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The Function of Hyaluronic Acid Metabolism in the Development of Breast Cancer Brain Metastases

Dissertation

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TABLE OF CONTENTS

1 INTRODUCTION

1.1 BREAST CANCER

Breast cancer is the most common cancer in women worldwide. In Germany about 70.000 new cases in women and about 610 new cases in men are diagnosed each year. In comparison to other tumours, breast cancer often occurs at a young age. One in four women is under 55 and one in ten women is under 45 years old at the time of diagnosis. Each year about 24 of 100.000 women die of breast cancer (Robert-Koch-Instutute, 2010). Most breast cancer related deaths are due to metastases, which most frequently spread to the liver, lung, bone and brain (Hu et al., 2009). The characteristics of breast cancer, including invasiveness and aggressiveness, and the outcome depend on the histopathological and molecular subtype as well as on the tumour grade and stage at the time of diagnosis.

1.2 BREAST CANCER CLASSIFICATIONS

Breast cancer is a heterogeneous disease with respect to its histopathological type, tumour stage, tumour grade and molecular subtype (Parker et al., 2009). The two major histopathological breast cancer types, the ductal and lobular carcinoma, are further divided into in situ and invasive carcinomas. The invasive ductal carcinoma is the most common subtype with 70-80% of all invasive breast cancers (Breast-Cancer.org, 2013). Tumour grading and staging are pathological and clinical parameters, which describe the magnitude of tumour development at time of diagnosis and are used as predicting factors for treatment options and outcome (Cancer-Research-UK, 2012). However, patient outcome and tumour cell response to treatment is not only determined by the tumour type, stage, and grade but also by its molecular subtype. On the basis of gene expression, breast tumours are classified in molecular subtypes, which are simulated via immunohistochemistry (IHC) in clinical routine. Here hormone receptors, such as oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), as well as markers like antigen KI67 (KI67), cytokeratin (CK), and epidermal growth factor receptor (EGFR) play an important role (Dai et al., 2015). While past research has detected the

diversity and intricacy of the molecular characteristics in breast cancer cells (Blows et al., 2010), Sorlie et al. emphasised five intrinsic breast cancer subtypes with distinct clinical outcomes. These subtypes are the luminal A, luminal B, HER2 enriched, basal-like, and normal-like subtype (Perou et al., 2000, Sorlie et al., 2001). The IHC status, the associated tumour grade, and outcome as well as the prevalence of these subtypes are shown in Table 1.

Table 1: Molecular subtypes of breast cancer. Detailed IHC expression patterns, tumour grade, outcome, and prevalence. Data from this table are taken and altered from the the listes publications (Badve et al., 2011b, Cheang et al., 2009, Dai et al., 2015, Smid et al., 2008).

Molecular subtypes	IHC status	Grade	Outcome	Prevalence
Luminal A	ER+, PR+, HER2-, KI67-	1-2	good	23,7%
Luminal B	ER+, PR+, HER2-, KI67+	2-3	intermediate	38,8%
	ER+, PR+, HER2+, KI67+		poor	14%
HER2 enriched	ER-, PR-, HER2+	2-3	poor	11,2%
Basal-like	ER-, PR-, HER2- CK5/6+, and/or CK14+, and/or CK17+ ER-, HER2-, CK5/6+ and/or EGFR ER-, PR-, HER2-, CK5/6+ and/or EGFR+	3	poor	12,3%
Normal-like	ER+, PR+, HER2-, KI67-	1-3	intermediate	7,8%

Furthermore, Badve et al. highlighted the differences between the triple negative subtype, which is defined by negativity of ER, PR, and HER2, and the basal-like subtype (Badve et al., 2011a). While basal-like tumours account for 60-90% of triple negative cases (Fan et al., 2006), basal-like tumour have a more complex IHC definition as shown in table 1. Hence, these two subtypes are individual and should not be treated as one entity. As mentioned before, the intrinsic subtypes differ in treatment options and patient outcome. Patients with a luminal A subtype have a more favourable outcome than patients with a luminal B subtype (Sorlie et al., 2003). Both

luminal A and B respond well to hormone therapy, however poorly to chemotherapy (Brenton et al., 2005). Moreover, HER2-enriched tumours are often of high grade and are associated with poor prognosis (Sorlie et al., 2001, Sorlie et al., 2003, Sotiriou et al., 2003). However, they respond to chemotherapy and can be treated with anti-HER2 monoclonal antibody trastuzumab (Brenton et al., 2005). Similar to HER2-enriched tumours, basal-like tumours are likely to be of high grade and follow an aggressive clinical course (O'Brien et al., 2010, Sorlie et al., 2001). Compared with other molecular subtypes, basal-like tumours more often occur in young patients, in African-American woman, and pre-menopausal women (Carey et al., 2006). Interestingly, HER2-enriched and basal-like tumours are associated with aggressiveness, invasiveness and subsequent brain metastasis (Berghoff et al., 2012, Hung et al., 2014).

1.3 METASTASES

Metastases from breast carcinoma distribute mainly haematogenous in a process consisting of multiple steps as shown in figure 1. While the basement membrane is intact in the in situ carcinoma, it is broken down in the invasive carcinoma. This allows tumour cells to invade the surrounding breast tissue and to interact with the tumour microenvironment (TME) (Guo and Giancotti, 2004). The TME, an active participant in tumourigenesis, promotes several hallmarks of cancer such as angiogenesis, invasiveness, and resistance to apoptosis (Hanahan and Weinberg, 2011). The tumour cell itself undergoes a process called epithelial-mesenchymal transition (EMT), where cell polarity and cell-cell adhesion is lost, facilitating the separation of tumour cells from their primary mass and the migration towards blood vessels (Moreno-Bueno et al., 2008). For the process of tumour cell intravasation, factors play a role that cause the disruption of the endothelial basement membrane and allow tumour cells to enter the circulation. During transport through circulation, tumour cells use intelligent mechanisms such as tumour immune evasion, where tumour cells suppress the immune system in order to enhance their own survival, until they have reached the target tissue (Zindl and Chaplin, 2010). Rolling, tethering, loose and tight adhesion to endothelial cells facilitates extravasation and tumour cells migrate into the new organ.

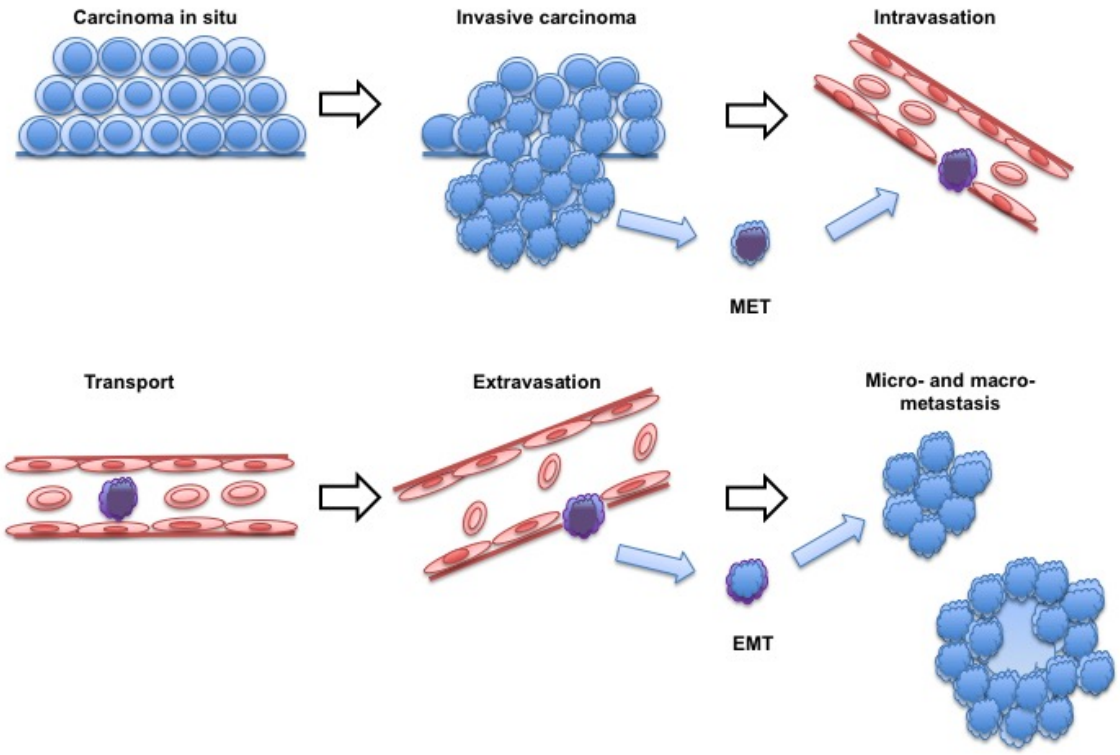

Figure 1: Process of metastasis. MET = mesenchymal-epithelial transition; EMT = epithelial-mesenchymal transition. The content of this figure is derived from the work of Moreno-Bueno et al., Nguyen et al., and Zindl and Chaplin (Moreno-Bueno et al., 2008, Nguyen et al., 2009, Zindl and Chaplin, 2010).

Via mesenchymal-epithelial transition (MET), tumour cells regain their original properties and form micro-and macro metastasis in their new environment (Nguyen et al., 2009) (Fig. 1). Breast cancer secondary growth is not random. In fact, breast cancer cells only grow in certain organs, which provide a more fertile environment for secondary tumour growth than others. This phenomenon agrees with the “seed and soil theory”, which has been announced by Paget 1889. The theory claims that a metastatic tumour cell (the seed) needs certain fertile tissue (the soil) for successful implantation and proliferation (Paget, 1989). While it remains elusive which factors lead to the secondary growth pattern in breast cancer, it is known that breast cancer most often metastasises to the bone, lung, liver, and brain (National-Cancer-Institue, 2013). Interestingly, the pattern of breast cancer metastases varies by hormone receptor status. Kennecke et al. stated that HER2-enriched tumours are associated with significantly higher rates of metastases to the brain, liver, and lung in comparison

to other breast cancer subtypes. Moreover, this study revealed that basal-like tumours are associated with significantly higher rates of brain, lung, and lymphatic node metastases and lower rates of bone and liver metastases in comparison to the other subtypes (Kennecke et al., 2010).

1.4 THE BLOOD-BRAIN BARRIER AND METASTASES TO THE BRAIN

In the event of metastasis to the brain, tumour cells have to overcome a special form of resistance, the blood-brain barrier (BBB). It forms a neurovascular unit, consisting of brain microvascular endothelial cells, connected via tight- and adherence junctions, pericytes, astrocytes, and brain extracellular matrix (ECM). This interface between blood circulation and central nervous system, with its highly selective permeability, allows a bidirectional control of passage of nutrients, electrolytes, toxins, regulatory proteins, and many other substances (Arshad et al., 2010). In breast cancer the BBB is a challenging demand on clinicians as it prevents effective delivery of chemotherapy, however cannot stop tumour cells from invasion (Hu et al., 2009). In fact, 10-43% of patients with metastatic breast cancer develop brain metastases and the prevalence has increased over the past years due to improved systemic treatment for stage IV patients (Hung et al., 2014). Of the breast cancer brain metastases (BCBM), 78% are multiple, 14% are solitary, and 8 % are leptomeningeal (Lin et al., 2004, Weil et al., 2005). It has been shown that chances of BCBM are higher in younger, premenopausal patients and in patients with ER-negative, PR-negative, but HER2-positive primary tumours (Pestalozzi et al., 2006, Tham et al., 2006). Furthermore, studies reported, that BCBM develop more likely and earlier in patients with triple-negative tumours than in others, who subsequently experience shorter overall survival (Dawood et al., 2009, Heitz, 2008). Interestingly, HER2-positive tumours have been reported to metastasise to the brain more frequently. Here, systemic metastases are still responding to trasuzumab treatment, while BCBM become manifest (Bendell et al., 2003, Burstein et al., 2005, Clayton et al., 2004). This is attributed to the fact that trastuzumab is not able to cross the BBB (Pestalozzi and Brignoli, 2000). It has become clear that BCBM constitute a difficult clinical problem and BCBM are considered to be the main cause of death in more than half of the patients (Pestalozzi et al., 2008). More specific, the median survival of BCBM patients

has been reported to be 4.6 months, and only 20-40% of patients were alive one year after diagnosis (Dawood et al., 2008, Pestalozzi et al., 2008). Despite the powerful impact of brain metastases, it remains unclear how tumour cells manage to invade the central nervous tissue and which tumour cell intrinsic and extrinsic factors play a role.

1.5 THE TUMOUR MICROENVIRONMENT AND THE ROLE OF HYALURONIC ACID METABOLISM

As a tumour progresses, its surrounding microenvironment, consisting of ECM, fibroblasts, endothelial cells, pericytes, and leukocytes, co-evolves. A continuous signalling circuitry between tumour cells and tumour-associated stroma promotes tumour growth, progression, and invasion (Pietras and Ostman, 2010). Supposably, all members of the TME act in concert to foster the tumour. However, one ECM component, the hyaluronic acid (HA), plays a special role in tumour development and progression. A HA rich tumour stroma provides a favourable microenvironment for malignant progression by maintaining osmotic balance and hydration while facilitating cell proliferation and migration (Koyama et al., 2007, Tan et al., 2011a). It has been shown that HA accumulates in the tumour microenvironment of many malignant tumours and is associated with tumour aggressiveness, invasiveness, angiogenesis, and metastasis (Csoka et al., 2001, Jiang et al., 2012, Schwertfeger et al., 2015, Sironen et al., 2011, Tammi et al., 2008). Moreover, in breast cancer tumour progression, invasiveness, and aggressiveness is associated with altered HA metabolism and increased deposition of HA, especially in the TME of the tumours invading edges (Auvinen et al., 2000, de la Torre et al., 1993). Therefore, HA and its synthesising and degrading enzymes evolved to be an interesting subject in breast cancer research.

1.6 HYALURONIC ACID

HA is a non-processed and non-sulfated linear glucosaminoglycan consisting of 2.000-25.0000 disaccharides of glucoronic acid and N-acetylglucosamin (Fig. 2). As a regular component of the ECM, HA is distributed ubiquitously within the body and often found to be up-regulated in solid tumours (Csoka et al., 2001). HA and HA-fragments

have wide-ranging and often opposing functions, which amongst others depend on its size. Some of the most universal properties are the ability to increase volume and viscoelasticity in solutions, resulting from mutual repulsion of carboxyl groups. When external pressure is applied, increased repulsion leads to the development of a swelling pressure within the HA-meshwork. With a pressure drop, the meshwork reshapes or adjusts to new restrictions. Additionally, HA facilitates tissue movement and cell proliferation. Hence HA plays an important role in tissue homeostasis and hydrodynamics, however, also in tumourgenesis (Stern et al., 2006, Toole, 2004). For the existence and diversity in function of HA the synthesising enzyme hyaluronic acid synthase (HAS) and degrading enzyme hyaluronidase (HYAL) come into account. Furthermore, specific isoforms of these enzymes, hyaluronic acid synthase 2 (HAS2) and hyaluronidase 1 (HYAL1), have been associated with tumour aggressiveness and invasiveness in breast cancer (Auvinen et al., 2014b, Schwertfeger et al., 2015). Interestingly, a recent study conducted by Milde-Langosch et al. based on microarray data, showed a correlation between high expression levels of HAS2 and HYAL1 in primary breast cancer and poor patient outcome as well as increased incidences of brain metastases formation (Milde-Langosch et al., 2014).

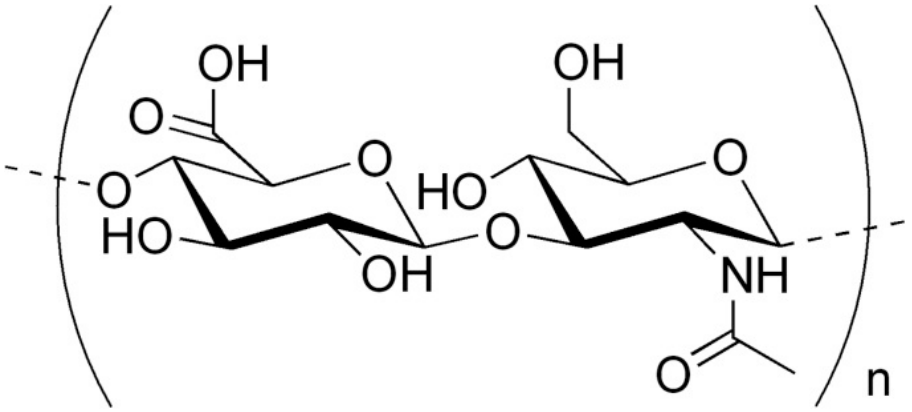

Figure 2: Chemical structure of hyaluronic acid (HA) taken from Wikipedia.de (Wikipedia, 2016).

1.7 HYALURONIC ACID SYNTHASE

In humans three isoenzymes of HAS are known: HAS1, HAS2, and HAS3. HAS is a transmembrane protein with its active domain on the inner site of the plasma membrane (Toole, 2004). Regulation of HAS activity is complex and occurs at both, transcriptional and posttranscriptional level, the latter for example via growth factors (Auvinen et al., 2014b, Itano et al., 1999). While HA is synthesised on the inner cell surface, it is simultaneously extruded to the outside of the cell via ABC-transporters (Toole, 2004). The newly synthesised HA is either released into the ECM or remains attached to the plasma membrane, retained there by either HAS itself or specific HA receptors (Auvinen et al., 2014b).

Previous studies show that HAS1-3 have different enzymatic characteristics, as HAS2 knock out is embryonically lethal, while HAS1 and HAS3 deletions only have minor effects on the phenotype (Camenisch et al., 2000). Furthermore, HAS2 up-regulation in breast cancer is associated with aggressiveness and invasiveness (Auvinen et al., 2014b), and, as mentioned before, associated with poor patient outcome and increased development of brain metastases (Milde-Langosch et al., 2014). Hence of the different HAS enzymes HAS2 is the focus of this study.

1.8 HYALURONIDASE

The human genome has six hyaluronidase-like genes, supposedly resulting from gene duplication. HYAL1, HYAL2, and HYAL3 are clustered on chromosome 3 (3p21.3) and HYAL4, HP-20/SPAM1, and HYALP1 are clustered on chromosome 7 (7p31.3). Little is known about HYAL3, HYAL4 only occurs in the placenta and skeletal muscles, HP-20 plays its role in fertilisation, and HYALP1 is not expressed in humans. Hence, HAYL1 and HYAL2 seem to be the major human hyaluronidases. It is suggested that HYAL1 and HYAL2 act in concert to degrade high molecular weight HA into low molecular weight oligosaccharides. The process starts with HA binding to cell surface receptors, such as cluster of differentiation 44 (CD44) and is followed by endocytosis and HA degradation through HYAL2 into 20-kDa fragments. In later steps HYAL1 and beta-glycuronidases degrade HA into small oligosaccharides. However, a weakness

of this proposal is the pH-optimum of HYAL2, which does not accord with the pH of it's here suggested environment (Csoka et al., 2001).

