breast cancer prognosis.* Jpn J Cancer Res **89**: 283-90 (1998)
194. Trichopoulos, D., Adami, H.O., Ekbom, A., Hsieh, C.C., and Lagiou, P.: *Early life events and conditions and breast cancer risk: from epidemiology to etiology.* Int J Cancer **122**: 481-5 (2008)
195. Tsunoda, Y., Sakamoto, M., Sawada, T., Sasaki, A., Yamamoto, G., and Tachikawa, T.: *Characteristic genes in luminal subtype breast tumors with CD44+CD24-/low gene expression signature.* Oncology **81**: 336-44 (2011)
196. van der Gun, B.T., Melchers, L.J., Ruiters, M.H., de Leij, L.F., McLaughlin, P.M., and Rots, M.G.: *EpCAM in carcinogenesis: the good, the bad or the ugly.* Carcinogenesis **31**: 1913-21 (2010)
197. Visvader, J.E.: *Cells of origin in cancer.* Nature **469**: 314-22 (2011)
198. Wang, H., Wang, L., Song, Y., Wang, S., Huang, X., Xuan, Q., Kang, X., and Zhang, Q.: *CD44(+)/CD24(-) phenotype predicts a poor prognosis in triple-negative breast cancer.* Oncol Lett **14**: 5890-5898 (2017)
199. Wang, Y., Chen, M., Wu, Z., Tong, C., Dai, H., Guo, Y., Liu, Y., Huang, J., Lv, H., Luo, C., Feng, K.C., Yang, Q.M., Li, X.L., and Han, W.: *CD133-directed CAR T cells for advanced metastasis malignancies: A phase I trial.* Oncoimmunology **7**: e1440169 (2018)
200. Wei, X.L., Dou, X.W., Bai, J.W., Luo, X.R., Qiu, S.Q., Xi, D.D., Huang, W.H., Du, C.W., Man, K., and Zhang, G.J.: *ERalpha inhibits epithelial-mesenchymal transition by suppressing Bmi1 in breast cancer.* Oncotarget **6**: 21704-17 (2015)
201. Weichert, W., Knosel, T., Bellach, J., Dietel, M., and Kristiansen, G.: *ALCAM/CD166 is overexpressed in colorectal carcinoma and correlates with shortened patient survival.* J Clin Pathol **57**: 1160-4 (2004)
202. Weinstein, B.: *Relevance of the concept of oncogene addiction to hormonal carcinogenesis and molecular targeting in cancer prevention and therapy.* Adv Exp Med Biol **617**: 3-13 (2008)
203. Went, P.T., Lugli, A., Meier, S., Bundi, M., Mirlacher, M., Sauter, G., and Dirnhofer, S.: *Frequent EpCam protein expression in human carcinomas.* Hum Pathol **35**: 122-8 (2004)
204. Wiiger, M.T., Gehrken, H.B., Fodstad, O., Maelandsmo, G.M., and Andersson, Y.: *A novel human recombinant single-chain antibody targeting CD166/ALCAM inhibits cancer cell invasion in vitro and in vivo tumour growth.* Cancer Immunol Immunother **59**: 1665-74 (2010)
205. Willett, W.C., Tamimi, R.M., Hankinson, S., Hazra, A., Eliassen, A.H., and Colditz, G.A.: *Chapter 18: nongenetic factors in the causation of breast cancer.* . In: Harris, J.R., Lippman, M.E., Morrow, M., and Osborne, C.K. (Ed.): Diseases of the breast. 211-267, Wolters Kluwer Health, Philadelphia, (2014)

