UNIVERSITÄTSKLINIKUM HAMBURG-EPPENDORF

Zentrum für Experimentelle Medizin

Institut für Tumorbiologie

Institutsdirektor: Prof. Dr. med. Klaus Pantel

Potential involvement of Jagged1, integrin alpha5 beta1 and VCAM1 proteins in metastatic progression of human breast carcinomas

Dissertation

zur Erlangung des Doktorgrades an der Fakultät für Mathematik, Informatik, Naturwissenschaften

Fachbereich Chemie

der Universität Hamburg

vorgelegt von:

Antonia Efstathiou

Hamburg 2016

UNIVERSITÄTSKLINIKUM HAMBURG-EPPENDORF

Zentrum für Experimentelle Medizin

Institut für Tumorbiologie

Institutsdirektor: Prof. Dr. med. Klaus Pantel

Potential involvement of Jagged1, integrin alpha5 beta1 and VCAM1 proteins in metastatic progression of human breast carcinomas

Dissertation

zur Erlangung des Doktorgrades an der Fakultät für Mathematik, Informatik, Naturwissenschaften

Fachbereich Chemie

der Universität Hamburg

vorgelegt von:

Antonia Efstathiou

Hamburg 2016

Evaluators of the dissertation:

Prof. Dr. med. Klaus Pantel

Prof. Dr. rer nat Peter Heisig

Members of the examination commitee

- Frau Prof. Dr. Zoya Ignatova (Vorsitz),

- Herr Prof. Dr. Klaus Pantel (stellv. Vorsitz),

- Herr Prof. Dr. Bernd Meyer (einfaches Mitglied)

Date of dissertation

den 6. Januar 2017

I hereby declare that the doctoral procedure was performed at the Institute of Tumor Biology, in the Center for Experimental Medicine of the University Medical Center Hamburg-Eppendorf (UKE) between 15 November, 2012- 31 January 2016.

Parts of the thesis have been already published:

Potential Involvement of Jagged1 in Metastatic Progression of Human Breast Carcinomas. Bednarz-Knoll N., **Efstathiou A.**, Gotzhein F., Wikman H., Mueller V., Kang Y., Pantel K. Clincal Cancer Research, 2015.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