HYAL1, the enzyme of interest here, is an acid-active hyaluronidase consisting of a single polypeptide chain of 57-kDa. High levels of HYAL1 have been found in parenchymal organs such as heart, liver, spleen, and kidney. Moreover, HYAL1 has been detected in serum and two isoenzymes of HYAL1 with different sizes, however equal specificities, have been found in urine (Csoka et al., 2001). Previous research delivers converse data about HYAL1 and its influence on tumourgenesis. Some are stating that HYAL1 plays a role as tumour suppressor, where lack of HYAL1 leads to a HA rich tumour microenvironments and therefore tumour aggressiveness and invasiveness (Lokeshwar et al., 2005). Others are stating, that elevated HA and HYAL1 expression levels promote proliferation, migration, angiogenesis, and aggressiveness in several cancers including breast cancer (Poola et al., 2008, Schwertfeger et al., 2015). Moreover, HYAL1 expression in non-invasive ductal hyperplasia correlates with subsequent development of invasive breast cancer (Schwertfeger et al., 2015). And high HYAL1 levels in primary breast cancer showed a significant association with poor patient outcome and brain metastases formation (Milde-Langosch et al., 2014).

1.9 RESEARCH QUESTION AND AIMS OF THIS STUDY

The incidence rates of BCBM have increased over the past decades. This is a result of improved breast cancer diagnostic methods and enhanced molecular subtype adapted systemic treatment options, facilitating a longer patient survival, however also facilitating more time for brain metastases to develop. This current state is intensified by the lack of clarity about the molecular mechanisms involved in BCBM formation, including BBB crossing and central nervous tissue colonisation, as well as the lack of BCBM accessibility for drugs. As elucidated above, HA often accumulates in the TME of breast cancer and is associated with invasiveness and aggressiveness. Moreover, a recent microarray study by Milde-Langosch et al. associated the HA synthesising and degrading enzymes HAS2 and HYAL1 with poor patient outcome as well as increased BCBM formation (Milde-Langosch et al., 2014). In this regard, the aim of this study was:

1) To investigate the prognostic role of HAS2 and HYAL1 in breast cancer at protein level.
2) And to explore the role of HAS2 and HYAL1 in BCBM formation.

To achieve this aim, protein expression levels of HA-binding protein (HA-bp), HAS2, and HYAL1 were detected via IHC in four different collectives. These collectives were comprised of primary breast cancer tissue with and without corresponding brain metastases, BCBM tissue as well as breast cancer metastatic tissue of different locations such as brain, bone, skin, liver, and lung. The evaluated HA-bp, HAS2, and HYAL1 expression levels were statistically analysed, compared, and correlated with clinical and pathological parameters.

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 PATIENT COHORTS

The patient population for the IHC analysis is composed of four different patient cohorts. All patients included in this study have signed an informed consent. The study has been approved via the ethics commission and the study conduction followed the principles of the declaration of Helsinki (World-Medical-Association, 2015). The histopathological and clinical data provide information about the patients age, histological tumour type, tumour grade, tumour stage, status of lymphatic node, bone marrow status, lymphangoisis, vascular invasion, hormone receptor status, recurrence and survival. Further details are listed in Table 7-10 (Results).

Primary breast cancer

The first patient cohort comprises a tissue microarray (TMA) of 411 primary breast cancers and 89 corresponding lymphatic node metastases. The TMA, as well as corresponding histopathological and clinical data, have been provided by Dr. habil. Harriet Wikman-Kocher, Institute for Tumourbiology, Universitätsklinikum Hamburg-Eppendorf.

Brain metastases from breast cancer

The second patient cohort consists of a TMA of 137 BCBM. The TMA as well as corresponding histopathological and clinical data have been provided by Prof. Dr. med. Markus Glatzel and Priv.-Doz. Dr. med. Jakob Matschke, Institute of Neuropathology, Universitätsklinikum Hamburg-Eppendorf.

Breast cancer metastases

The third patient cohort comprises 117 paraffine sections of distant breast cancer metastases (Bone: n=36; Liver: n=24; Brain: n=21; Lung: n= 19 und Skin: n=17). The paraffine sections with corresponding histopathological and clinical data are property of the Department of Gynaecology, Universitätsklinikum Hamburg-Eppendorf.

Primary breast cancer with matching corresponding brain metastases

The fourth patient cohort includes 12 paraffine sections of primary breast cancer with 12 paraffine sections of corresponding brain metastases and 14 paraffine sections of primary breast cancer without brain metastases as control. The former tissue sections as well as the corresponding histopathological and clinical data have been provided by Prof. Dr. med. Markus Glatzel and Priv.-Doz. Dr. med. Jakob Matschke, Institute of Neuropathology, Universitätsklinikum Hamburg-Eppendorf. The latter tissue sections with corresponding histopathological and clinical data are property of the Department of Gynaecology, Universitätsklinikum Hamburg-Eppendorf.

2.1.2 MATERIALS FOR IHC STAINING AND ANALYSIS

Table 2: Technical equipment

	Company	Order number
Camera	Leica	DFC320 Camera
Microscope	Zeiss	Axioscope 40
Microtome	Leica	SM 2000R

Table 3: Primary antibodies or binding protein, secondary antibodies, and isotype controls.

	Company	Order number
Primary antibody		
Anti-Habp	Calbiochem	385911
Anti-HAS2 antibody	Abcam	ab140671
Anti-HYAL1 antibody	Abcam	ab103977
Secondary antibody		
Biotinylated anti-mouse IgG for HAS2	Vector Laboratories	BA-2000
Biotinylated anti-rabbbit IgG for HYAL1	Vector Laboratories	BA-1000
Isotype control		
Mouse IgG1 for HAS2	Dako	X0931
Rabbit IgG1 for HYAL1	Dako	X0903

Table 4: Reagents, Solutions and Buffers

	Components	Company	Order number
Reagents			
ABC kit elit	20µl A (Avidin) 20µl B (biotinylated enzyme)	Vectastain Laboratories	PK-6100
	1ml TBS-work solution	see below	see below
ABC kit standard	20µl A (Avidin) 20µl B (biotinylated enzyme)	Vectastain Laboratories	PK-4000
	1ml TBS-work solution	see below	see below
Bovine Serum Albumin, 2% (BSA)	2g BSA 100ml destilled water	Sigma	A7030
DAB-peroxidase substrate kit (3,3'-diaminobenzine)	2 drops of buffer stock 4 drops hydrogen peroxide	Vectastain Laboratories	SK-4100
Eosine		Carl Roth	3137.1
Haematoxylin		Merck	HX080645
H2O2 (0.5% and 3%)		Merck	K29827697
Solutions			
Antibody dilutent		Dako	S0809
Ethanol (80%, 96%, and 100%)		Th. Geyer	118700/55
Xylen (dimethylbenzene)		Sigma-Alorich	16446-1L
Buffers			
Citrate buffer (10mM; pH 6.0)	2,9g tri-sodiumcitrate-dihydrate 1l distilled water	Carl Roth	5110.1

TBS buffer (50mM Tris buffered saline; pH 7.8)	6,057g TRIS	Sigma	T1503-1KG
	8,709g NaCl	Carl Roth	3957.1
	1l distilled water		
TEC buffer (20mM Tris, 13mM EDTA, 10mM citrate; pH 7.8)	2.9g tri-sodiumcitrate-dihydrate	Carl Roth	5110.1
	2.4g TRIS	Sigma	T1503-1KG
	4.8g Titriplex III (EDTA)	Merck	K12510518
	1l distilled water		

2.2 METHODS

2.2.1 IMMUNOHISTOCHEMISTRY

The tissue processing was identical for all tissue sections. Previously positive tested breast cancer tissue sections were used as positive controls and isotype controls within each staining cycle. The isotype controls were made in the same species and applied in same concentrations as the primary antibodies that were tested. Further details referring the IHC staining methods are provided in table 5.

Table 5: IHC and histochemistry staining protocol

Protocol	**HAbp**	**HAS2**	**HYAL1**
Pre-treatment: The formalin fixed, paraffine-embedded tissue sections were cut to 4µm, mounted on superfrost slides, dewaxed with xylene, and greadually hydrated.	✔	✔	✔
Step 1: Heat introduced epitope retrieval	✔ (Over night with citrate buffer at 60°C)	✔ (20 min at 60°C with Citrate buffer)	✔ (5 min at 60°C with Citrate buffer)
Step 2: Rinse	✔ 1 x 5 min aq dist. and 2 x 5 min TBS	✔ 3 x 5 min with water	✔ 3 x 5 min with water

Protocol	HAbp	HAS2	HYAL1
Step 3: Inhibition of the endogenous peroxidase with H_2O_2	✗ Instead protein block with 1% BSA in TBS for 30 min at RT	✔ 0,5% H_2O_2 for 30 min at RT	✔ 0,5 H_2O_2 for 30 min at RT
Step 4: Incubation with primary antibody or binding protein	✔ 1:75 with antibody diluent diluted for 1h at RT	✔ 1:1000 with antibody diluent diluted over night at 4°C	✔ 1:500 with antibody diluent diluted over night at 4°C
Step 5: Rinse	✔ 2 x 5 min with TBS	✔ 3 x 5 min with TBS	✔ 3 x 5 min with TBS
Step 6: Incubation with secondary antybody	✗	✔ 50µl anti-mouse antibody with 10ml TBS for 30 min at RT	✔ 50µl anti-rabbit antibody with 10ml TBS for 30 min at RT
Step 7: Rinse	✗	✔ 3 x 5 min with TBS	✔ 3 x 5 min with TBS
Step 8: Incubation with ABC reagent	✔ 2 drops A and 2 drops B with 5 ml TBS for 30 min at RT	✔ 2 drops A and 2 drops B with 5 ml TBS for 30 min at RT	✔ 2 drops A and 2 drops B with 5 ml TBS for 30 min at RT
Step 9: Rinse	✔ 3 x 5 min with TBS	✔ 3 x 5 min with TBS	✔ 3 x 5 min with TBS
Step 10: Incubation with DAB/permanent red	✔ Permanent red for 30 min in the dark at RT	✔ 2 drops of buffer stock, 4 drops of DAB, and 2 drops of hydrogen peroxide with 5ml aq dist. for 3 min at RT	✔ 2 drops of buffer stock, 4 drops of DAB, and 2 drops of hydrogen peroxide with 5ml aq dist. for 3 min at RT
Step 11: Rinse	✔ 1 x 5 min with water	✔ 2 x 5 min with water	✔ 2 x 5 min with water
Step 12: Counterstain with haematoxylin, gradual dehydration and mounting.	✔	✔	✔

2.2.2 MICROSCOPIC EVALUATION

The IHC staining was evaluated independently by a medical student and a scientist, using the immunoreactive score (IRS). The IRS is a product of staining intensity and percentage of positive tumour cells, as shown in table 6, and has been introduced by Remmele and Stegner in 1987 (Remmele and Stegner, 1987).

Table 6: Methos of evaluation of the IRS in accordance with Remmele and Stegner (Remmele and Stegner, 1987). (A) Evaluation of score 1. (B) Evaluation of score 2. (C) Evaluation of the IRS.

Intensity of stain	Score 1
None	0
Weak	1
Meadium	2
Strong	3

Percentage of positive cells	Score 2
0%	0
1-19%	1
20-49%	2
50-79%	3
80-100%	4

IRS (Score 1 x Score 2)	Expresion level
0-2	negative - weak
3-6	medium
6-12	medium-strong

Depended on the distribution of the IRS of HAS2, HAYL1, and HAbp within the individual patient cohorts, different score groups have been formed for further statistical analysis. Details are provided in the result section.

2.2.3 STATISTICAL ANALYSIS

The statistical analyses were performed by IBM SPSS, version 20.0 (SPSS, 2014) and Microsoft Excel, version 15.11.2 (Microsoft-Excel, 2014). Frequency, mean, and standard deviation (SD) of HAS2, HAYL1, and HAbp IRS expression levels were computed. Associations between the IRS patterns and histopathological and clinical data as well as the type of metastasis were analysed by Chi-squared test. Survival was calculated by Kaplan-Meier curves and log-rak test. HAS2 and HYAL1 IRS expression levels within the different cohorts were analysed by two-sample t-test.

3 RESULTS

3.1 COHORT CHARACTERISTICS

In this study four different patient cohorts were analysed: The first cohort was comprised of 411 primary breast cancer samples, the second cohort consisted of 132 BCBM, the third cohort was a compilation of breast cancer brain-, bone-, skin-, liver-, and lung metastases, and the fourth cohort was composed of 14 primary breast cancer samples and 14 corresponding brain metastases. The first cohort served to verify the prognostic relevance of HAS2 and HYAL1 in breast cancer. The first, second, and fourth cohort were analysed in terms of the influence of HA metabolism on the development of brain metastases from breast cancer. And the third cohort served to evaluate the role of HA- metabolism in BCBM in comparison to other breast cancer metastases. All four cohorts are characterised in table 7, table 8, table 9, and table 10 respectively. No follow-up data was evaluable for the second and the fourth cohort.

Table 7: Cohort characteristics of primary breast cancer patients analysed by HAS2 and HYAL1 Immunohistochemistry. Percentages in brackets.

n=		441
Age at time of surgery (y)	mean (median)	57.7(58.5)
Histological type	invasive ductal	279 (67.9)
	invasive lobular	80 (19.5)
	invasive ductolobular	16 (3.9)
	other/unknown	36 (8.8)
Grade	grad 1	32 (7.8)
	grade 2	222 (54.0)
	grade 3	147 (35.8)
	unknown	10 (2.4)
Stage	T1	218 (53.0)
	T2	156 (38.0)
	T3	20 (4.9)
	T4	13 (3.2)
	unknown	4 (1.0)

Nodal involvement	negative	322 (78.3)
	positive	89 (21.7)
Bone marrow status	no tumour cells	302 (73.5)
	1-2 tumour cells	103 (25.1)
	unknown	6 (1.5)
Lymphangiosis	negative	310 (75.4)
	positive	30 (7.3)
	unknown	71 (17.3)
vascular invasion	negative	342 (83.2)
	positive	33 (8.0)
	unknown	36 (8.8)
Hormone receptor status	triple negative	26 (6.3)
	positive	374 (91.0)
	unknonw	11 (2.7)
ER	negative	83 (20.2)
	positive	325 (79.1)
	unknown	3 (0.7)
PR	negative	121 (29.4)
	positive	286 (69.6)
	unknown	4 (1.0)
HER2	negative	134 (32.6)
	positive	221 (53.8)
	unknown	56 (13.6)
Recurrence	no	296 (72.0)
	yes	90 (21.9)
	unknown	25 (6.1)
Metastasis	no	385 (93.7)
	yes	15 (3.6)
	unknown	11 (2.7)
Died of breast cancer or breast cancer metastases	no	340 (82.7)
	yes	62 (15.1)
	unknown	9 (2.2)

Table 8: Cohort characteristics of patients with BCBM analysed by HAS2, HYAL1, and Habp Immunohistochemistry. Percentages in brackets.

n=		132
Age at time of surgery (y)	mean (median)	56.4 (55.0)
Histological type	invasive ductal	16 (12.1)
	invasive lobular	2 (1.5)
	other/unknown	114 (86.4)
Grade	grad 1	2 (1.5)
	grade 2	5 (3.8)
	grade 3	14 (10.6)
	unknown	111 (84.1)
Stage	T1	4 (3.0)
	T2	9 (6.8)
	T3	4 (3.0)
	T4	4 (3.0)
	unknown	111 (84.1)
Nodal involvement	negative	8 (6.1)
	positive	14 (10.6)
	unknown	110 (83.3)
ER primary tumour	negative	24 (18.2)
	positive	29 (22.0)
	unknown	79 (59.8)
PR primary tumour	negative	41 (31.1)
	positive	14 (10.6)
	unknown	77 (58.3)
HER2 primary tumour	negative	8 (6.1)
	positive	42 (31.8)
	unknown	82 (621)
ER brain metastases	negative	76 (57.6)
	positive	41 (31.1)
	unknown	15 (11.4)

PR brain metastases	negative	103 (78.0)
	positive	13 (98)
	unknown	16 (12.1)
HER2 brain metastases	negative	60 (45.5)
	positive	59 (44.7)
	unknown	13 (9.8)
Molecular subtype of brain metasitases	triple negative	46 (34.8)
	lumanal	20 (15.2)
	HER2 positive	55 (41.7)
	unknown	11 (8.3)
Died of breast cancer or breast cancer metastases	no	13 (9.8)
	yes	13 (9.8)
	unknown	106 (80.3)

Table 9: Cohort charactristics of patients with breast cancer metastases spread to different locations analysed by HAS2, HYAL1, and HAbp Immunohistochemistry. Percentages in brackets.

		total	**brain**	**bone**	**skin**	**liver**	**lung**
n=		130	26	39	23	23	19
Age at time of surgery (y)	mean (median)	62.4 (63.0)	58.1 (56.0)	61.9 (61.5)	66.2 (62.5)	62.6 (62.5)	63.8 (66.5)
Histological type	invasive ductal	56 (43.1)	8 (30.8)	9 (23.1)	13 (56.5)	16 (69.6)	10 (52.6)
	invasive lobular	5 (3.8)	0 (0.0)	2 (5.1)	3 (13.0)	0 (0.0)	0 (0.0)
	other/unknown	69 (53.1)	18 (69.2)	28 (71.8)	7 (30.4)	7 (30.4)	9 (47.4)

Grade	grad 1	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	grade 2	27 (20.8)	0 (0.0)	8 (20.5)	6 (26.1)	11 (47.8)	2 (10.5)
	grade 3	38 (29.2)	0 (0.0)	16 (41.0)	9 (39.1)	4 (17.4)	9 (47.4)
	unknown	65 (50.0)	26 (100)	15 (38.5)	8 (34.8)	8 (34.8)	8 (42.1)
ER	negative	33 (25.4)	14 (53.8)	5 (12.8)	5 (21.7)	5 (21.7)	4 (21.1)
	positive	49 (37.7)	7 (26.9)	10 (25.6)	10 (43.5)	12 (52.2)	10 (52.6)
	unknown	48 (36.9)	5 (80.8)	24 (61.5)	8 (34.8)	6 (26.1)	5 (26.3)
PR	negative	54 (41.5)	17 (65.4)	11 (28.2)	7 (30.4)	12 (52.2)	7 (36.8)
	positive	27 (20.8)	4 (15.4)	4 (10.3)	8 (34.8)	5 (21.7)	6 (31.6)
	unknown	49 (37.7)	5 (19.2)	24 (61.5)	8 (34.8)	6 (26.1)	6 (31.6)
HER2	negative	28 (21.5)	0 (0.0)	7 (17,9)	10 (43.5)	4 (17.4)	7 (36.8)
	positive	16 (12.3)	0 (0.0)	3 (7.7)	4 (17.4)	4 (17.4)	5 (26.3)
	unknown	86 (66.2)	26 (100)	29 (74.4)	9 (39.1)	15 (65.2)	7 (36.8)

Table 10: Cohort characteristics of two groups: patients with breast cancer and corresponding brain metastasis (Primary + Brain Met.) and patients with breast cancer only (Primary) as a control. Both groups were analysed by HAS2, HYAL1, and HAbp Immunohistochemistry. Percentages in brackets.