206. Wockel, A., Festl, J., Stuber, T., Brust, K., Stangl, S., Heuschmann, P.U., Albert, U.S., Budach, W., Follmann, M., Janni, W., Kopp, I., Kreienberg, R., Kuhn, T., Langer, T., Nothacker, M., Scharl, A., Schreer, I., Link, H., Engel, J., Fehm, T., Weis, J., Welt, A., Steckelberg, A., Feyer, P., Konig, K., Hahne, A., Kreipe, H.H., Knoefel, W.T., Denkinger, M., Brucker, S., Luftner, D., Kubisch, C., Gerlach, C., Lebeau, A., Siedentopf, F., Petersen, C., Bartsch, H.H., Schulz-Wendtland, R., Hahn, M., Hanf, V., Muller-Schimpfle, M., Henscher, U., Roncarati, R., Katalinic, A., Heitmann, C., Honegger, C., Paradies, K., Bjelic-Radisic, V., Degenhardt, F., Wenz, F., Rick, O., Holzel, D., Zaiss, M., Kemper, G., Budach, V., Denkert, C., Gerber, B., Tesch, H., Hirsmuller, S., Sinn, H.P., Dunst, J., Munstedt, K., Bick, U., Fallenberg, E., Tholen, R., Hung, R., Baumann, F., Beckmann, M.W., Blohmer, J., Fasching, P.A., Lux, M.P., Harbeck, N., Hadji, P., Hauner, H., Heywang-Kobrunner, S., Huober, J., Hubner, J., Jackisch, C., Loibl, S., Luck, H.J., von Minckwitz, G., Mobus, V., Muller, V., Nothlings, U., Schmidt, M., Schmutzler, R., Schneeweiss, A., Schutz, F., Stickeler, E., Thomssen, C., Untch, M., Wesselmann, S., Bucker, A., and Krockenberger, M.: *Interdisciplinary Screening, Diagnosis, Therapy and Follow-up of Breast Cancer. Guideline of the DGGG and the DKG (S3-Level, AWMF Registry Number 032/045OL, December 2017) - Part 1 with Recommendations for the Screening, Diagnosis and Therapy of Breast Cancer.* Geburtshilfe Frauenheilkd **78**: 927-948 (2018)
207. Wolff, A.C., Hammond, M.E., Schwartz, J.N., Hagerty, K.L., Allred, D.C., Cote, R.J., Dowsett, M., Fitzgibbons, P.L., Hanna, W.M., Langer, A., McShane, L.M., Paik, S., Pegram, M.D., Perez, E.A., Press, M.F., Rhodes, A., Sturgeon, C., Taube, S.E., Tubbs, R., Vance, G.H., van de Vijver, M., Wheeler, T.M., and Hayes, D.F.: *American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer.* J Clin Oncol **25**: 118-45 (2007)
208. Wright, M.H., Calcagno, A.M., Salcido, C.D., Carlson, M.D., Ambudkar, S.V., and Varticovski, L.: *Brca1 breast tumors contain distinct CD44+/CD24- and CD133+ cells with cancer stem cell characteristics.* Breast Cancer Res **10**: R10 (2008)
209. Xu, H., Tian, Y., Yuan, X., Liu, Y., Wu, H., Liu, Q., Wu, G.S., and Wu, K.: *Enrichment of CD44 in basal-type breast cancer correlates with EMT, cancer stem cell gene profile, and prognosis.* Onco Targets Ther **9**: 431-44 (2016)
210. Yerushalmi, R., Woods, R., Ravdin, P.M., Hayes, M.M., and Gelmon, K.A.: *Ki67 in breast cancer: prognostic and predictive potential.* Lancet Oncol **11**: 174-83 (2010)
211. Yin, A.H., Miraglia, S., Zanjani, E.D., Almeida-Porada, G., Ogawa, M., Leary, A.G., Olweus, J., Kearney, J., and Buck, D.W.: *AC133, a novel marker for human hematopoietic stem and progenitor cells.* Blood **90**: 5002-12 (1997)
212. Zardavas, D., Irrthum, A., Swanton, C., and Piccart, M.: *Clinical management of breast cancer heterogeneity.* Nat Rev Clin Oncol **12**: 381-94 (2015)
213. Zhao, P., Lu, Y., and Han, W.: *Clinicopathological significance and prognostic value of leukemia-related protein 16 expression in invasive ductal breast carcinoma.* Cancer Sci **101**: 2262-8 (2010)

214. Zhao, P., Lu, Y., Jiang, X., and Li, X.: *Clinicopathological significance and prognostic value of CD133 expression in triple-negative breast carcinoma.* Cancer Sci **102**: 1107-11 (2011)
215. Zheng, Z., Shao, N., Weng, H., Li, W., Zhang, J., Zhang, L., Yang, L., and Ye, S.: *Correlation between epidermal growth factor receptor and tumor stem cell markers CD44/CD24 and their relationship with prognosis in breast invasive ductal carcinoma.* Med Oncol **32**: 275 (2015)

8 Appendix

8.1 Ethics permit

The study was approved by the local ethical review board (Universität zu Lübeck) on March 12th, 2008; Aktenzeichen Nr. 08-012: „Erforschung von Brustkrebs für verbesserte (Früh-)Diagnose, Therapie, Nachsorge und Prognose“.