		Primary + Brain Met.	**Primary**
n=		12	14
Age at time of surgery (y)	mean (median)	55.33 (55.95)	unknown
Histological type of primary tumour	invasive ductal	8 (66.7)	8 (57,1)
	invasive lobular	0 (0.0)	1 (7.2)
	other/unknown	4 (33.4)	5 (35.7)
Grade	grad 1	0 (0.0)	0 (0.0)
	grade 2	1 (8.3)	6 (42.9)
	grade 3	6 (50.0)	6 (42.9)
	unknown	5 (41.7)	2 (14,2)
Stage	T1	2 (16.7)	0 (0.0)
	T2	6 (50.0)	7 (50,0)
	T3	0 (0.0)	3 (21.4)
	T4	0 (0.0)	1 (7.2)
	unknown	4 (33.3)	3 (21.4)
Nodal involvement	negative	1 (8.3)	0 (0.0)
	positive	6 (50.0)	12 (85.7)
	unknown	5 (41.7)	2 (14.3)
ER primary tumour	negative	7 (58.3)	3 (21.4)
	positive	3 (25.0)	9 (64.3)
	unknown	2 (16.7)	2 (14.3)
PR primary tumour	negative	10 (83.3)	5 (35.7)
	positive	0 (0.0)	7 (50.0)
	unknown	2 (16.7)	2 (14.3)
HER2 primary tumour	negative	5 (41.7)	8 (57.1)
	positive	5 (41.7)	2 (14.3)
	unknown	2 (16.6)	4 (28.6)

3.2 HAS2 EXPRESSION IN BREAST CANCER AND ITS METASTASES

HAS2 IHC was performed on tissue sections of primary breast cancer, BCBM, and other breast cancer metastases in order to identify the role of HAS2 in the development of brain metastases from breast cancer.

3.2.1 HAS2 EXPRESSION IN PRIMARY BREAST CANCER

HAS2 immunohistochemistry in primary breast cancer

Of the 411 HAS2 stained primary breast cancer samples included in the TMA, 201 were analysable. Criteria for the exclusion were, for example, insufficient quality of stain or artefacts. HAS2 was expressed very frequently in breast cancer cells, mostly localised in the cell cytoplasm but in some cases also in the cell membrane or nucleus (Figure 3B and C).

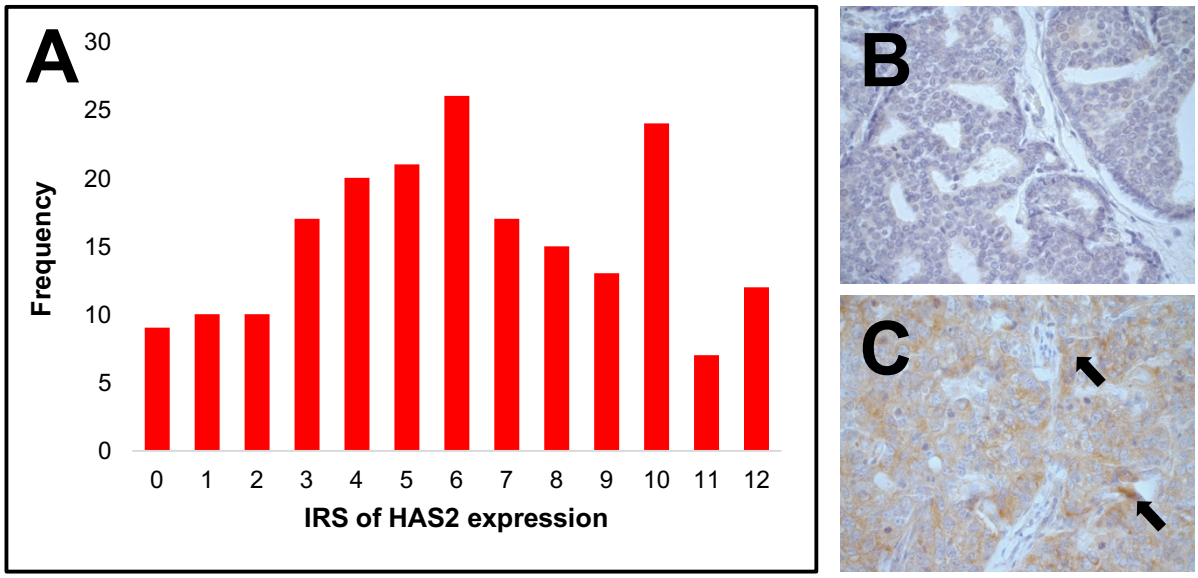

Figure 3: HAS2 expression in primary breast cancer. (A) IRS distribution of HAS2 with n = 201; mean = 6.16; Standard deviation = 3.292. (B-C) HAS2 staining pattern in primary breast cancer. (B) 40x, IRS 0-3/negative-weak. (C) 40x, IRS 6/medium with some cells that have high HAS2 expression levels (arrow).

The average IRS was 6.16 with a SD of 3.29. About a quarter (23%) of all tissue samples were considered to be HAS2 negative or only weakly stained (IRS 0-3) (Figure 3B). The greater proportion (77%) was stained medium to strong (IRS 4-12) (Figure 3C). For further statistical analyses the cohort was arranged into two groups

according to the IRS expression levels: negative to weak HAS2 expression levels (IRS 0-3) and medium to strong HAS2 expression levels (IRS 4-12).

The association of HAS2 expression in primary breast cancer with prognostic factors

No significant correlations between HAS2 expression levels and the age at time of surgery, tumour stage, nodal involvement, hormone receptor status, ER status, PR status, HER2 status, and recurrence were noted (Table 11).

Table 11: Correlations of HAS2 IRS in primary breast cancer with clinical and pathological data. Significant result ($p<0.05$) are shown in red.

	Groups	P-value	n
Age at time of surgery	above median below median	0.274	194
Histological type	invasive ductal invasive lobular invasive ductolobular	< 0.001	201
Grade	grad 1-2 grade 3	0.022	197
Stage	T1 T2-3 T4	0.352	200
Nodal involvement	negative positive	0.855	201
Bone marrow status	no tumour cells 1-2 tumour cells	0.033	200
Lymphangiosis	negative positive	0.037	160

Hormone receptor status	triple negative positive	0.299	196
ER	negative positive	0.351	200
PR	negative positive	0.900	200
HER2	negative positive	0.690	168
Recurrence	no yes	0.793	160

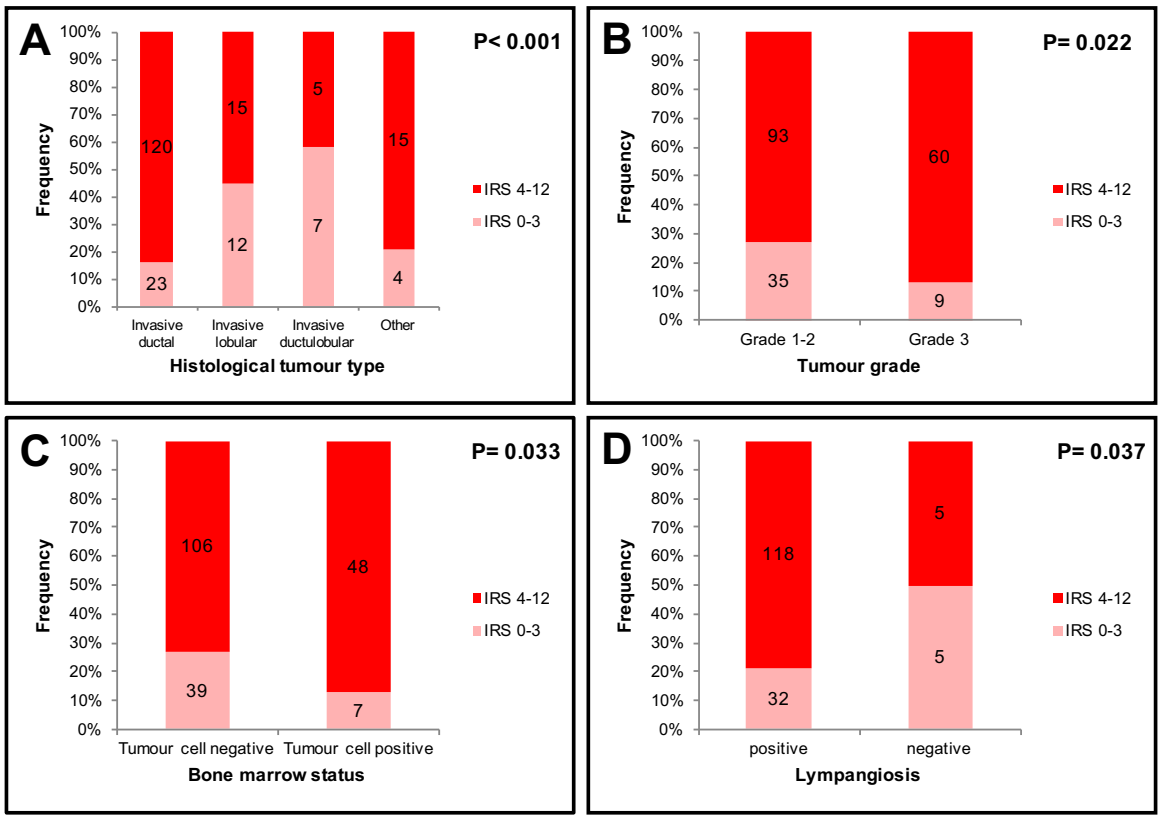

Figure 4: HAS2 expression levels in primary breast cancer in correlation to clinical and pathological data. (A) Histological tumour type, n = 201. (B) Tumour grade, n = 197. (C) Bone marrow status, n = 200. (D) Lymphangiosis, n = 160. Data in columns represent case numbers. P-values as shown in graph.

However, chi-square analysis including HAS2 expression levels and the histological tumour type, tumour grade, bone marrow status, and lymphangiosis showed significant associations (Table 11 and Figure 4). Medium to high HAS2 expression levels were recorded in 84% of invasive ductal carcinoma cases, however only in 56% of invasive lobular carcinoma cases ($p < 0.001$) (Figure 4A). Medium to high HAS2 expression levels were significantly associated with high tumour grade (87%). In comparison, only 73% of low tumour grade cases showed medium to high HAS2 expression levels ($p = 0.022$) (Figure 4B). Strong HAS2 staining was associated with a positive bone marrow status ($p = 0.033$). Here, 87% of bone marrow positive cases had medium to high HAS2 expression levels, while only 73% of bone marrow negative cases had medium to high HAS2 expression levels (Figure 4C). Strong HAS2 staining correlated with lymphangiosis, where 79% of lymphangiosis positive but only 50% of lymphangiosis negative cases had medium to high HAS2 expression levels ($p = 0.037$) (Figure 4D).

HAS2 expression levels in primary tumours and corresponding lymphatic nodes

A total of 89 lymphatic nodes, where each lymphatic node corresponds to a primary breast cancer case in this collective, were stained for HAS2. Of those, 62 were analysable and showed on average slightly weaker HAS2 staining than the primary breast cancer cells, with a mean of 5.63. Within 18 matched pairs that were available, the staining pattern seemed to be random and no trend of either stronger staining or weaker staining in lymphatic nodes in comparison to the staining in the primary tumour could be detected (data not shown).

HAS2 expression and patient survival

By Kaplan-Meier analysis and log-rank test, HAS2 expression levels in primary breast cancer showed no significant associations to relapse or overall survival ($p = 0.708$ and 0.136) (Figure 5A and B). In both groups, HAS2 IRS 0-3 and HAS2 IRS 4-12, around 20% suffered a relapse (Figure 5A). However, there was a difference between the two groups in the overall survival. 22% of cases with negative to weak HAS2 expression levels died and only 12% of cases with medium to high HAS2 expression levels died (Figure 5B).

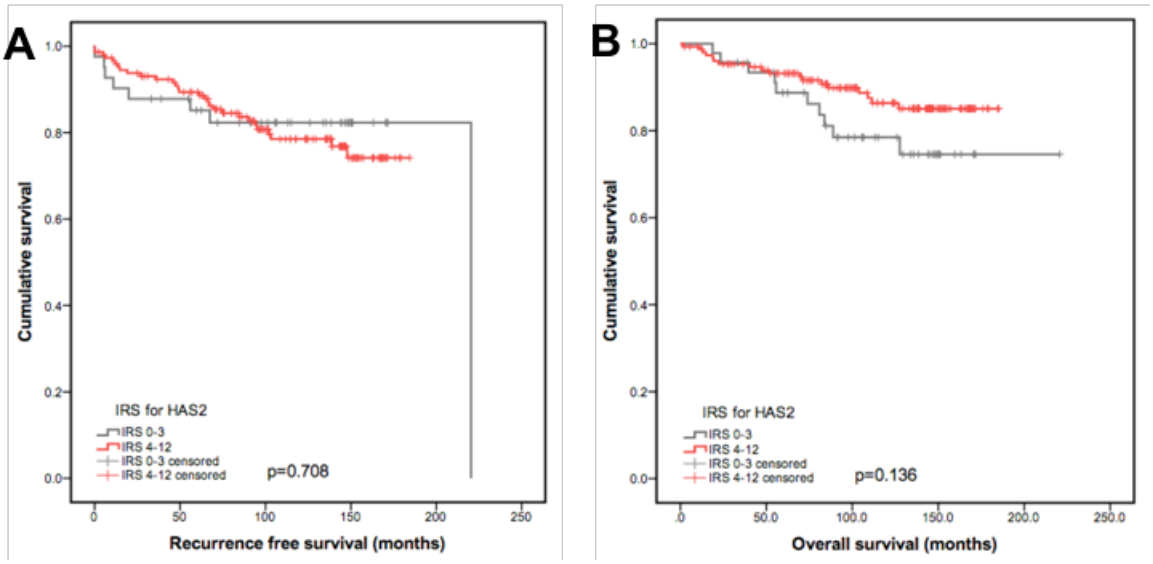

Figure 5: Kaplan-Meier analysis of primary breast cancer patients under the consideration of HAS2 expression. (A) Recurrence free survival. (B) Overall survival. P-values as shown in graph.

3.2.2 HAS2 EXPRESSION IN BREAST CANCER BRAIN METASTASES

HAS2 immunohistochemistry in breast cancer brain metastases

Amongst the 132 HAS2 stained BCBM samples included in the TMA, 87 were analysable. Overall, the HAS2 staining strength was weaker than in the primary tumour samples with a mean of 4.41 (Figure 6).

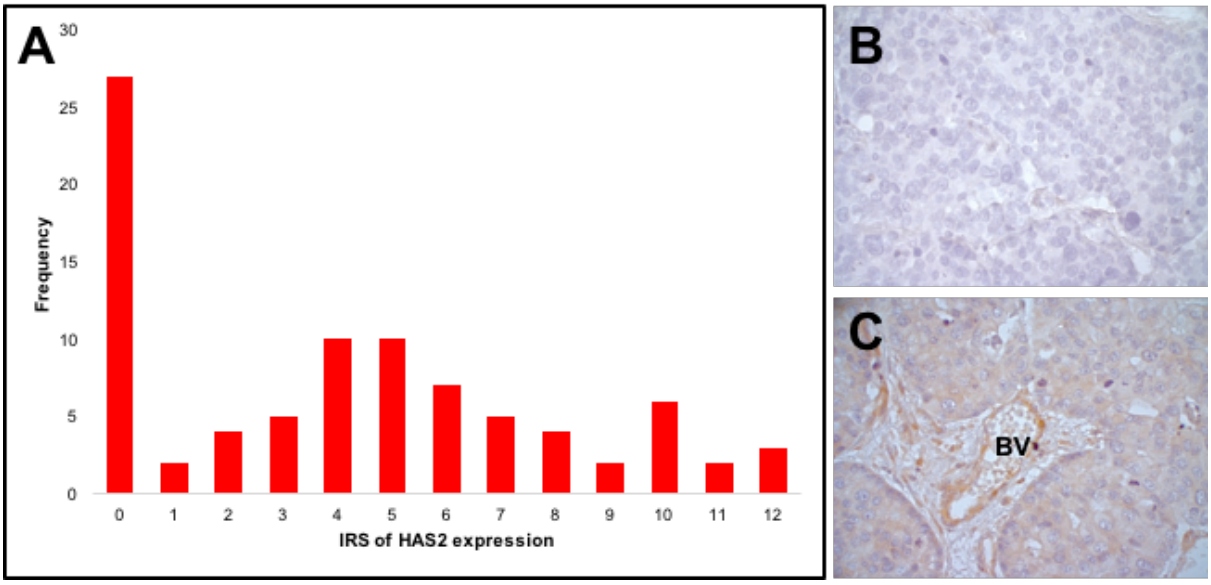

Figure 6: HAS2 expression in BCBM. (A) IRS distribution of HAS2 with n = 87; mean = 4.14. (B-C) HAS2 staining pattern in BCBM. (B) 40x, IRS 0/negative. (C) 40x, IRS 6-8/ medium HAS2 expression with a HAS2 stained blood vessel (BV).

Nearly one third (31%) of the samples were HAS2 negative (IRS 0) (Figure 6A). In the other two thirds, the HAS2 IRS distribution was quite regular. For further statistical analyses, cases were grouped according to the IRS expression levels in three groups: negative HAS2 expression levels (IRS 0), weak to medium expression levels (IRS 1-5), and medium to high expression levels (IRS 6-12).

The association of HAS2 in BCBM with prognostic factors

No significant correlations between HAS2 expression levels and the age at time of surgery, ER and PR status in the primary tumour, histological subtypes of brain metastases, and the PR and HER2 status of brain metastases were found (Table 12).

Table 12: Correlations of HAS2 IRS in BCBM cancer with clinical and pathological data. Significant result ($p<0.05$) are shown in red.

	Groups	P-value	n
Age at time of surgery	above median below median	0.160	91
ER primary tumour	negative positive	0.684	35
PR primary tumour	negative positive	0.369	36
HER2 primary tumour	negative positive	0.030	33
Histological subtype of brain metastases	triple negative luminal HER2 positive	0.782	84
ER brain metastases	negative positive	0.031	83
PR brain metastases	negative positive	0.920	82
HER2 brain metastases	negative positive	0.923	83

However, chi-square analysis including HAS2 expression levels and the HER2 status of the primary tumour as well as the ER status of the brain metastases showed significant correlations (Table 12 and Figure 7). Medium to high HAS2 expression levels were significantly associated with HER2-negativity in the primary tumours of the brain metastases (p = 0.030) (Figure 7A). Strong HAS2 staining correlated with ER-positivity of the brain metastases, where 34% of ER positive but only 22% of ER negative cases had medium to high HAS2 expression levels (p = 0.031) (Figure 7B).