8.2 Material

Instruments

Hyrax M 55…….	Carl Zeiss, Jena, D
Cool Cut UKK…….	Carl Zeiss, Jena, D
Hyrax STS Section-Transfer-System.	Carl Zeiss, Göttingen, D
Tissue Arrayer……	Pathology Devices Inc., Westminster, USA
Pannoramic DESK ……..	3DHISTECH, Budapest, H
Phase microscope ……	Carl Zeiss Microscopy, LLC, Thornwood, USA
Incubator…….	Melag, Berlin, D
Jung SM 2000 R……	Leica Instruments GmbH, Wetzlar, D
Paraffin water bath……	Medax GmbH, Kiel, D
EMB 2000-2……	Kern & Sohn GmbH, Balingen, D
Mettler PM 3000……	Mettler-Toledo-Inc., Columbus, USA
Custom Pipette Eppendorf Reference…….. 10, 100, 1000, 2500	Eppendorf AG, Hamburg, D
PIPETMAN Classic™……...	Gilson S.A.S., Villiers le Bel, F
Centrifuge 5417 R…….	Eppendorf AG, Hamburg, D
Avanti® J-25……..	Beckman Coulter Inc., Brea, USA

Consumables

SuperFrost® plus……..	Gerhard Menzel GmbH, Braunschweig, D
SuperFrost ultra Plus®…….	Gerhard Menzel GmbH, Braunschweig, D
Cover glass 24x60 mm, 24x40mm…..	Gerhard Menzel GmbH, Braunschweig, D
Reaction tube 1.5 ml……	Greiner Bio-One BVBA/SPRL, Wemmel, BE
Pipette tips…….	Greiner Bio-One BVBA/SPRL, Wemmel, BE
Tubes 5 ml, 15 ml, 50 ml……..	Greiner Bio-One BVBA/SPRL, Wemmel, BE
Dako Pen……	Dako Cytomation A/S, Glostrup, DK

Chemicals

Meyer's Hämalaun, soure…….	Waldeck GmbH+Co. KG, Münster, D
Eosin G-solution 0.5%, diluted……	Merck KGaA, Darmstadt, D
Xylene 100%.....	J.T. Baker®, Avantor Performance Materials BV, Deventer, NL
Ethanol 100%.....	Pharmacy, UKSH, Campus Lübeck
Eukitt®…….	O. Kindler GmbH, Freiburg, D
Aquatex®…….	Merck KGaA, Darmstadt, D
Paraffin…….	Merck KGaA, Darmstadt, D

Buffer and Solutions

Dulbecco´s PBS pH 7.0-7.5 (without Ca & Mg)……..	PAA Laboratories GmbH, Pasching, AT
Target Retrieval Solution Ready-to-use……..	Dako Cytomation A/S, Glostrup, DK
Antibody Diluent with Background Reducing Components…….	Dako Cytomation A/S, Glostrup, DK

3% H_2O_2 in Methanol

9.9 ml 30% H_2O_2.....	Merck KGaA, Darmstadt, D
91.1 ml Methanol…….	Merck KGaA, Darmstadt, D

3% H_2O_2 in Antibody Diluent

9.9 ml 30% H_2O_2…….	Merck KGaA, Darmstadt, D
91.9 ml Antibody Diluent with Background Reducing Components…….	Dako Cytomation A/S, Glostrup, DK

Citrate-buffer, 10 mM, pH 6.0

2.1 g Tri- Natriumcitrat-Dihydrat…	Merck KGaA, Darmstadt, D
1000 ml deionized water	

Blocking solution:

10% Goat Serum in PBS pH 7.4

40 µl Goat Serum (normal)……	Dako Cytomation A/S, Glostrup, DK
360 µl PBS…….	PAA Laboratories GmbH, Pasching, AT

Streptavidin-Horseradish-peroxidase-solution:

1% Streptavidin-Horseradish-peroxidase in Antibody Diluent

4 µl Streptavidin/HRP…….	Dako Cytomation A/S, Glostrup, DK
396 µl Antibody Diluent with Background Reducing Components…….	Dako Cytomation A/S, Glostrup, DK

Kits

Vectastain® Elite ABC Universal Kit..	Biologo, Dr. Hartmut Schultheiß, Kronshagen, D