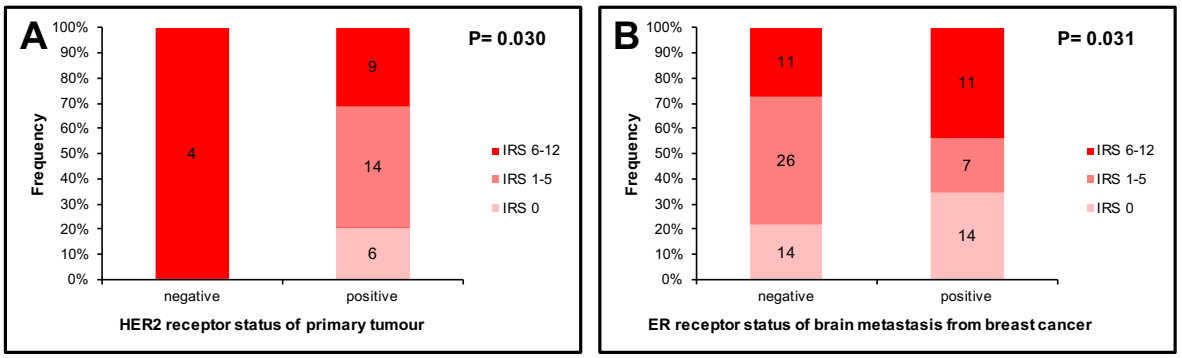

Figure 7: HAS2 expression levels in BCBM in correlation to clinical and pathological data. (A) HER2 receptor status of primary tumours, n = 33. (B) ER receptor status of BCBM, n = 83. Data in columns represent case numbers. P-values as shown in graph.

3.2.3 HAS2 EXPRESSION IN BREAST CANCER METASTASES VARIOUS LOCATIONS

HAS2 immunohistochemistry in breast cancer metastases samples from different sites

Of the 130 HAS2 stained samples of breast cancer metastases various locations 106 were analysable. 26% of those were negative (IRS 0) and 32% were only weakly stained (IRS 1-3). Hence the overall HAS2 expression level was weaker than in the other collectives with a mean of 3.45 (data not shown). For further statistical analyses, cases were grouped according to the IRS expression levels in four groups: negative HAS2 expression levels (IRS 0), weak HAS2 expression levels (IRS 1-3), medium HAS2 expression levels (IRS 4-7), and strong HAS2 expression levels (IRS 8-12).

HAS2 expression in breast cancer brain metastases in comparison to HAS2 expression in breast cancer metastases from other sites

In order to learn more about the role of HAS2 in the development of brain metastases, HAS2 expression levels in BCBM were compared with HAS2 expression levels in breast cancer metastases derived from the bone, skin, liver, and lung. Chi-square analyses, where HAS2 expression levels of all different metastatic sites were studied, showed significant differences amongst brain, bone, liver, and lung metastases ($p < 0.001$) (Figure 8). Medium to high HAS2 expression levels were significantly higher in skin (53%), liver (67%), and lung metastases (88%) in comparison to brain (14%) and bone metastases (17%). Amongst all metastases, brain metastases had the highest percentage of negative to weak HAS2 expression levels with 86% (Figure 8).

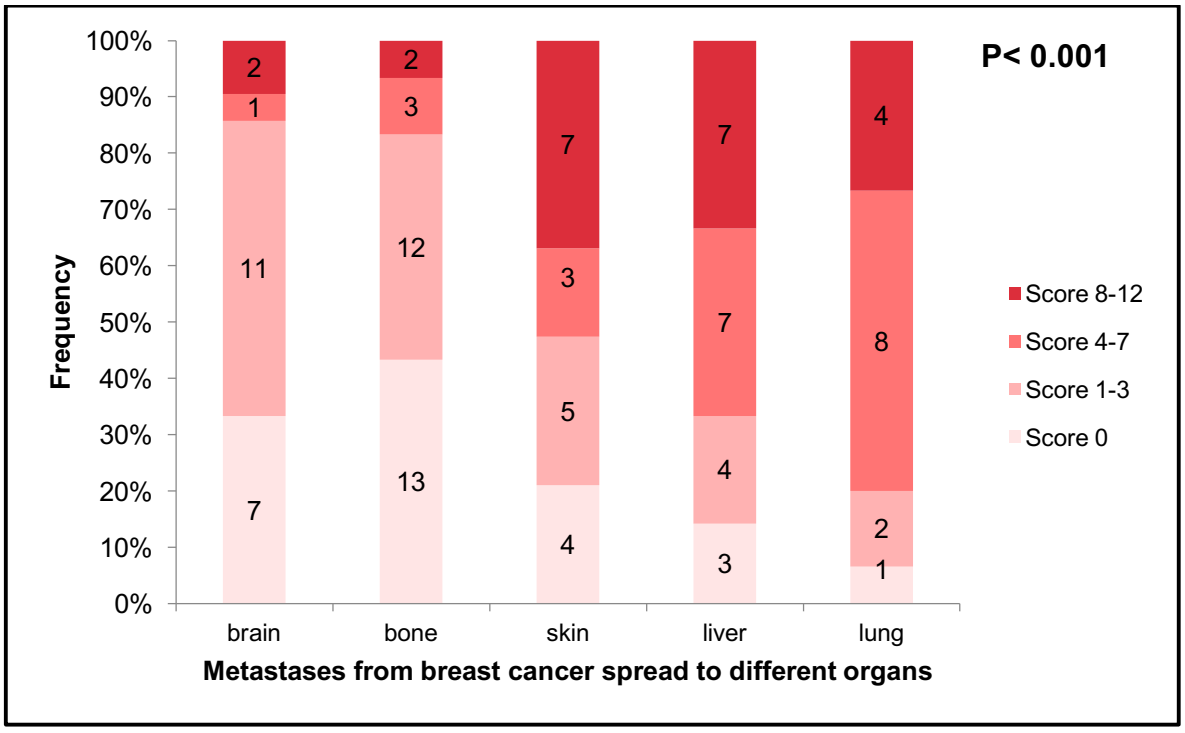

Figure 8: HAS2 expression levels in metastases from breast cancer spread to the brain (n = 21), bone (n = 30), skin (n = 19), liver (n = 21), and lung (n = 15). Total n = 106. Data in columns represent case numbers. P-value as shown in graph.

The microscopic analysis revealed that in all breast cancer metastases HAS2 was located in the tumour cell cytoplasm rather than in the membrane or nucleus (Figure 9). However, HAS2 staining was also found in some surrounding structures, such as in blood vessels (Figure 9A-F), neurons (not shown), inflammatory cells (Figure 9C and 9E), epithelium (Figure 9D) and glands (not shown) of the skin, and hepatocytes (Figure 9E). Interestingly, in BCBM, tumour cells at the edge of the

tumour, next to non-tumourous surrounding brain tissue, often displayed higher HAS2 expression levels, than tumour cells in the centre of the tumour (Figure 9B).

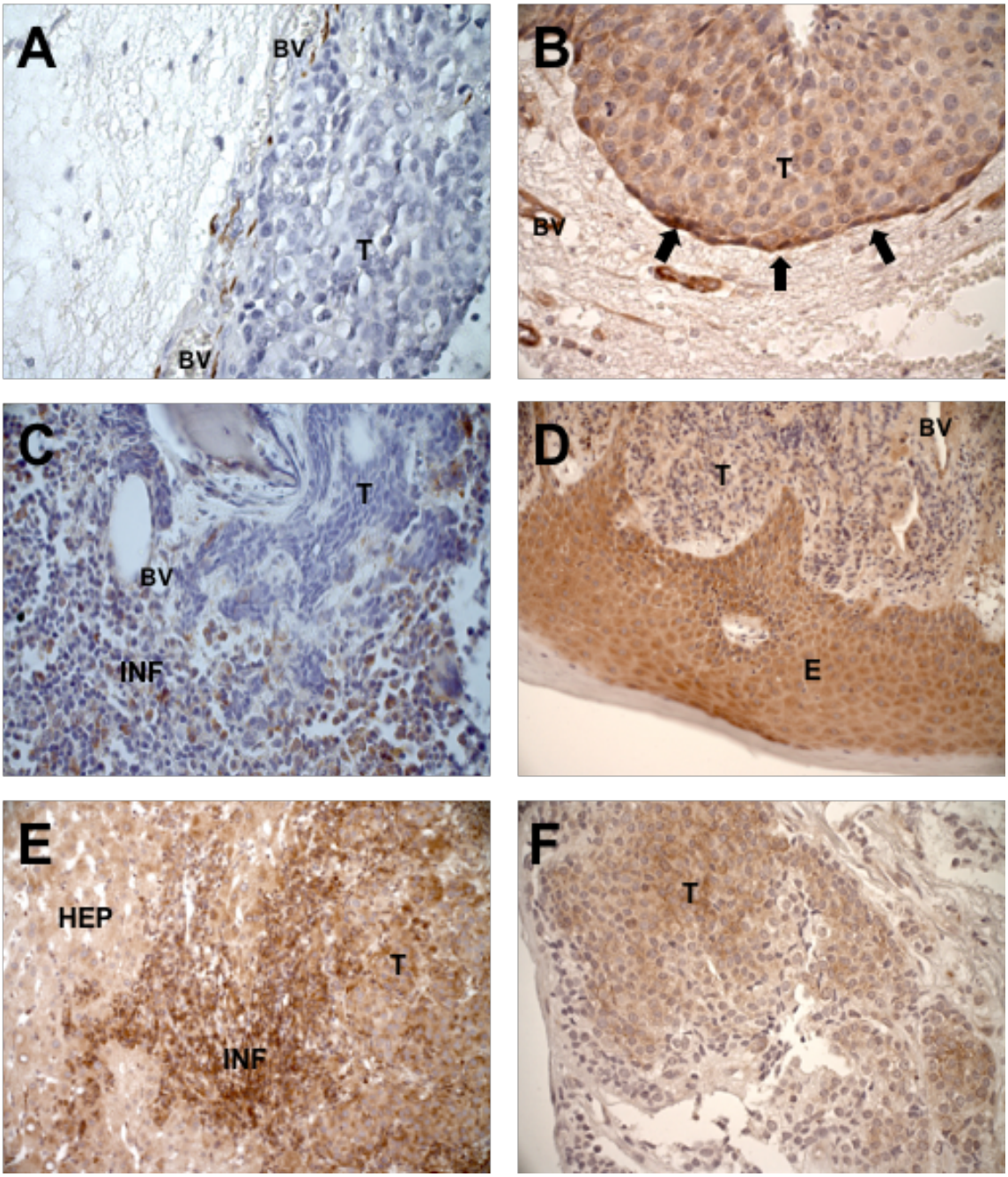

Figure 9: HAS2 staining pattern in breast cancer metastases various locations. (A) Brain metastasis with HAS2-negative tumour cells (T) and HAS2 negative blood vessels (BV) (20x, IRS 0). (B) Brain metastases with HAS2 positive tumour cells (T) which show higher HAS2 expression levels at the tumour invasion front (arrow), and HAS2 positive blood vessels (BV) (20x, IRS 6-11). (C) Bone metastases with HAS2 negative tumour cells (T), HAS2 positive inflammatory cells (INF), and HAS2 negative blood vessels (BV) (20x, IRS 0 and IRS 10). (D) Skin metastases with HAS2 positive tumour cells (T), HAS2 positive epithelium (E), and HAS2 positive blood vessels (BV) (10x IRS 5 and IRS 10). (E) Liver metastases with HAS2 positive tumour cells (T), HAS2 positive hepatocytes (HEP), and HAS2 positive inflammatory cells (INF) (10x, IRS 6-8, 4, and 11-12). (F) Lung metastases with HAS2 negative and positive tumour cells (T) (20x, IRS 0-8).

3.2.4 HAS2 EXPRESSION IN PRIMARY BREAST CANCER WITH AND WITHOUT CORRESPONDING BRAIN METASTASES

To detect the influence of HAS2 prevalence in primary breast cancer on the formation of BCBM, primary breast cancer cases with corresponding brain metastases as well as primary breast cancer samples of patient cases without brain metastases were stained for HAS2. The means of the HAS2 IRSs for all three groups were between 4.7 and 5.6, hence no great difference was noted (Figure 10). Still, primary breast cancer samples of patients with corresponding brain metastases had higher IRSs than the primary breast cancer samples of patients without brain metastases (Figure 10).

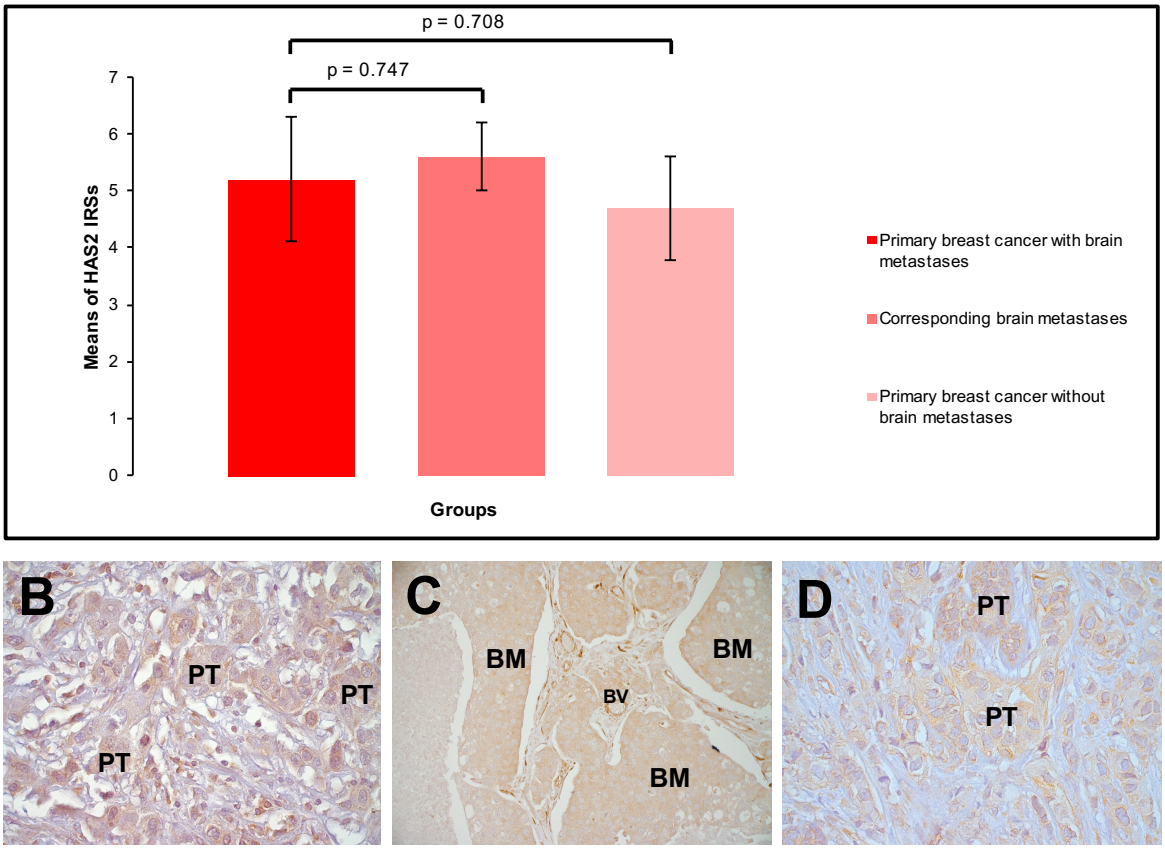

Figure 10: HAS2 expression levels in pairs of primary breast cancer and corresponding BCBM, both from the same patient. (A) Means of the HAS2 IRSs in three groups: primary breast cancer, which formed brain metastases (dark red), the corresponding brain metastases (middle light red), and primary breast cancer, which did not form brain metastases (light red). The error bars represent the standard error in each group. P-values as shown in graph. (B-C) HAS2 staining pattern in the primary tumour (PT) (40x, overall IRS = 4), and its corresponding brain metastasis (BM) (20x, overall IRS = 5). B and C both derived from the same patient. (D) HYAL1 staining pattern of primary breast cancer which did not metastasis (40x, IRS = 4). BV = blood vessel.

3.3 HYAL1 EXPRESSION IN BREAST CANCER AND ITS METASTASES

HYAL IHC was performed on tissue sections of primary breast cancer, BCBM, and other breast cancer metastases in order to identify the role of HYAL1 in the development of brain metastases from breast cancer.

3.3.1 HYAL1 EXPRESSION IN PRIMARY BREAST CANCER

HYAL1 immunohistochemistry in primary breast cancer

Of the 411 HYAL1 stained primary breast cancer samples included in the TMA, 283 were analysable. The overall HYAL1 expression levels were relatively high in the primary breast cancer cells and mostly localised in the cytoplasm but in some cases also in the nucleus. The average IRS was 8.68 with a SD of 2.32 (Figure 11).

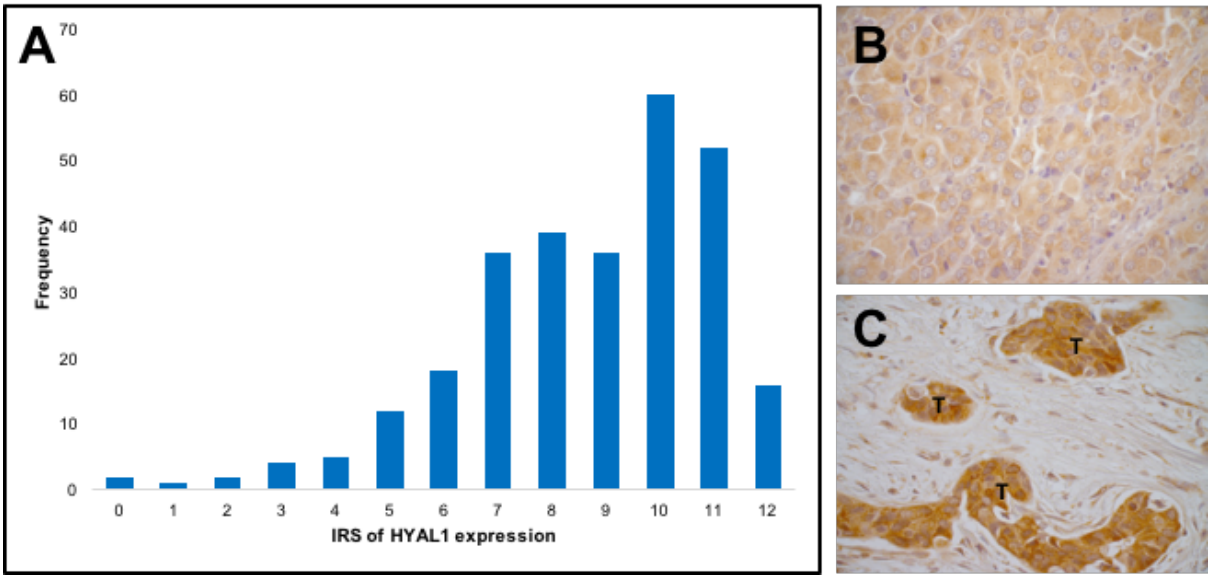

Figure 11: HYAL1 expression in primary breast cancer. (A) IRS distribution of HYAL1 with n = 283; mean = 8.68; Standard deviation = 2.317. (B-C) HYAL1 staining pattern in primary breast cancer. (B) 40x, IRS 8-9/medium. (C) 40x, IRS 12/ high HYAL1 expression, T = Tumour.

More than half (58%) of all tissue samples had fairly strong HYAL1 expression levels (IRS 9-12) (Figure 11C). Only 26% had medium HYAL1 expression levels (Figure 11B) and only 16% had negative to weak HYAL1 expression levels. For further statistical analysis, the cohort was arranged into four groups according to the IRS expression levels: negative to weak HYAL1 expression levels (IRS 0-6), medium HYAL1

expression levels (IRS 7-8), medium to strong HYA1 expression levels (IRS 9-10), and strong HYAL1 expression levels (IRS 11-12).

The association of HYAL1 expression in primary breast cancer with prognostic factors

No significant correlations between HYAL1 expression levels and the age at time of surgery, histological tumour type, tumour stage, nodal involvement, bone marrow status, lymphangiosis, hormone receptor status, HER2 status, and recurrence were noted (Table 13). However, chi-square analysis including HYAL1 expression levels and the tumour grade, ER status, and PR status showed significant associations (Table 13 and Figure 12).

Table 13: Correlations of HYAL1 IRS in primary breast cancer with clinical and pathological data. Significant result ($p<0.05$) are shown in blue.

	Groups	P-value	n
Age at time of surgery	above median below median	0.813	274
Histological type	invasive ductal invasive lobular invasive ductolobular	0.807	283
Grade	grad 1-2 grade 3	0.031	279
Stage	T1 T2-3 T4	0.658	283
Nodal involvement	negative positive	0.995	280
Bone marrow status	no tumour cells 1-2 tumour cells	0.263	280
Lymphangiosis	negative positive	0.483	229

Hormone receptor status	triple negative positive	0.423	275
ER	negative positive	0.009	283
PR	negative positive	0.019	282
HER2	negative positive	0.329	243
Recurrance	no yes	0.319	269

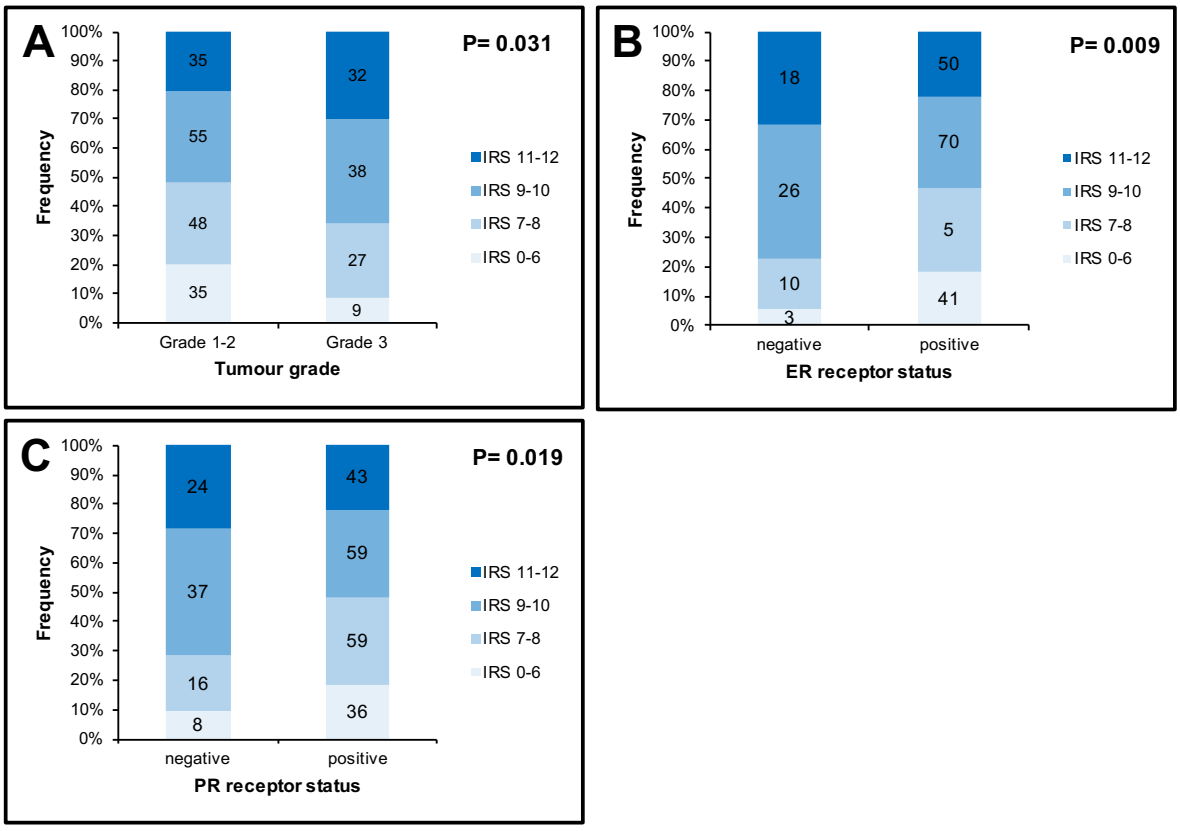

Figure 12: HYAL1 expression levels in primary breast cancer in correlation to clinical and pathological data. (A) Tumour grade, n = 279. (B) ER receptor status, n = 283. (C) PR receptor status, n = 282. Data in columns represent case numbers. P-values as shown in graph.

High HYAL1 expression levels correlated with high tumour grade (p = 0.031). Thus, 66% of all grade 3 cases had medium to high HYAL1 expression levels but only 51% of grade 1-2 cases had medium to high HYAL1 expression levels (Figure 12A). High HYAL1 expression levels were associated with ER-negativity (p = 0.009). Here, more than three quarters (78%) of all ER-negative cases had medium to high HYAL1 expression levels. In comparison, only 53% of all ER-positive cases had medium to high HYAL1 expression levels (Figure 12B). Strong HYAL1 staining correlated with PR-negativity (p = 0.019). Nearly three quarters (72%) of all PR-negative cases had medium to high HYAL1 expression levels, while only 52% of all PR-positive cases had medium to high HYAL1 expression levels (Figure 12C).

HYAL1 expression levels in primary tumours and corresponding lymphatic nodes

A total of 89 lymphatic nodes, that each corresponded to a primary breast cancer case in this collective, were stained for HYAL1 to detect any association between HYAL1 expression levels in the primary tumour and in the corresponding lymphatic nodes. 70 of those were analysable, which showed a similar IRS distribution to the primary breast cancer cells, with a mean of 8.54. Of the analysable primary breast cancer sections and the analysable lymphatic node sections 55 pairs were available. In this 55 pairs the staining pattern seemed to be random and no trend of stronger staining or weaker staining in lymphatic nodes in comparison to the staining in the primary tumour could be detected (results are not shown).

HYAL1 expression and patient survival

By Kaplan-Meier analysis and log-rank test, HYAL1 expression levels in primary breast cancer showed no significant associations to relapse and overall survival (p = 0.442 and 0.659) (Figure 13). Here, 26% of all HYAL1 IRS 9-12 cases, 18% of all HYAL IRS 6-8 cases, and only 15% of all IRS 0-5 cases suffered a relapse. Hence, the higher the HYAL1 expression levels in tumour cells, the more patients suffered a relapse (Figure 13A). Additionally, 16% of all HYAL1 IRS 9-12 cases, 14% of all IRS 6-8 cases, and only 8% of all IRS 0-5 cases died. Therefore, the higher the HYAL1 expression in tumour cells, the more patients succumbed to the disease (Figure 13B).

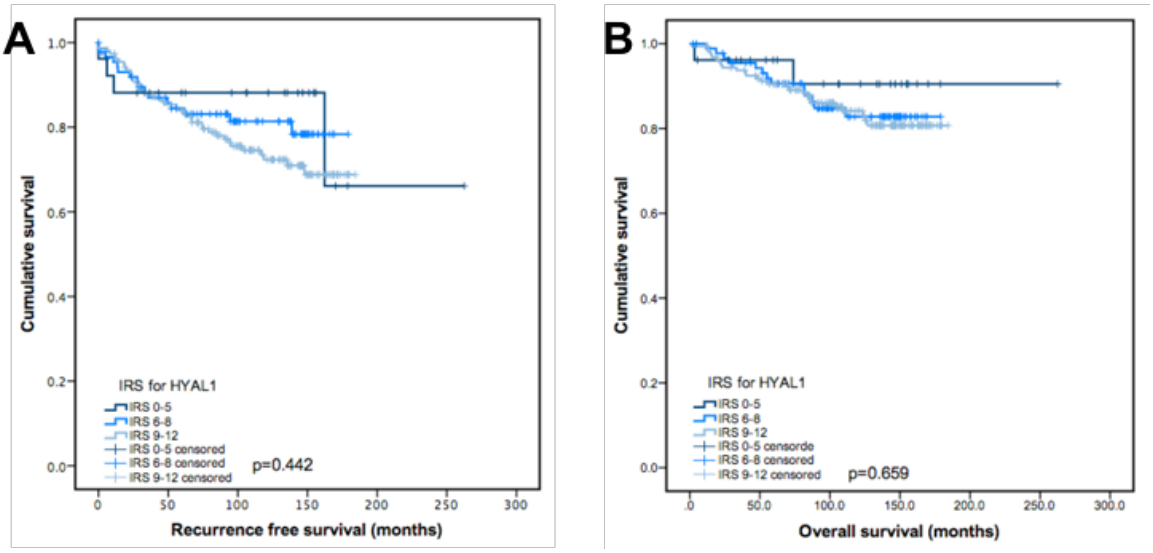

Figure 13: Kaplan-Meier analysis of primary breast cancer patients under the consideration of HYAL1 expression. (A) Recurrence free survival. (B) Overall survival. P-values as shown in graph.

3.3.2 HYAL1 EXPRESSION IN BREAST CANCER BRAIN METASTASES

HYAL1 immunohistochemistry in breast cancer brain metastases

Of the 132 HYAL1 stained BCBM samples included in the TMA, 87 were analysable. Overall, the HYAL1 staining pattern in brain metastases matched the HYAL1 staining pattern of the primary tumours, with a mean of 8.87 (Figure 14).

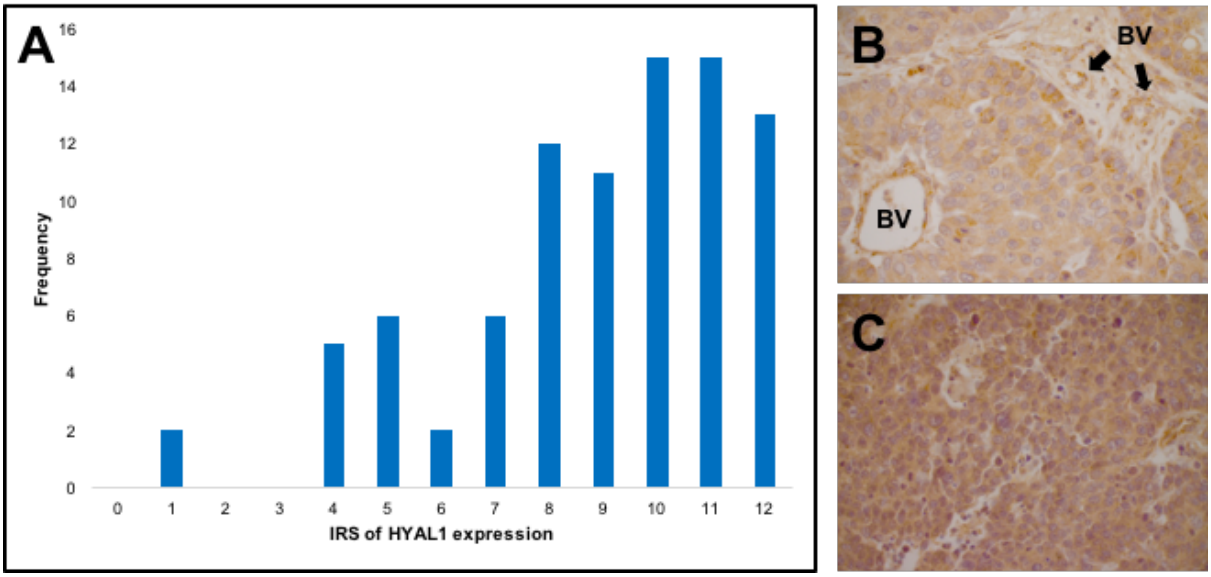

Figure 14: HYAL1 expression in BCBM. (A) IRS distribution of HYAL1 with n = 87; mean = 8.87. (B-C) HYAL1 staining pattern in BCBM. (B) 40x, IRS 6-8/medium with some strongly stained tumour cells and stained blood vessels (BV). (C) 40x, IRS 11-12/high HYAL1 expression.

Here, 83% of all brain metastases had medium to high HYAL1 expression levels (IRS 7-12) (Figure 14B and C), while only 17% had negative to weak HYAL1 expression levels (IRS 0-6).

The association of HYAL1 in BCBM with prognostic factors

No significant correlations between HYAL1 expression levels and the age at time of surgery, ER, PR, and HER2 status in the primary tumour, histological subtype of brain metastases, and ER, PR, and HER2 status of brain metastases were found (Table 14).

Table 14: Correlations of HYAL1 IRS in BCBM with clinical and pathological data.

	Groups	P-value	n
Age at time of surgery	above median below median	0.083	87
ER primary tumour	negative positive	0.445	36
PR primary tumour	negative positive	0.761	37
HER2 primary tumour	negative positive	0.622	33
Histological subtype of brain metastases	triple negative luminal HER2 positive	0.857	79
ER brain metastases	negative positive	0.678	78
PR brain metastases	negative positive	0.379	77
HER2 brain metastases	negative positive	0.778	78

3.3.3 HYAL1 EXPRESSION IN BREAST CANCER METASTASES VARIOUS LOCATIONS

HYAL1 immunohistochemistry in breast cancer metastases samples from different sites

Of the 130 HYAL1 stained samples of breast cancer metastases various locations 89 were analysable. The overall HYAL1 expression levels were weaker than in the other collectives and relatively homogeneously distributed with a mean of 6.08 (data not shown). For further statistical analyses, cases were grouped according to the IRS values in four groups: negative HYAL1 expression levels (IRS 0), weak HYAL1 expression levels (IRS 1-3), medium HYAL1 expression levels (IRS 4-8), and strong HYAL1 expression levels (IRS 9-12).

HYAL1 expression in BCBM in comparison to breast cancer metastases from other sites

In order to learn more about the role of HYAL1 in the development of especially brain metastases, HYAL1 expression levels in BCBM were compared with HYAL1 expression levels in breast cancer metastases derived from the bone, skin, liver, and lung. Chi-square analyses, where HYAL1 expression levels of all different metastatic sites were analysed, showed significant differences between the different kind of metastases ($p < 0.001$) (Figure 15). High HYAL1 expression levels were significantly more prevalent in bone (17%), skin (44%), liver (41%), and lung metastases (76%) than in brain metastases (0%). Almost all brain metastases had negative to weak HYAL1 expression levels (86%) and only a few (14 %) had medium HYAL1 expression levels (Figure 15).

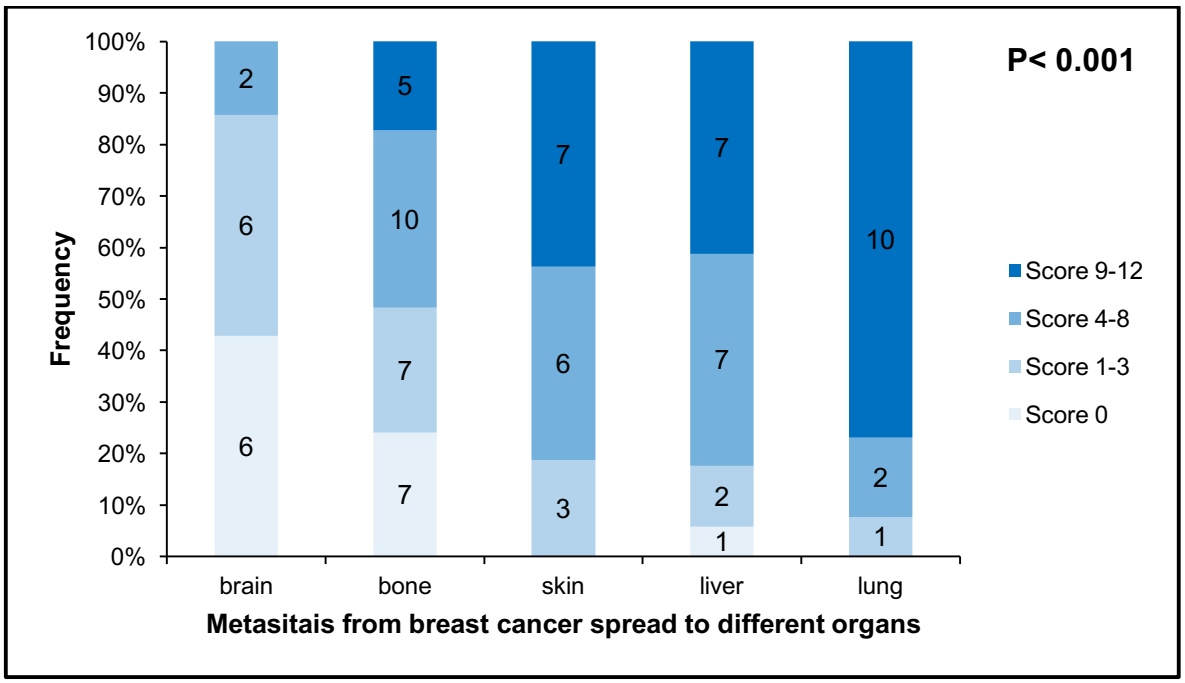

Figure 15: HYAL1 expression levels in metastases from breast cancer spread to the brain (n = 14), bone (n = 29), skin (n = 16), liver (n = 17), and lung (n = 13). Total n = 89. Data in columns represent case numbers. P-value as shown in graph.

The microscopic analysis revealed that in all breast cancer metastases HYAL1 was located in the tumour cell cytoplasm, however in a few cases also in the nucleus (Figure 16). Furthermore, HYAL1 staining was also detected in some tumour surrounding structures, such as blood vessels (Figure 16A and C), neurons (not shown), inflammatory cells (Figure 16A, B, D, E, and F), epithelium and glands of the skin (not shown), and hepatocytes (Figure 16E). Interestingly, in BCBM tumour cells at the edge of the tumour, next to the surrounding non-tumourous brain tissue, often displayed higher HYAL1 expression levels, than tumour cells in the centre of the tumour (Figure 16A).