Universal Färbekit AEC……. Biologo, Dr. Hartmut Schultheiß, Kronshagen, D
Biotin/Streptavidin-System…….. Biologo, Dr. Hartmut Schultheiß, Kronshagen, D
Liquid DAB+ Substrate Chromogen.. System Dako Cytomation A/S, Glostrup, DK

Software

NEXUS/PAS
NEXUS/Pathologie…… NEXUS AG, Villingen-Schwenningen, D
Microsoft® Office Excel®……. Microsoft® Corporation, Redmond, USA
IBM® SPSS®……. IBM®, Armonk, USA

Antibodies

Primary Antibody	Clone	Host	Specificity	Manufacturer
Anti-CD 326 (IgG1)	monoclonal, Ber-EP4	Mouse	Human	Dako Cytomation A/S, Glostrup, DK (M0804)
Anti-CD 166 (IgG2a)	monoclonal, MOG/07	Mouse	Human	Abcam®, Cambridge, UK (ab49496)
CD 133 (IgG1)	monoclonal, AC133	Mouse	Human	Miltenyi Biotec Inc., Auburn, USA (130-090-422)
CD 44 (IgG2b)	monoclonal, G44-26	Mouse	Human	BD Biosciences, Franklin Lakes, USA (550392)

Table 13 Primary antibodies used in the immunohistochemistry staining

Secondary Antibody	Dilution	Host	Specificity	Manufacturer
Goat Anti Mouse-IgG	1:100	Goat	Mouse - IgG	Dako Cytomation A/S, Glostrup, DK
Horse Anti Mouse-IgG	1:50	Horse	Mouse - IgG	Biologo, Kronshagen, D

Table 14 Secondary antibodies used in the immunohistochemistry staining.

8.3 Protocols

Protocol No.1: Haematoxylin and Eosin staining

1) 3x Xylene, 5 min. each
2) Descending ethanol series: 2x 100 % each 3 min.
   2x 96 % each 3 min.
   1x 70 % 3 min.
3) Wash with deionized water
4) Mayer's Haematoxylin 5 min.
5) Wash with running tab water 10 min.
6) Eosin 0,5% 20-40 sec.
7) Wash briefly with tab water
8) Ascending ethanol series: 1x 70 % for 1 min
   2x 96 % each 3 min.
   2x 100 % each 3 min.
9) Wash briefly 3x in Xylene
10) Cover with Eukitt

Protocol No. 2: Immunohistochemistry staining for estrogen and progesterone receptors, Her2-neu and Ki67

1) 3x Xylene, 10 min. each
2) Descending ethanol series: 2x 99.9%, wash briefly
   1x 96%, wash briefly
   1x 70%, wash briefly
3) Wash for 5 min. with Tris-buffer (pH 7.2-7.6) + Tween 0.3%
4) Heat samples in antibody specific buffer in microwave oven at 850 watts for 5 min. and 2x at 150 watts for 15 min.
5) Wash in Tris-buffer
6) Automated stainer (Dako Stainer) is loaded with primary and secondary antibodies, blocking reagent, haematoxylin and chromogen
7) Start the automated staining program and wait for completion of the process
8) Briefly wash with tab water
9) Ascending ethanol series: 1x 70%, wash thoroughly
   1x 96%, wash thoroughly
   2x 99.9%, wash thoroughly
10) 1x 100% isopropyl alcohol
11) Wash thoroughly 3x in xylene
12) Start automated covering program and wait for completion of the process

Protocol No. 3: Fluorescence in-situ hybridization for Her2-neu

1) 2x Xylene, 10 min. each
2) 2x Xylene, 5 min each
3) Wash thoroughly in 100% ethanol

4) Descending ethanol series: 1x 100%, 2 min.
   1x 85%, 2 min.
   1x 70%, 2 min.
5) Wash in distilled water for 5 min.