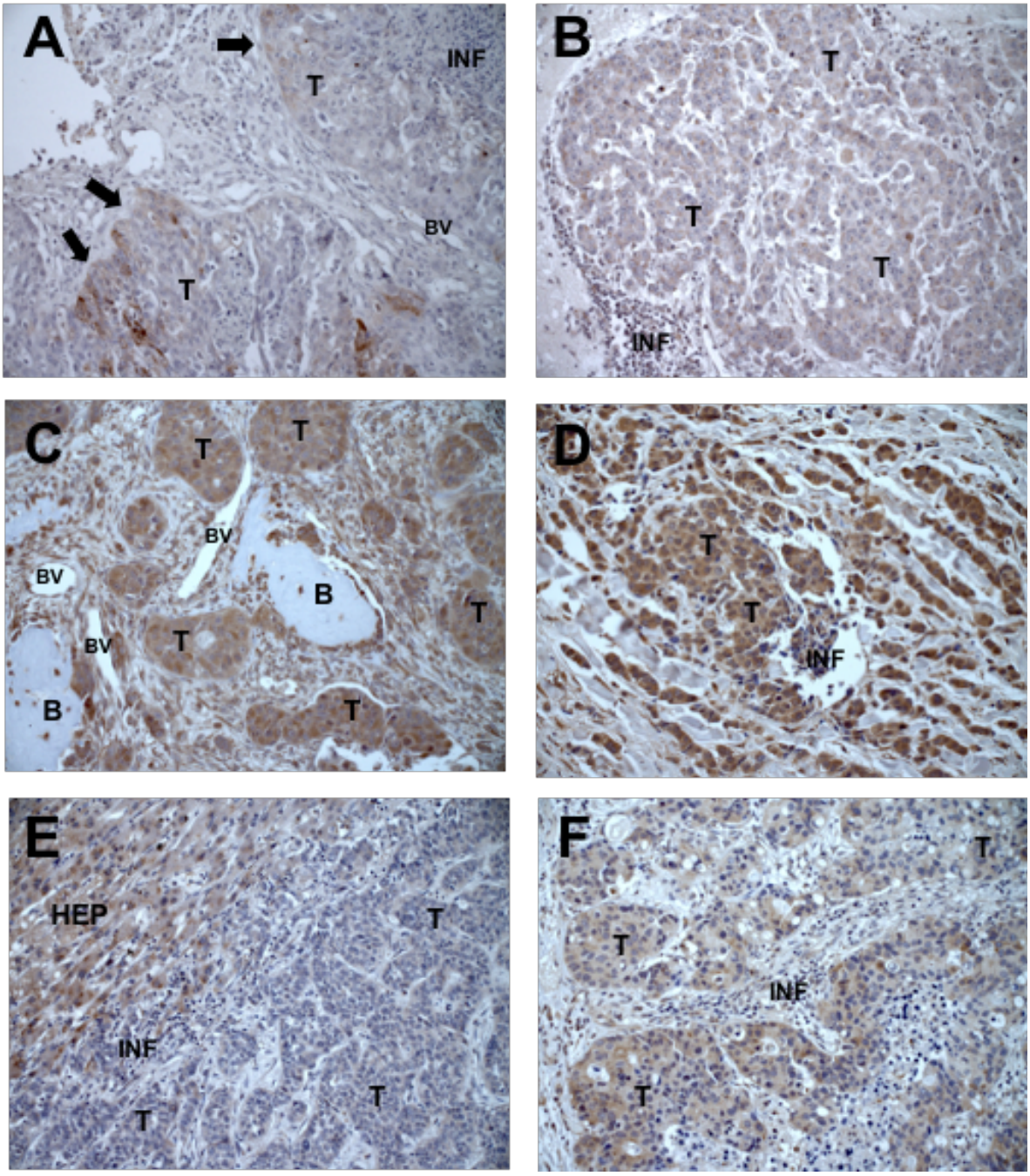

Figure 16: HYAL1 staining pattern in breast cancer metastases various locations. (A) Brain metastases with HYAL1-negative to positive tumour cells (T) which show higher HYAL1 expression levels at the tumour invasion front (arrow), HYAL1 negative inflammatory cells (INF), and HYAL1 negative blood vessels (BV) (20x, IRS 0-8). (B) Brain metastases with HYAL1 positive tumour cells (T), and HYAL1 positive inflammatory cells (INF) (20x, IRS 4-8). (C) Bone metastases with HYAL1 positive tumour cells (T), HYAL1 negative bone tissue (B), and HYAL1 positive blood vessels (BV) (20x, IRS 0 and IRS 11). (D) Skin metastases with HYAL1 positive tumour cells (T) and HYAL1 positive inflammatory cells (INF) (20x IRS 10-12). (E) Liver metastases with HYAL1 negative and weakly stained tumour cells (T), HYAL1 positive hepatocytes (HEP), and HYAL1 positive inflammatory cells (INF) (20x, IRS 0-2, and 4-6). (F) Lung metastases with HYAL1 positive tumour cells (T) and HYAL1 negative inflammatory cells (20x, IRS 4-5 and IRS 0).

3.3.4 HYAL1 EXPRESSION IN PRIMARY BREAST CANCER WITH AND WITHOUT CORRESPONDING BRAIN METASTASES

To detect the influence of HYAL1 prevalence in primary breast cancer on the formation of BCBM, primary breast cancer cases with corresponding brain metastases as well as primary breast cancer samples of patient cases without brain metastases were stained for HYAL1. Interestingly, HYAL1 expression levels in primary breast cancer samples of patients that suffered from brain metastases was higher than in samples from patients without brain metastases. By t-tests a significant difference between these two groups could be confirmed ($p = 0.001$). Further, HYAL1 IRSs in primary breast cancer samples of patients with metastases was significant higher than IRS in the corresponding brain metastases samples ($p = 0.003$) (Figure 17).

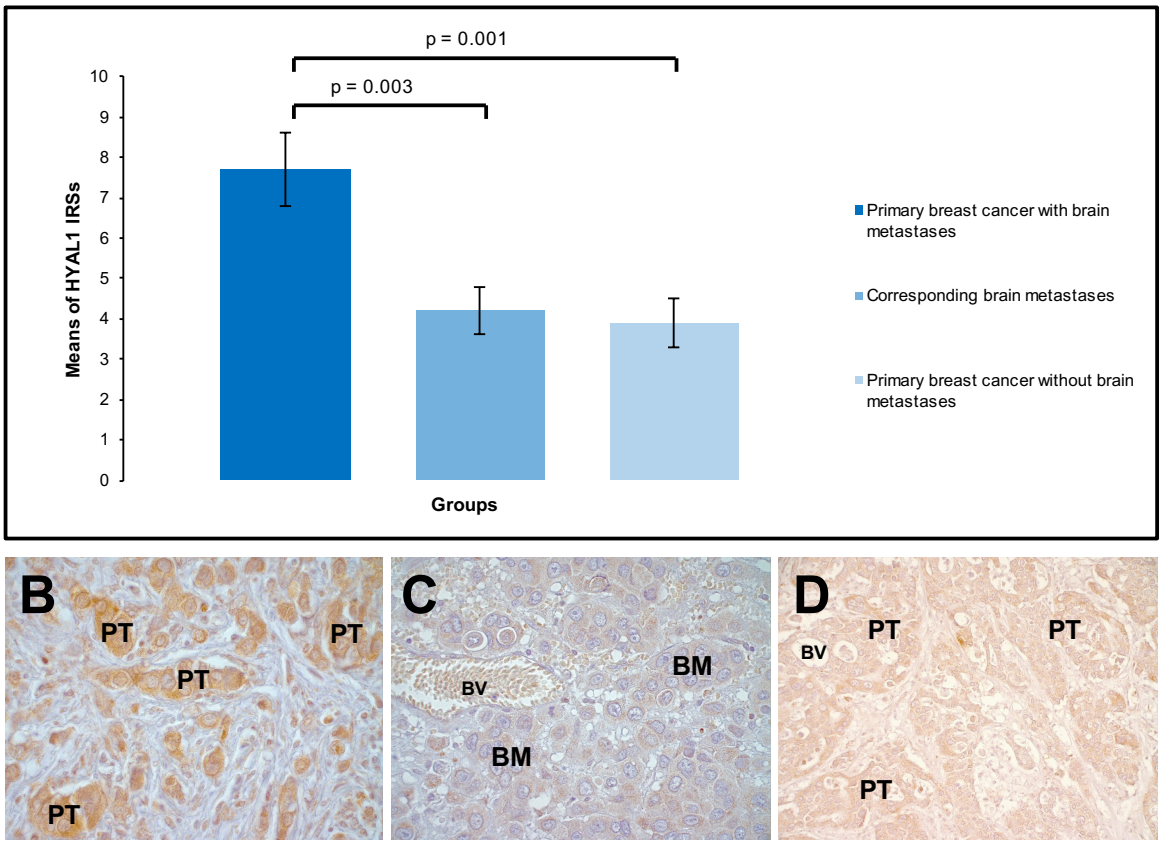

Figure 17: HYAL1 expression levels in pairs of primary breast cancer and corresponding BCBM, both from the same patient. (A) Means of HAYL1 IRSs in three groups: primary breast cancer, which formed brain metastases (dark blue), the corresponding brain metastases (middle light blue), and primary breast cancer, which did not form brain metastases (light blue). The error bars represent the standard error in each group. P-values as shown in graph. (B-C) HYAL1 staining pattern in the primary tumour (PT) (20x, overall IRS = 7), and its corresponding brain metastasis (BM) (40x, overall IRS = 3). B and C both derived from the same patient. (D) HYAL1 staining pattern in primary breast cancer, which did not metastasis (20x, IRS = 4). BV = blood vessel.

3.4 HA EXPRESSION IN BREAST CANCER METASTASES

Tissue sections of BCBM and other breast cancer metastases were stained for HAbp in order to detect the influence of the HA synthesising and degrading enzymes, HAS2 und HYAL1, on the expression of HA in metastases from breast cancer. In microscopic analysis, HAbp stained tumour cells and TMEs were evaluated separately. This way, it can be distinguished between two mechanisms: First, HA production by tumour cells and secondly, HA production by tumour associated fibroblasts, which closely interact with the tumour cells via cross-talk.

3.4.1 HA EXPRESSION IN BREAST CANCER BRAIN METASTASES

HA histochemistry in breast cancer brain metastases

In 90 of the 132 HAbp stained BCBM samples included in the TMA, the tumour cells were analysable. Here, more than three quarter (78%) of the tumour cells were stained negative to weak (IRS 0-3) and only a few tissue samples had medium to high HA expression levels. The mean was 1.98 (Figure 18A, B, and D). For further statistical analyses, cases were grouped accordingly in three groups: negative HA expression levels in tumour cells (IRS 0), weak HA expression levels in tumour cells (IRS 1-3), and medium to high HA expression levels in tumour cells (IRS 4-12).

In comparison to the tumour cells, the HAbp staining in the TMEs was much stronger. In 75, of the 132 HAbp stained BCBM samples included in the TMA, the TME could be evaluated. Of those cases only over a third (37%) were negative or weakly stained (IRS 0-3), the other two thirds had medium to strong HA expression levels. The mean was 5.35 (Figure 18B, C, and D). For further statistical analyses, cases were grouped accordingly in three groups: negative to weak HA expression levels in the TMEs (IRS 0-3), medium HA expression levels in the TMEs (IRS 4-8), and strong HA expression levels in the TMEs (IRS 9-12).

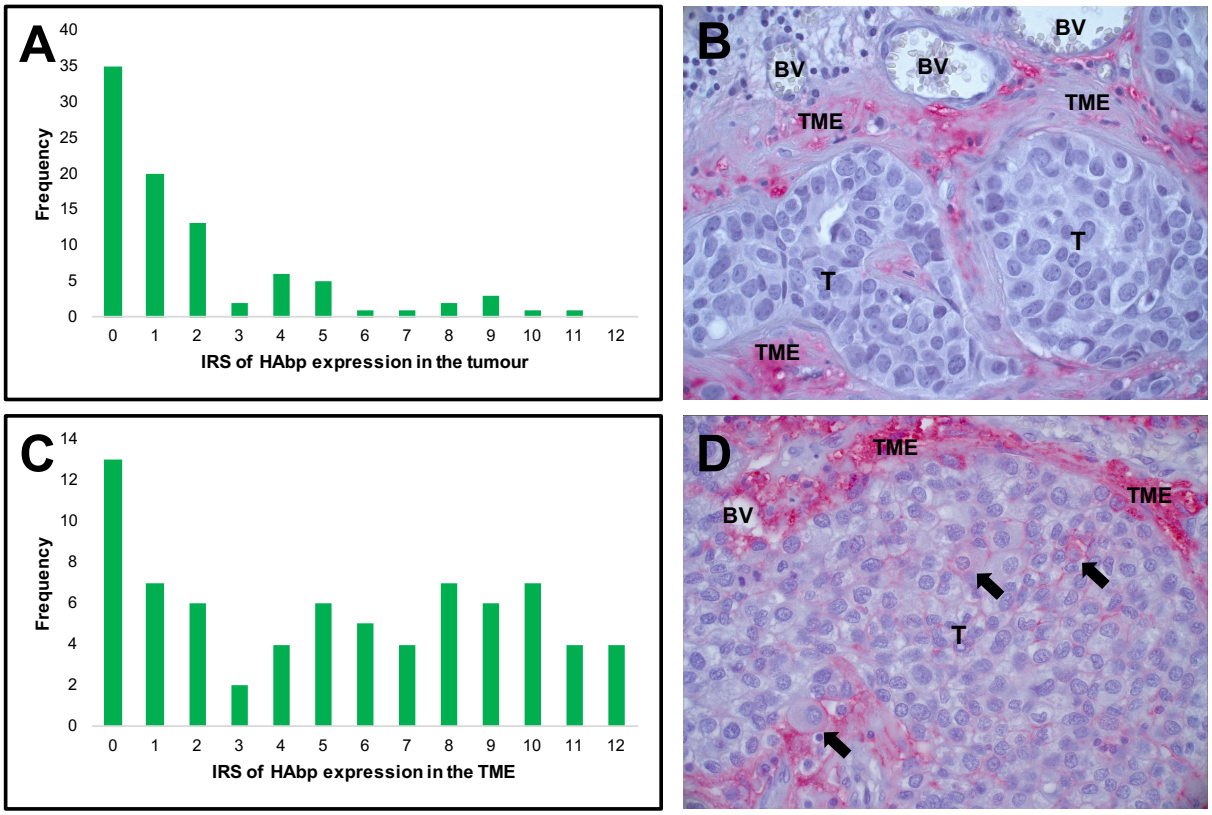

Figure 18: HA expression levels in tumours and TMEs of BCBM. (A) IRS distribution of HA expression levels in tumours with n = 90; mean = 1.98. (C) IRS distribution of HA expression levels in TMEs with n = 75; mean = 5.35. (B and D) HAbp staining pattern in BCBM. T = tumour; TME = tumour micro environment; BV = blood vessel. (B) 40x, IRS of the tumour 0/negative, IRS of the TME 8-12/medium-strong. (D) 40x, IRS of the tumour 4-8/weak-medium, IRS of the TME 8-12/medium-strong. In all tumour cells the membrane is stained. Some tumour cells also show positive HA expression in the cytoplasm (arrow).

The association of HA in breast cancer brain metastases with prognostic factors

No significant correlations between HA expression levels in tumour cells and the age at time of surgery, ER status in the primary tumour, and ER and HER2 status in brain metastases were found (Table 15). However, chi-square analyses including HA expression levels in tumour cells and the PR and HER2 status of the primary tumour, histological subtype of brain metastasis, and the PR status of the brain metastases showed significant correlations (Table 15 and Figure 19). Medium to high HA expression levels in tumour cells were associated with ER-negativity in the primary tumours of the brain metastases ($p = 0.050$) (Figure 19A). Also medium to high HA expression levels in tumour cells correlated with PR-negativity in the primary tumours of brain metastases ($p = 0.037$), where 29% of all PR-negative cases had medium to high HA expression levels, while in PR-positive cases it was only 20% (Figure 19B).

Table 15: Correlations of HAbp IRS in the tumour and TME of BCBM with clinical and pathological data. Significant result (p<0.05) are shown in green.

	Groups	P-value Tumour	n Tumour	P-value TME	n TME
Age at time of surgery	above median below median	0.154	90	0.006	75
ER primary tumour	negative positive	0.050	37	0.814	29
PR primary tumour	negative positive	0.037	38	0.359	30
HER2 primary tumour	negative positive	0.041	34	0.180	27
Histological subtype of brain metastases	triple negative luminal HER2 positive	0.023	83	0.075	69
ER brain metastases	negative positive	0.066	81	0.236	67
PR brain metastases	negative positive	0.028	80	0.935	66
HER2 brain metastases	negative positive	0.068	82	0.066	68

Strong HAbp staining correlated with HER2-negativity in the primary tumours of brain metastases, where 60% of all HER2-negative cases but only 14% of all HER2-positive cases had medium to high HA expression levels (p = 0.041) (Figure 19C). Medium to high HA expression levels in tumour cells correlated with the triple negative histological brain metastasis subtype (p = 0.023). Here, 42% of all triple negative brain metastases had medium to strong HA expression levels, however in luminal and HER2 positive cases it was only 13% and 11% (Figure 19D). Medium to high HA expression levels in tumour cells were associated with ER- negativity in brain metastases (p = 0.066)

(Figure 19E). Medium to high HA expression levels in tumour cells correlated with PR-negativity in brain metastases (p = 0.028), where 28% of all PR-negative cases had medium to high HA expression levels, while in PR-positive cases none were stained medium to high (Figure 19F). Additionally, no significant correlations between HA expression levels in the TMEs and ER, PR, and HER2 status in the primary tumour, histological subtype of brain metastasis, and the ER, PR, and HER2 status in brain metastases were found (Table 15). However, chi-square analysis including HA expression levels in the TMEs and the age at time of surgery showed significant correlations (Table 15).

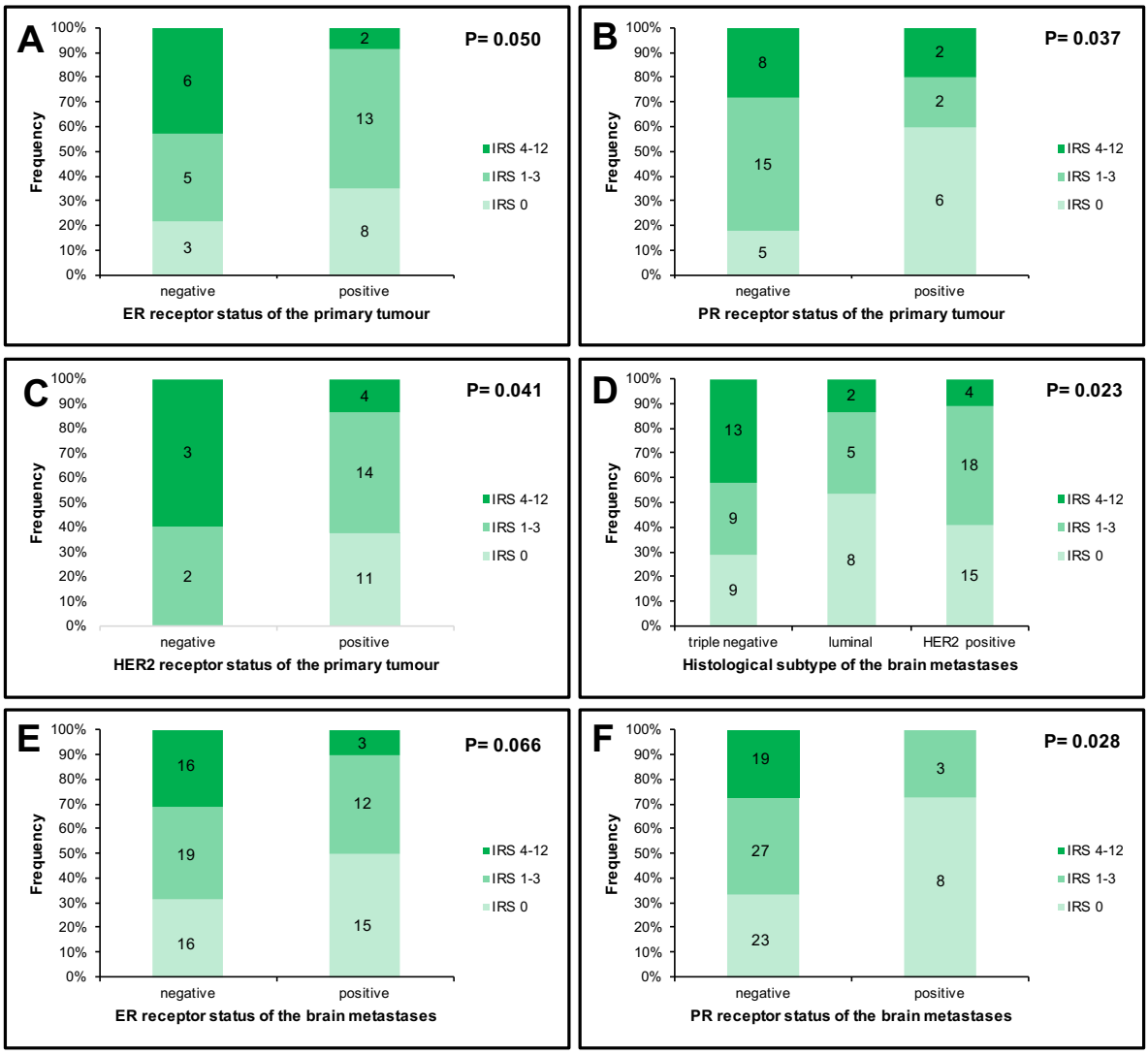

Figure 19: HA expression levels in the tumour cells of BCBM in correlation to clinical and pathological data. (A) ER receptor status of the primary tumour, n = 37. (B) PR receptor status of the primary tumour, n = 38. (C) HER2 receptor status of the primary tumour, n = 34. (D) Histological subtype of brain metastases, n = 83. (E) ER receptor status of brain metastases, n = 81. (F) PR-receptor status of brain metastases, n = 80. Data in columns represent case numbers. P-values as shown in graph.

3.4.2 HA EXPRESSION IN BREAST CANCER METASTASES VARIOUS LOCATIONS

HA histochemistry in breast cancer metastases samples from different sites

In 80 samples of the 130 HAbp stained breast cancer metastases derived from different sites the tumour cells were analysable. Here, 21% of the tumour cells were HAbp negative, more than half of the tumour cells (58%) had weak HA expression levels and another 21% had medium HA expression levels. None of the tumours had strong HA expression levels. The mean was 2.05 (data not shown). For further statistical analysis, the HAbp stained tumour cell cases were grouped according to the IRS expression levels in three groups: negative HA expression levels (IRS 0), weak HA expression levels (IRS 1-3), and medium HA expression levels (IRS 4-8).

In 76 samples of the 130 HAbp stained breast cancer metastases derived from different sites the TMEs were analysable. Interestingly, the IRS distribution in the TMEs was quite different to the IRS distribution in the tumour cells: only 8% of TME cases were HAbp negative, 20% were stained weakly, 20% were stained at medium strength, and 40% were stained strongly. The mean of the TME IRS was with 6.71 much higher than the mean of the tumour cell IRS (data not shown). For further statistical analysis, the HAbp stained TME cases were grouped accordingly into four groups: negative HA expression levels (IRS 0), weak HA expression levels (IRS 1-3), medium HA expression levels (IRS 4-7), and strong HA expression levels (IRS 8-12).

HA expression in breast cancer brain metastases in comparison to HA expression in breast cancer metastases from other sites

In order to learn more about the role of HA in the development of particularly brain metastases, HA expression levels in BCBM were compared with HA expression levels in breast cancer metastases derived from the bone, skin, liver, and lung. Chi-square analyses showed significant differences amongst the different kind of breast cancer metastases in both HA expression levels in tumour cells ($p < 0.005$) and HA expression levels in TMEs ($p = 0.045$) (Figure 20). In bone (15%), skin (22%), liver (10%), and lung metastases (17%) the percentages of medium HA expression levels in tumour cells were much lower than in brain metastases (43%). All brain metastases had positive HAbp tumour cells, while in all other metastases HAbp-negative tumours were

also found (Figure 20A). In comparison the HAbp TME evaluation revealed quite the opposite picture: In bone (36%), skin (26%), liver (78%), and lung metastases (64%) the percentages of high HA expression levels in tumour cells were much higher than in brain metastases (17%). Off all metastases, the TME of brain metastases had the lowest HA expression levels (Figure 20B).

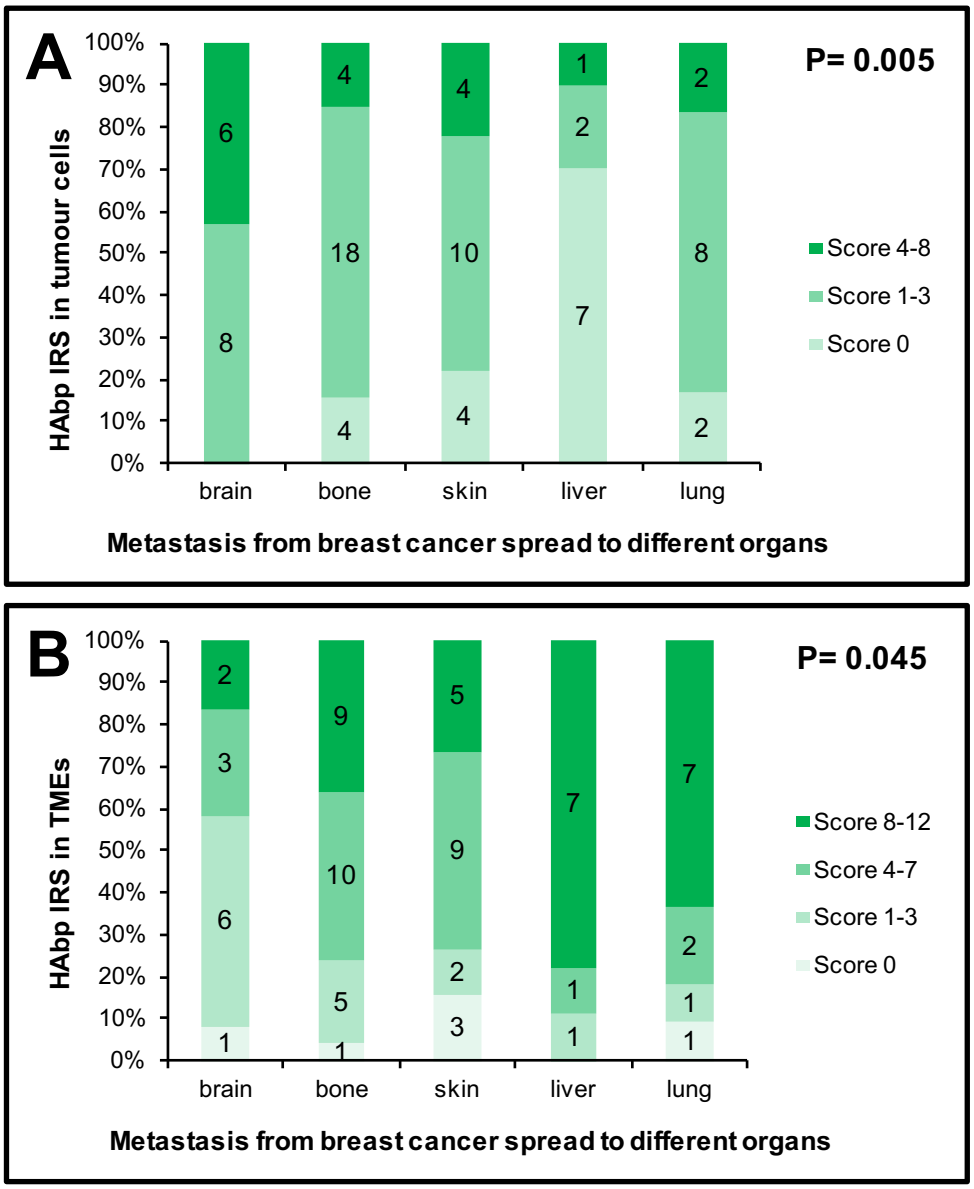

Figure 20: HA expression levels in tumour tissue and TME of metastases from breast cancer spread to different organs. (A) Ha expression levels in tumour cells of metastases to the brain (n = 14), bone (n = 26), skin (n = 18), liver (n = 10), and lung (n = 12). Total n = 80. (B) Ha expression levels in TMEs of metastases to the brain (n = 12), bone (n = 25), skin (n = 19), liver (n = 9), and lung (n = 11). Total n = 76. Data in columns represent case numbers. P-value as shown in graph.

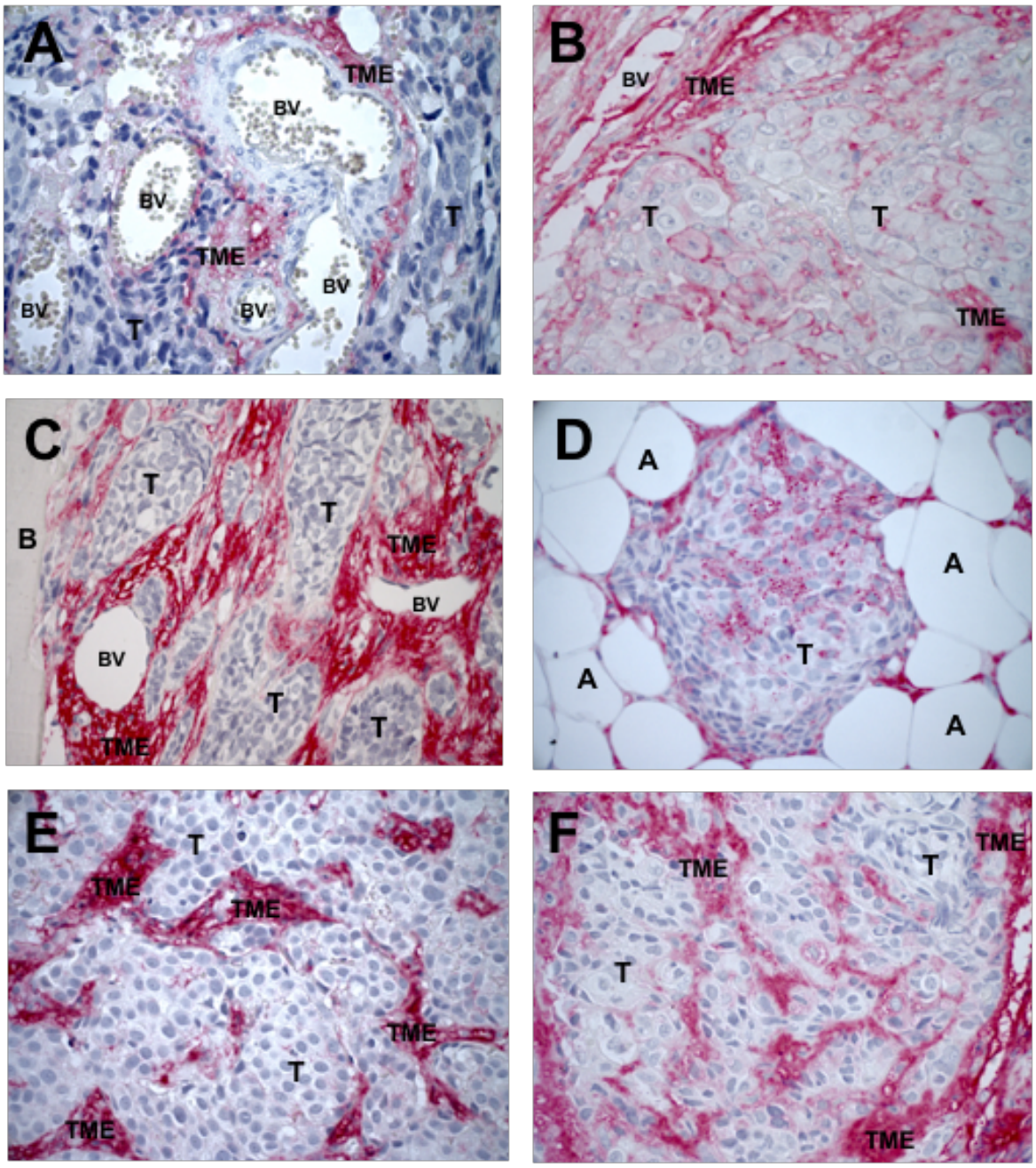

Figure 21: HAbp staining pattern of tumour and TME in breast cancer metastases various locations. (A) Brain metastases with HAbp-negative to positive tumour cells (T), HAbp negative to positive TME and HAbp negative to positive blood vessels (BV) (20x, IRS 0-8). (B) Brain metastases with HAbp positive tumour cells (T) and HAbp positive TME. All tumour cells show HA expression in the membrane and some in the cytoplasm (20x, IRS 2-8 and IRS 6-10). (C) Bone metastases with HAbp negative tumour cells (T), HAbp positive TME, and HAbp positive blood vessles (BV) (20x, IRS 0 and IRS 11-12). (D) Skin metastases with HAbp positive tumour cells (T) and HAbp positive adipocytes (A) (20x IRS 4-8 and IRS 10-12). (E) Liver metastases with HAbp negative and weakly stained tumour cells (T) and HAbp positive TME (20x, IRS 0-4, and 10-12). (F) Lung metastases with HAbp negative to positive tumour cells (T) and HAbp positive TME (20x, IRS 0-4 and IRS 8-12).

The microscopic analysis revealed that in all breast cancer metastases HAbp was most often located in the membrane, however at times also in the cytoplasm of the tumour cell (Figure 21). In most tissue samples the TME was HAbp positive and HAbp was found additionally in some tumour external structures, such as blood vessels (Figure 21A and C), epithelium and glands of the skin (not shown) and adipocytes of the skin (Figure 21D), and hepatocytes (not shown).

4 DISCUSSION

4.1 THE INFLUENCE OF HYALURONIC ACID METABOLISM ON THE DEVELOPMENT OF BREAST CANCER BRAIN METASTASES

During the past decades we have witnessed great improvement in breast cancer detection and treatment, which lead to improved patient outcomes. Nevertheless, 15-30% of breast cancer patients develop brain metastasis, which therefore has become a major life limiting and life quality limiting factor. It is well known that HA metabolism is altered in many different invasive tumours. However, the multistep process of brain metastasis formation itself is still poorly understood and, to our knowledge, the role of HA and its enzymes HAS2 and HYAL1 in the development of BCBM has not been investigated to date. In previous studies based on microarray data of breast cancer patients, we were able to demonstrate that mRNA expression of HAS2 and HYAL1 significantly correlated with brain metastases formation (Milde-Langosch et al., 2014, Milde-Langosch et al., 2015). In this study we showed that HYAL1 expression at protein level was significantly higher in primary breast tumours of patients which subsequently developed BCBM in comparison to primary breast tumours that did not metastasise into the brain. These results indicate the potential role of HYAL1 in breast cancer cell dissemination and brain-specific colonisation. Comparing HYAL1 expression levels in the primary breast cancer and corresponding BCBM, we detected higher HYAL1 levels in the primary tumour than in the corresponding brain metastases. Furthermore, we showed that HYAL1 and HAS2 expression levels were lower in BCBM than in breast cancer metastases of other locations such as skin, liver, lung, and bone. Additionally, we found significantly high HA expression levels in tumour cells of BCBM in comparison to breast cancer metastases in skin, liver, lung, and bone. Surprisingly the opposite was observed in the TME, where HA expression levels were the highest in BCBM in comparison to breast cancer metastases of the other tissues.

Several studies have demonstrated that high HYAL1 expression levels in breast cancer are associated with tumour cell proliferation, migration, invasion, and angiogenesis (Poola et al., 2008, Tan et al., 2011a, Tan et al., 2011b). However, no study has previously investigated HYAL1 expression levels in primary breast cancer

tissue with brain metastases in comparison to primary breast cancer tissue without brain metastases. We were able to show that HYAL1 expression levels in primary breast cancer tissue from which subsequently brain metastases developed are significantly higher than HYAL1 expression levels in primary breast cancer tissue, which did not metastasise. These novel results suggest that the HA degrading enzyme HYAL1 may play a crucial role in the process of breast cancer brain metastasis formation. Still, its exact function in the multi-step process of brain metastases formation remains to be discovered.

Invasiveness and aggressiveness in breast cancer is associated with altered HA metabolism and increased deposition of HA, especially in the TME of the tumours invading edges (de la Torre et al., 1993, Koyama et al., 2007). A HA rich tumour stroma provides a favourable microenvironment for malignant progression by maintaining osmotic balance and hydration while facilitating cell proliferation and migration (Koyama et al., 2007, Tan et al., 2011a). Interestingly, the function of HA is dependent on its molecular size and especially small fragments produced by HYAL1 have been reported to be generated primarily by highly invasive tumour cells and to promote tumour proliferation, migration, invasion, and angiogenesis (Jiang et al., 2011, Lepperdinger et al., 1998, Tan et al., 2011b, Udabage et al., 2005, Wu et al., 2015). Moreover, Tan et al. demonstrated that knock-down of HYAL1 in breast cancer cells reduces cell growth, adhesion, and invasion in culture and also decreases tumour growth in vivo (Tan et al., 2011a). Therefore, this cycle of increased HA deposition and HA degradation into small fragments by HYAL1 seems to be an intelligent way of tumour cells and their stromal partners to sustain tumour progression and invasion and may facilitate the development of metastases.

Multiple studies in the past revealed the impact of low molecular weight HA fragments on the interaction of tumor cells and the endothelial barrier. Recently, it has been shown that low molecular weight HA can activate specific HA-binding proteins during tumor progression, promoting disruption of endothelial cell–cell contacts. This is an essential initiating step for tumor angiogenesis and metastasis (Singleton, 2014). It also has been suggested that HA fragments are able to assemble at the cell surface to form a hyaluronan matrix, which in turn promotes adhesion to bone marrow endothelial cells (Simpson et al., 2001). Furthermore, low molecular weight HA has been reported to induce the integrin-mediated adhesion of colon cancer cells to

endothelial cells (Fujisaki et al., 1999). Whether HYAL1 overexpression in primary breast cancer leads to BBB disruption and enhanced tumour cell adhesion to brain endothelial cells and whether this potential effect is mediated by HYAL1-induced low molecular weight HA fragments needs to be clarified in further studies.