6) Heat samples in boiling 1mM EDTA-buffer in pressure cooker at maximum pressure for 2 min.
7) Cool with tab water and wash samples in distilled water for 2 min.
8) Samples are incubated for 30 min. at 37°C in 200 ml of 1% HCL solution + 1ml
pepsin
9) Wash in distilled water for 1 min.
10) Fix samples in 1% paraformaldehyde/PBS for 2 min.
11) Wash in distilled water for 1 min.
12) Ascending ethanol series: 1x 70%, 2 min.
1x 85%, 2 min.
1x 100%, 2 min.
13) Dry for 10 min.
14) Add 2 µl Vysis probe to area of interest
15) Cover with cover glass and seal with rubber cement
16) Incubate in wet chamber at 80°C for 30 min.
17) Incubate at 37°C for 72 h

Protocol No. 4: Immunohistochemistry staining for CD166 (ALCAM)

1) 3x Xylene, 10 min. each
2) Descending ethanol series: 2x 100%, 3 min.
2x 96%, 3 min.
1x 70%, 3 min.
3) Wash 3x in deionized water for 3 min. each
4) Heat in citrate-buffer in a microwave oven at 900 watts for 5 min. and at 360 watts for 2x 7 min.
5) Let samples cool down and wash 3x in deionized water for 3 min. each
6) Incubate samples with 3% H_2O_2 in Antibody Diluent at room temperature for 10 min.
7) Wash 3x in PBS for 5 min. each
8) Incubate with 10% goat serum for 30 min.
9) Incubate with CD166 antibody (1:100) at 4°C for 12 h in wet chamber
10) Wash 3x in PBS for 5 min.
11) Incubate with secondary antibody (goat-anti-mouse) for 30 min. at room temperature
12) Wash 3x in PBS for 5 min.
13) Incubate with streptavidin-horseradish-peroxidase solution for 30 min. at room
14) temperature
15) Wash 3x in PBS for 3 min.
16) Incubate with Universal Färbekit AEC chromophore for 6 min.
17) Wash with tab water for 10 min.
18) Stain with Haematoxylin for 1.5 min.
19) Wash with tab water for 10 min
20) Cover with cover glass

Protocol No. 5: Immunohistochemistry staining for CD44

1) 3x Xylene, 10 min. each
   Descending ethanol series: 2x 100%, 3 min.
   2x 96%, 3 min.
   1x 70%, 3 min.
2) Wash 3x in deionized water for 3 min. each
3) Heat in citrate-buffer in a microwave oven at 900 watts for 5 min. and at 360 watts for 2x 7 min.
4) Let samples cool down and wash 3x in deionized water for 3 min. each
5) Incubate samples with 3% H_2O_2 in methanol at room temperature for 10 min.
6) Wash 3x in PBS for 3 min. each
7) Incubate with blocking solution of Vectastain® Elite ABC Universal Kit for 30 min.
8) Incubate with CD44 antibody (1:150) at 4°C for 12 h in wet chamber
9) Wash 3x in PBS for 3 min.
10) Incubate with secondary antibody (horse-anti-mouse) of Vectastain® Elite ABC Universal Kit for 30 min. at room temperature
11) Wash 3x in PBS for 3 min.
12) Incubate with streptavidin-horseradish-peroxidase solution of Vectastain® Elite ABC Universal Kit for 30 min. at room temperature
13) Wash 3x in PBS for 3 min.
14) Incubate with Universal Färbekit AEC chromophore for 6 min.
15) Wash with tab water for 10 min.
16) Stain with Haematoxylin for 1.5 min.
17) Wash with tab water for 10 min
18) Cover with cover glass