A number of studies associated high HAS2 levels in breast cancer with tumour aggressiveness, invasiveness, and angiogenesis (Bernert et al., 2011, Koyama et al., 2007, Li et al., 2007). In previous studies, we were able to show a significant correlation between mRNA expression of HAS2 and brain metastases formation (Milde-Langosch et al., 2014, Milde-Langosch et al., 2015). However, in this study we were not able to show a significant difference in HAS2 expression at protein level in primary breast cancer tissue which formed brain metastases in comparison to primary breast cancer tissue which did not form metastases. This might result from small sample size or lack of correlation between RNA and protein levels for example due to post transcriptional regulations. Therefore, the impact of HAS2 on BCBM formation remains unclear and subject to future research.

Compared with high HYAL1 expression levels found in primary tumours from patients with BCBM, we observed significantly lower HYAL1 expression levels in corresponding brain metastases. Additionally, we detected significantly lower HAS2 and HYAL1 staining in BCBM than in breast cancer skin, liver, lung, or bone metastases. While HYAL1 may play an important role in the process of tumour cell dissociation from the primary tumour mass, migration through the BBB, and implantation into the brain tissue, our findings implicate that high HYAL1 or HAS2 expression levels may not be a requirement for subsequent metastatic growth of breast cancer cells within the brain parenchyma. It has been shown that brain metastases from breast cancer grow in two different ways, either as leptomeningeal lesions or as parenchymal lesions. The latter were mainly observed around small blood vessels, which suggests that these brain blood vessels aid as “soil” for tumour growth (Witzel et al., 2016). Taking under consideration that HYAL1 is often up-regulated in primary breast cancer (Tan et al., 2011a) and that HYAL1 produces small pro-angiogenic HA fragments, it can be reasoned that HAYL1 contributes to the disruption of brain blood vessel integrity and the process of tumour cells “seeding” into the brain parenchyma. However, HYAL1 up-regulation may not be further needed to promote metastatic growth once the process of colonisation has been completed.

In this study we found significantly higher HA expression levels in tumours cells of breast cancer brain metastasis in comparison to breast cancer metastases in skin, liver, lung, and bone. Surprisingly the opposite was detected in the TME, where HA expression levels were the lowest in BCBM in comparison to breast cancer metastases of the other tissues. It can be assumed, that the HA found within the tumour cells is produced by the tumour cells themselves, while the HA found within the TME might either be produced by tumour cells or by tumour associated stroma cells. HA influences the tumour cell behaviour, the interaction with its environment, and cancer progression by modulating the hydration and osmotic balance within the TME (Udabage et al., 2005). Furthermore, HA actively regulates tumour cell proliferation, adhesion, migration, as well as neovascularisation (Auvinen et al., 2000, Tan et al., 2011a). Possibly, intricate crosstalk between tumour and stroma cells takes place, where tumour cells stimulate stroma cells to produce HA or vice versa. Several studies suggested that increased HA levels in breast cancer are not just the result of increased HA synthesis by tumour cells but also by tumour cell stimulated stromal cells (Asplund et al., 1993, Auvinen et al., 2000, Knudson et al., 1984, Schor et al., 1989). These observations show how specific the conditions and how complex the extracellular influences on breast cancer cells are in the process of colonisation and proliferation within the brain tissue. It furthermore suggests, that the fundamental TME conditions of a tumour nest vary amongst the different tissue types. Hence, breast cancer tumour cells require different properties for colonisation and proliferation within the brain tissue in comparison to skin, lung, liver, or bone tissue.

Interestingly, we often observed HA enriched stroma especially on the invading edges of the tumour or metastasis, which also has been observed by other study groups (Auvinen et al., 2000, Li et al., 2007, Udabage et al., 2005). Because of relatively high tumour cell associated HA expression levels in BCBM and relatively low HA expression levels in the TME of BCBM, our results suggest that BCBM might depend more on HA produced by tumour cells in particular compared with breast cancer metastasis of other tissues. Taking furthermore under consideration, that HAS2 and HYAL1 levels in tumour cells of BCBM were relatively low, however the HA expression levels relatively high, other HA synthesising and degrading enzymes may be responsible for the high HA expression levels within the tumour cells of BCBM.

4.2 THE PROGNOSTIC SIGNIFICANCE OF HYALURONIC ACID METABOLISM IN BREAST CANCER

HA has been determined as one major ECM component associated with breast cancer progression and invasion, promoting cell proliferation, cell movement, and neovascularisation (Auvinen et al., 2000). In the past years the HA synthesising and degrading enzymes HAS2 and HYAL1 in particular have been associated with invasiveness and aggressiveness in breast cancer (Schwertfeger et al., 2015). Previous studies in our laboratory, concerned with microarray analysis of 194 breast cancer cases with long-term follow-up information, demonstrated that high RNA expression levels of HAS2 and HYAL1 in primary breast cancer are associated with poor prognosis (Milde-Langosch et al., 2014). Based on these novel results, it was our interest to validate the prognostic relevance of HAS2, and HYAL1 expression in primary breast cancer on protein level by performing HAS2 and HYAL1 IHC on a TMA with 411 primary tumours and by analysing the HAS2 and HYAL1 expression in relation to long-term follow-up data. We could show a tendency between high HYAL1 expression in primary breast cancer and shorter overall survival and recurrence free survival as well as a tendency between HAS2 expression and shorter overall survival. However, in this study data lacked statistical significance, hence the prognostic role of HAS2 and HYAL1 that we observed in our previous work on cDNA microarray data of breast cancer patients could not be confirmed. Whether this might be based on a lack of correlation between protein and mRNA levels could not be clarified in our study due to the fact that the two cohorts, TMA and the previous cDNA microarray, only included a few common patients.

Although in many studies HYAL1 has been associated with invasiveness and tumour progression in breast cancer and HYAL1 expression in non-invasive ductal hyperplasia has been demonstrated to correlate with subsequent development of invasive breast cancer (Poola et al., 2008), this is the first attempt to determine the prognostic significance of HYAL1 expression in breast cancer relating recurrence free survival and overall survival on protein level. Previous studies indicated that increased amounts of HA in tumour cells or stroma predict poor survival in patients with breast cancer (Auvinen et al., 2000), bladder cancer (Kramer et al., 2011), colorectal cancer (Ropponen et al., 1998), gastric cancer (Setala et al., 1999), pancreatic cancer (Cheng

et al., 2013), and prostate cancer (Posey et al., 2003). Furthermore, increased expression of HYAL1 has been shown to be associated with poor patient outcome. For example, Kramer et al. demonstrated that HYAL1 is a predictor for disease specific mortality and that HYAL1 and HAS2 combined predict shorter recurrence free survival (Auvinen et al., 2000). Posey et al. demonstrated a correlation between high HYAL1 levels in prostate tumour and poor prognosis (Posey et al., 2003) and Ekici et al. as well as Gomez et al. showed that HYAL1 in prostate cancer specimen acts as an independent predictor of recurrence following surgery (Ekici et al., 2004, Gomez et al., 2009). While all those studies support our proposition that HYAL1 in breast cancer may as well be a factor that promotes short recurrence free survival and overall survival, previous research also delivered opposing results. In pancreatic cancer it has been proposed that weak HYAL1 levels are associated with short overall survival (Cheng et al., 2013). While in current research there is a shortfall of information concerning the association of HYAL1 expression in breast cancer with patient survival and disease recurrence, there is much evidence that high HYAL1 levels in cancer implicate shorter overall survival and recurrence free survival and therefore it seems reasonable to assume that HYAL1 also influences patient survival in breast cancer.

This study only showed a loose association between HYAL1 in breast cancer and patient survival on protein level but no statistical significance, another study conducted in our laboratory however confirms our previous findings. Here, HYAL1 levels in protein extract from 150 primary tumour samples were quantified in western blot. We state that high HYAL1 levels tend to correlate with short patient survival (data not published yet). Despite this data not being of statistical significance, there is a clear trend of high HYAL1 levels in breast cancer and poor patient survival. Furthermore, high HYAL1 expression levels in the primary breast cancer TMA correlated with high tumour grade as well as ER- and PR-negativity which are all factors that are associated with poor patient outcome.

In breast cancer, high HA levels in tumour cells and associated stromal cells are strongly associated with malignancy and poor patient outcome (Auvinen et al., 2000). Former research revealed that the three HA producing isoenzymes, HAS1, HAS2, and HAS3, have different expression patterns and properties. Still their functional importance in tumourgenesis remains unclear (Weigel and DeAngelis, 2007). Strikingly, only HAS2 knockdown is embryonically lethal and also HAS2 has been

claimed to plays a critical role in the development of a pro-metastatic microenvironment (Okuda et al., 2012, Tammi et al., 2011). Furthermore, culture studies show that invasive breast cancer cells synthesise and accumulate larger amounts of HA than normal tissue and preferentially express more HAS2 mRNA than less aggressive tumour cells (Li et al., 2007). In line with that, previous studies in our laboratory showed a significant association between high RNA expression levels of HAS2 in primary breast cancer and poor patient prognosis (Milde-Langosch et al., 2014). However, this study only showed a tendency between HAS2 expression and shorter overall survival with lack of statistical significance.

Despite the current ambiguity about the influence of HAS2 on patient survival, we can report a correlation between high HAS2 levels in primary breast cancer and high tumour grade, positive bone marrow status, and lymphangiosis. With these results we are not the only study group to report an association of HAS2 with unfavourable prognostic factors in breast cancer. High HAS2 expression levels are associated with triple-negative and basal-like breast cancer subtypes and moreover with reduced overall survival (Lien et al., 2014). Furthermore, high HAS2 expression levels were related to large tumour size, lymph node positivity, and ER negativity (Auvinen et al., 2014a). Additional studies associated HAS2 with cancer progression and shorter tumour cell survival in breast cancer cell lines as well as high HYA1 and HAS2 expression levels in bladder cancer with high recurrence rates (Kramer et al., 2011, Udabage et al., 2005).

While in our study we evaluated the HAS2 expression levels in tumour cells, HAS2 expression levels in cells of the TME might be of equal or greater importance. Auvienen et al. showed that expression levels of all HAS isoforms correlated with stromal HA staining, high relapse rate, and short overall survival of patients. These data suggest that increased HAS enzyme levels contribute to the accumulation of HA in breast cancer, synthesised by carcinoma cells and stromal cells. It furthermore indicates a relationship between HAS enzyme levels and tumour aggressiveness and poor patient outcome (Auvinen et al., 2014a). Whether it is HAS2 playing the major role of the three HAS enzymes and whether its occurrence within tumour cells or within the TME is of greater prognostic relevance in breast cancer still needs to be clarified in future research.

Over the past decades, HA has been determined as one major ECM component, associated with breast cancer progression and invasion, promoting cell proliferation, cell movement, and neovascularisation (Auvinen et al., 2000). While our main focus of attention applied to the role of HAS2 and HYAL1 as prognostic factors in breast cancer we also evaluated HA expression levels in primary breast cancer samples and brain metastases. Here, we were able to show an association of high HA expression levels in primary tumour cells with ER, PR, and HER2 negativity as well as an association of high HA expression levels with triple negative receptor status, ER, and PR negativity in tumour cells of BCBM. There results show the importance of HA metabolism in breast cancer progression and furthermore underline the importance of identifying the function of HA and its synthesising and degrading enzymes as future prognostic factors in breast cancer.

A limitation in this study was a low comparability of the results of one cohort with the results of another. This might be due to a discrepancy between the IRSs of the TMAs and the standard tumor slides due to a heterogeneous distribution pattern of HAS2 and HYAL1 expression levels within the tumour tissues. Furthermore, survival analysis was limited by a lack of follow-up data in the cohorts.

However, the role of HA metabolism in breast cancer, particularly its influence on the development of BCBM has been demonstrated in this study. Our results suggest that HYAL1 plays an essential role in tumour dissemination and brain specific colonization, rather than in subsequent metastatic growth. Our findings further highlight that a fine balance between HA synthesis and degradation plays an integral role facilitating tumour invasiveness and aggressiveness, thereby highlighting potential future biological targets with potential therapeutic value in the treatment of breast cancer.

5 SUMMARY

Breast cancer is the most common cancer in women worldwide. Over the past decades, enhanced breast cancer detection methods and treatment options have led to improved patient outcome and overall survival. However, this process is currently limited by two factors: first by a lack of knowledge of how breast cancer cells manage to migrate through the blood-brain barrier (BBB) and to grow within brain parenchyma, and secondly by the inability of systemic therapy to cross the BBB and therefore to reach breast cancer brain metastasis (BCBM). 15-30% of breast cancer patients develop brain metastases, which have become a major life limiting and life quality limiting factor. Hence, new markers for BCBM incidences are urgently needed in order to detect high risk patients at an early breast cancer stage and furthermore to aid as a new drug target.

In previous studies based on microarray data of breast cancer patients, we were able to demonstrate that mRNA expression of the hyaluronic acid (HA) synthesising and degrading enzymes, hyaluronic acid synthase 2 (HAS2) and hyaluronidase 1 (HYAL1), significantly correlated with brain metastases formation and poor overall survival. In order to investigate the role of HAS2 and HYAL1 in the development of BCBM and their impact on patient outcome, protein expression levels of HA, HAS2, and HYAL1 were detected via immunohistochemistry (IHC) in four different collectives and correlated with clinical and pathological data.

HYAL1 expression levels were significantly higher in primary breast cancers of patients which subsequently developed BCBM in comparison to primary cancers that did not metastasise into the brain. These results indicate the potential role of HYAL1 in breast cancer cell dissemination and brain-specific colonisation. In this cohort we also detected higher HYAL1 expression levels in the primary tumour than in the corresponding brain metastases. Furthermore, we showed that HYAL1 and HAS2 expression levels were lower in BCBM than in breast cancer metastases of other locations such as skin, liver, lung, and bone. Additionally, we found significantly high HA expression levels in tumours cells of BCBM in comparison to breast cancer metastases in skin, liver, lung, and bone. Surprisingly, the opposite was observed in

the tumour micro environment (TME), where HA expression levels were the lowest in BCBM in comparison to breast cancer metastases of the other tissues.

We could not confirm the prognostic role of HAS2 and HYAL1, that we observed in our previous work on cDNA microarray data of breast cancer patients.

In conclusion, these novel results suggest that HYAL1 plays a role in the process of brain specific tumour cell migration and colonisation, however may not be responsible for subsequent metastatic growth.

ZUSAMMENFASSUNG

Brustkrebs ist der häufigste maligne Tumor bei Frauen weltweit. In den letzten Jahrzehnten haben diagnostische und therapeutische Fortschritte den Patienten-Outcome und die Gesamtüberlebenszeit deutlich verbessert. Weitere Erfolge werden durch zwei Faktoren erschwert: Zum einen ist der Mechanismus weitgehend unbekannt, der es einzelnen Brustkrebszellen ermöglicht, die Blut-Hirn-Schranke (blood-brain barrier [BBB]) zu überqueren und das Hirngewebe zu kolonisieren. Zum anderen können die angewandten systemischen Therapeutika die BBB nicht überwinden und somit Brustkrebs-Hirnmetastasen (breast cancer brain metastases [BCBM]) nicht erreichen. 15-30% aller Brustkrebspatientinnen entwickeln Hirnmetastasen, die ein wesentlicher Faktor für eingeschränkte Lebenszeit und -qualität geworden sind. Es bedarf daher neuer Marker für BCBM-Vorkommen. Diese könnten helfen, Brustkrebs bei Hochrisikopatienten in frühen Krankheitsstadien zu diagnostizieren und neue Medikamente zu entwickeln.

In früheren Studien konnten wir basierend auf Microarray-Daten von Brustkrebspatienten zeigen, dass die mRNA-Expression von Hyaluronsäure (HA) und deren auf- und abbauenden Enzymen – Hyaluronsäure Synthase 2 (HAS2) und Hyaluronidase 1 (HYAL1) – signifikant mit der Entwicklung von Hirnmetastasen und einer verminderten Gesamtüberlebenszeit korreliert. Um diesen Einfluss von HA, HAS2 und HYAL1 auf Proteinebene zu eruieren, wurden vier verschiedenen Kohorten via Immunohistochemie (IHC) untersucht und mit klinisch-pathologischen Daten korreliert.

Wir konnten zeigen, dass die HYAL1-Expressionslevel in Primärtumorgeweben von Brustkrebspatientinnen mit Gehirnmetastasen signifikant höher sind als bei solchen ohne Gehirnmetastasen. Diese Ergebnisse suggerieren, dass HYAL1 die Streuung von Brustkrebszellen und Entwicklung von BCBM beeinflusst. Ferner ist die HYAL1-Expression in den Primärtumoren signifikant höher als in den zugehörigen Gehirnmetastasen. Des Weiteren konnten wir zeigen, dass HYAL1 und HAS2 in BCBM geringer exprimiert werden als in Brustkrebsmetastasen der Haut, Leber, Lunge und des Knochens. Die HA-Expression ist in Tumorzellen von BCBM signifikant höher als in Tumorzellen von Brustkrebsmetastasen der Haut, Leber, Lunge und des

Knochens, während in der Tumormikroumgebung (tumor microenvironment [TME]) HA in BCBM signifikant geringer exprimiert wird als in Brustkrebsmetastasen anderer Organe.

Die prognostische Rolle von HAS2 und HYAL1, die wir in früheren Studien mit cDNA-Microarray-Daten von Brustkrebspatientinnen aufzeigten, konnten wir in dieser Studie nicht bestätigen.

Diese neuen Ergebnisse sprechen dafür, dass HYAL1 eine wichtige Rolle in der Entwicklung von BCBM spielt; sobald sich diese einmal gebildet haben, wird ihr weiterer Wachstum von HYAL1 aber wohl nicht weiter beeinflusst.

ABBREVIATIONS

Common abbreviations such as SI-units as well as non Si-units are not listed.

A	Avidin
Aq dist.	Distilled water
B	Biotin
BCBM	Breast cancer brain metastases
BBB	Blood-brain barrier
BSA	Bovine serum albumin
BV	Blood vessel
CD44	Cluster of differentiation 44
CK	Cytokreatin
DAB	3,3'-Diaminobenzidin
E	Epithelium
ECM	Extracellular matrix
EDTA	Ethylendiamintetraacetat
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ER	Oestrogen receptor
HA	Hyaluronic acid
HAbp	Hyaluronic acid binding protein
HAS2	Hyaluronic acid synthase 2
HEP	Hepatocytes
HER2	Human epidermal growth factor receptor 2

HYAL1	Hyaluronidase 1
IHC	Immunohistochemistry
INF	Inflammatory cells
IRS	Immunoreactive score
KI67	Antigene KI67
MET	Mesenchymal-epithelial transition
PR	Progesterone receptor
SD	Standard deviation
T	Tumour cells
TBS	Tris-buffered Saline
TEC	Tris EDTA Citrate
TMA	Tissue microarray
TME	Tumour micro environment

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EIDESSTATTLICHE VERSICHERUNG

Ich versichere ausdrücklich, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

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