Protocol No. 6: Immunohistochemistry staining for CD133

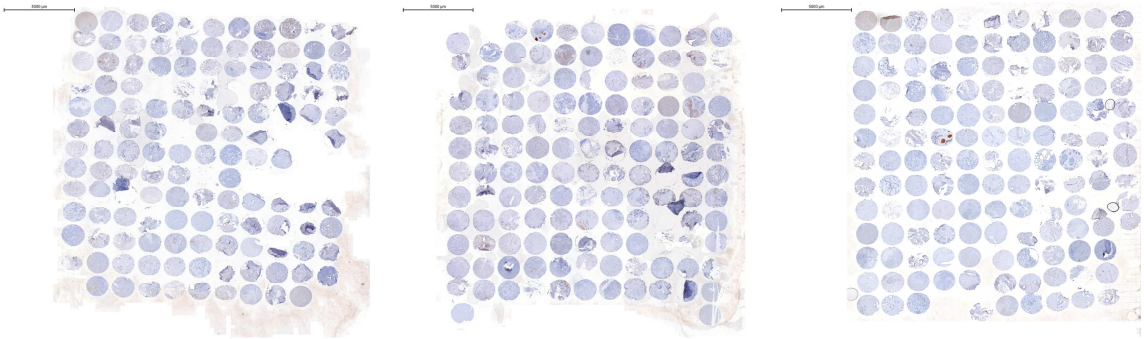
1) 2x Xylene, 5 min. each
2) Descending ethanol series: 2x 100%, 2 min.
   2x 96%, 2 min.
   2x 80%, 2 min.
   2x 70%, 2 min.
3) Wash in PBS for 5 min.
4) Heat in citrate-buffer in a microwave oven at 900 watts for 5 min. and at 360 watts for 2x 7 min.
5) Let samples cool down and wash in deionized water for 5 min.
6) Wash in PBS for 5 min.
7) Incubate samples with 3% H_2O_2 in Antibody Diluent at room temperature for 10 min.
8) Wash 2x in PBS for 3 min. each
9) Incubate with blocking solution of Vectastain® Elite ABC Universal Kit for 20 min.
10) Incubate with CD133 antibody (1:10) at 4°C for 12 h in wet chamber
11) Wash 2x in PBS for 3 min.
12) Incubate with secondary antibody (horse-anti-mouse) of Vectastain® Elite ABC Universal Kit for 30 min. at room temperature
13) Wash 2x in PBS for 3 min.
14) Incubate with streptavidin-horseradish-peroxidase solution of Vectastain® Elite ABC Universal Kit for 30 min. at room temperature

15) Wash 2x in PBS for 3 min.
16) Incubate with Liquid DAB for 10 min.
17) Wash with tab water for 10 min.
18) Stain with Haematoxylin for 1.5 min.
19) Wash with tab water for 10 min.
20) Cover with cover glass

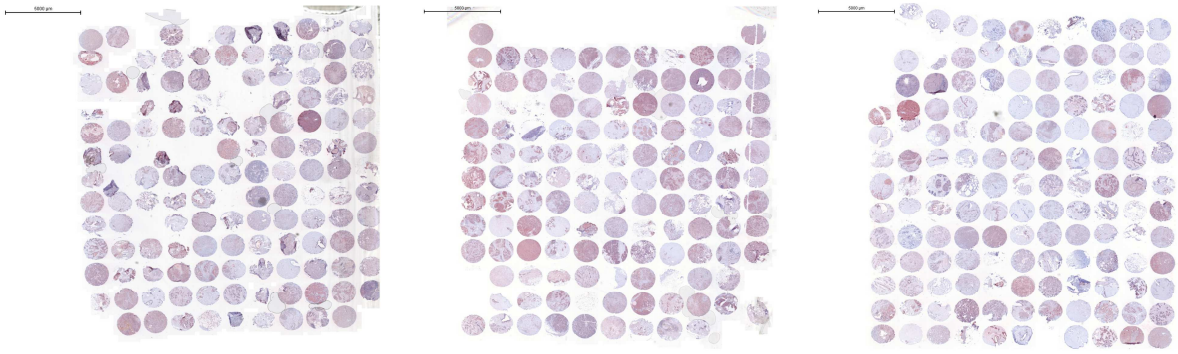
Protocol No. 7: Immunohistochemistry staining for CD326 (EpCAM)

1) 3x Xylene, 10 min. each
2) Descending ethanol series: 2x 100%, 3 min.
   2x 96%, 3 min.
   1x 70%, 3 min.
3) Wash 3x in deionized water for 3 min.
4) Heat in Antibody Retrieval solution in a microwave oven at 900 watts for 4x 5 min.
5) Let samples cool down and wash in PBS 3x for 3 min.
6) Incubate samples with 3% H_2O_2 in Antibody Diluent at room temperature for 10 min.
7) Wash 3x in PBS for 3 min. each
8) Incubate with blocking solution of Vectastain® Elite ABC Universal Kit for 10 min.
9) Incubate with CD326 antibody (1:50) at 4°C for 12 h in wet chamber
10) Wash 3x in PBS for 3 min.
11) Incubate with secondary antibody (horse-anti-mouse) of Vectastain® Elite ABC Universal Kit for 30 min. at room temperature
12) Wash 3x in PBS for 5 min.
13) Incubate with streptavidin-horseradish-peroxidase solution of Vectastain® Elite ABC Universal Kit for 30 min. at room temperature
14) Wash 3x in PBS for 3 min.
15) Incubate with Universal Färbekit AEC for 7 min.
16) Wash with tab water for 10 min.
17) Stain with Haematoxylin for 1.5 min.
18) Wash with tab water for 10 min.
19) Cover with cover glass

8.4 Supplementary Figures

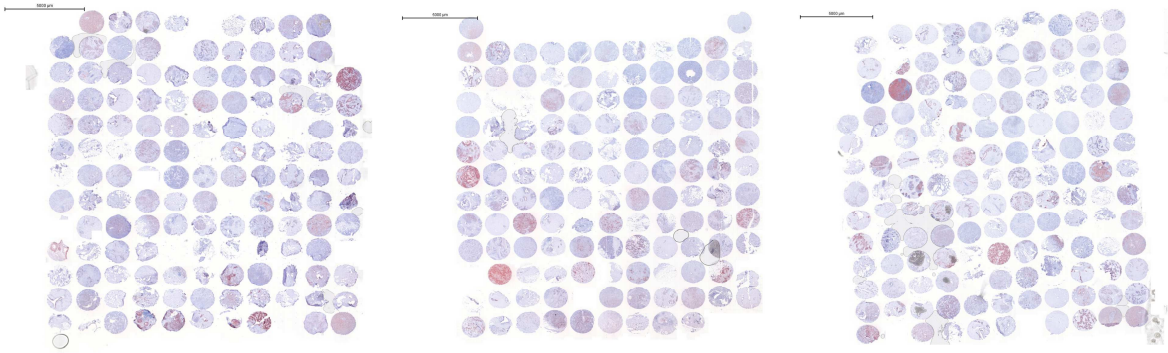


Supplementary Figure 1 Overview of the three TMA sections stained for CD133. The scale measures 5000 µm.



Supplementary Figure 2 Overview of the three TMA sections stained for CD44. The scale measures 5000 µm.

Supplementary Figure 3 Overview of the three TMA sections stained for EpCAM. The scale measures 5000 µm.



Supplementary Figure 4 Overview of the three TMA sections stained for CD166. The scale measures 5000 µm.

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10 Curriculum vitae

Hendrik Alkemade

Alter: 35 Jahre

Medical career:

Since August 2015	Residency, Department of Anesthesiology and Intensive Care Medicine, University Medical Center Schleswig-Holstein, Campus Lübeck (Director: Prof. Dr. med. Carla Nau)

University and Highschool Education:

May 2014	Second Medical Boards Exam (Zweite Ärztliche Prüfung, Universität zu Lübeck)
March 2009	First Medical Boards Exam (Erste Ärztliche Prüfung, Universität Witten/Herdecke)
February 2013 - January 2014	Clinical Electives (Praktisches Jahr, Universität zu Lübeck, King's College London, University of Melbourne)
June 2005	Abitur (Johannes-Keppler-Gymnasium Leonberg)

Scientific training and doctoral thesis:

Since February 2010	Section of Translational Surgical Oncology and Biobanking (Universität zu Lübeck)
February - September 2012	Section of Cancer Genomics (National Institutes of Health, USA)

Scholarships:

04/2010 – 08/2012	Doctoral student scholarship from the Ad Infinitum Foundation

11 Publications

Paper

1. Oberländer, M., Alkemade, H., Bünger, S., Ernst, F., Thorns, C., Braunschweig, T., Habermann, J.K.: *A 'waterfall' transfer-based workflow for improved quality of tissue microarray construction and processing in breast cancer research.* Pathol Oncol Res 20: 719-726 (2014)
2. Alkemade, H., Tübing, J., Freitag-Wolf, S., Bünger, S., Ernst, F., Schlesiger, S., Oberländer, M., Fritzsche, B., Thorns, C., Görg, S., Lindner, U., Rody, A., Widengren, J., Auer, G., Ried, T., Gemoll, T., Habermann, J.K.: *Stem cell markers predict prognosis in breast cancer overall (EpCAM), and in subgroups of large (CD133), triple-negative (CD44), and hormone receptor positive carcinomas (CD166).* Manuscript / in submission

Poster

1. Alkemade, H., Ernst, F., Bünger, S., Oberländer, M., Thorns, C., Gemoll, T., Habermann, J.K.: *Krebsstammzellen: Die Suche nach einem Therapieziel für Brustkrebs an der Wurzel allen Übels.* Poster at the doctoral students day, June 10th 2015