aFGF, fibroblast growth factor a

ALDH1, aldehyde dehydrogenase

ALDH1A1, aldehyde dehydrogenase 1 family, member A1

ATM, ataxia telangiectasia mutated

bFGF, fibroblast growth factor b

BM, bone marrow

BMP, bone morphogenetic protein

BRCA1, breast cancer 1

BRCA2, breast cancer 2

BRIP1, BRCA1-interacting protein1

CD24, cluster of differentiation 24

CD44, cluster of differentiation 44

CD45, cluster of differentiation 45

CD45, cluster of differentiation 46

CDH1, cadherin-1

CDK, cyclin-dependent kinase

cfDNA, cell free DNA

CHEK2, checkpoint kinase 2

CRD, Cystein-Rich Domain

CTC, circulating tumor cells

ctDNA, circulating tumor DNA

CXCR4 , C-X-C chemokine receptor type 4

DAB, 3,3'-Diaminobenzidine

DAPI, 4',6-diamidino-2-phenylindol

DCIS, ductal carcinoma in situ

DEP-FFF, dielectrophoretic field-flow fractionation

DFS, disease- free survival

DKK-1, dickkopf-1

DLL1, delta-like 1

DLL3, delta-like 3

DLL4, delta-like 4

DMEM, dulbecco's modified eagle's sterile filtered medium

DMSO, dimethyl sulfoxide

DNA, deoxyribonucleic acid

DPBS, dulbecco’s Phoshate buffered saline

DSL, Delta-Serrate-Lag-2

DTC, disseminated tumor cells

E-cadherin, epithelial cadherin

EDTA, ethylenediamine tetraacetic acid

EGF, epidermal growth factor

EGFR, epidermal growth factor receptor

EMT, epithelial-mesenchymal transition

EpCAM , epithelial cell adhesion Molecule

ER, estrogen receptor

LIST OF ABBREVIATIONS

FCS, fetal calf serum

FDA, food and drug administration

FGF, fibroblast growth factors

HER2, human epidermal growth factor receptor

HR, hormone receptor

HRP, horseradish peroxidase

HSC, hematopoietic stem cells

IC, invasive or infiltrating carcinoma

ICC, immunocytochemistry

IDC, invasive ductal carcinoma

IF, immunofluorescence

IHC, immunohistochemistry

IIC, invasive inflammatory carcinoma

ILC, invasive lobular carcinoma

ISC, in situ carcinoma

ITG α5β1, integrin alpha5 beta1

K, keratin

LCIS, lobular carcinoma in situ

LNM, lymph node metastasis

MBI, molecular breast imaging

M-CSF, macrophage colony stimulating factor

MET, mesenchymal-epithelial transition

MFS, metastasis-free survival

miR-141, micro RNA-141

miR-219, micro RNA-219

miRNA, micro RNA

MRI, magnetic resonance imaging

mTOR, mammalian target of rapamycin

N-cadherin, neural cadherin

NF- κB, nuclear factor kappa-light-chain-enhancer of activated B cells

OPG, osteoprotegerin

OS, overall survival

PALB2, partner and localizer of BRCA2

PBMC, peripheral blood mononuclear cell

PDGF, platelet-derived growth factor

PDL1Programmed death ligand 1

PFA, paraformaldehyde

PFS, progress- free survival

PR or PgR, progesterone receptor

PTEN, phosphatase and tensin homolog

PTHrP , parathyroid hormone-related peptide

RANKL, receptor activator of nuclear factor kappa-β

LIST OF ABBREVIATIONS

RANKL, receptor activator of nuclear factor kappa-β ligand

RBC, red blood cell

RNA, ribonucleic acid

RPMI, roswell park memorial institute sterile filtered medium

shRNA, short hairpin RNA

sICAM1, soluble intracellular adhesion molecule

STK11, serine/threonine kinase 11

TGF-α, transforming growth factor α

TGF- β, transforming growth factor β

TGF-β, transforming growth factor β

TMA, tissue microarray

TNF-α, tumor necrosis factor α

TNF-β, tumor necrosis factor β

TP53, tumor protein 53

UKE, Universitätsklinikum Hamburg-Eppendorf

VCAM-1, vascular cell adhesion molecule 1

VEGF, vascular endothelial growth factor

VPF, vascular permeability factor

ZUSAMMENFASSUNG

Brustkrebs gehört mit ~28 % zu der häufigsten Krebserkrankung der Frauen. Die Todesursache bei Krebserkrankungen stellt in der Regel nicht der Primärtumor selbst dar, sondern Metastasen, die sich in der Folge einer Erkrankung ausbilden können. Zirkulierende Tumorzellen (CTCs) sind Krebszellen, die sich vom Primärtumor abgelöst haben, in den Blutkreislauf gelangt sind und das Potential besitzen Metastasen zu induzieren. Das Vorkommen von CTCs wurde bei Brustkrebspatientinnen bereits mehrfach mit einer schlechten Prognose assoziiert. Proteine wie Jagged1, Integrin alpha5beta1 (ITG α5β1) und das *Vascular Cell Adhesion Molecule-1* (VCAM-1) scheinen zusätzlich prognostische und therapeutisch relevante Informationen für den Verlauf einer Erkrankung aufzeigen zu können. Das Ziel dieser Studie war es die Expression dieser Proteine auf CTCs, DTDs und / oder dem Primärtumorgewebe zu bestimmen, um ihre klinische Relevanz weiter aufzuschlüsseln.

1. Der Notch-Signalweg ist vielfach in Tumoren, wie Brustkrebs aktiviert und wurde bereits im Mausmodell mit osteolytischen Knochenmetastasen in Verbindung gebebracht. Jagged1 fungiert als Ligand für den Notch-Rezeptor und scheint ein funktionell relevanter Mediator für die Knochenmetastasierung zu sein. Dieser Marker wurde zusätzlich bereits mit einer schlechten Prognose assoziiert. Ziel dieser Studie war es die Expression von Jagged1 auf CTCs von Brustkrebspatientinnen zu untersuchen und mit klinisch-pathologischen Parametern zu korrelieren. CTCs wurden aus dem peripheren Blut von 100 Patientinnen mit progressiver, metastastasierter Tumorerkrankung angereichert und über pan-Keratin / Jagged1 / CD45 Immunfluoreszenzfärbung nachgewiesen. 21 der untersuchten Blutproben waren positiv für CTCs (21 %), wobei 85,7% der CTCs Signale für die Expression von Jagged1 aufzeigten. Hierbei war die Expression von Jagged1 auf CTCs lediglich bei Patientinnen mit Bisphosphonat-Behandlung zu finden, wobei dieser Marker zusätzlich auch mit einem kürzeren progressionsfreien Überleben in Verbindung gebracht werden konnte (P = 0,013). Jagged1 könnte daher ein interessantes Zielprotein darstellen, um Therapieantworten von Bisphosphonat-behandelten Patientinnen zu verfolgen und insbesondere Resistenzen gegen diese Art von Therapie festzustellen.

2. ITG α5β1 wird in Tumorzellen und Tumor-Neogefäßen exprimiert und scheint für die Tumorinvasion und -proliferation von Bedeutung zu sein. Die Überexpression von diesem Marker konnte bereits mit einer schlechten Prognose und der Metastasierung in Verbindung gebracht werden. Insgesamt wurden im Rahmen dieser Studie Gewebsproben von 411 Brustkrebspatientinnen untersucht. Des Weiteren konnten CTCs aus peripheren Blutproben von 31 metastasierten Brustkrebspatientinnen isoliert und auf die Expression von ITG α5β1 analysiert werden. In 268 auswertbaren Tumorgewebsproben konnten in 85 Fällen (31,7%) keine ITG α5β1-Signale nachgewiesen werden. 53 (19,8%), 53 (19,8%), und 50 (18,7%) Tumorproben zeigten hingegen eine schwache, mittelstarke und starke Expression dieses Markers. Die ITG α5β1-Expression korrelierte signifikant zu dem M-Status (p = 0,046), dem Mikro- und Makrometastase-Status (p = 0,007), der ALDH1-Positivität (p = 0,023), der Jagged1-

Positivität (p = 0,025) und dem Auftreten von DTCs im Knochen (p = 0,035). Keine Korrelation konnte hinsichtlich des verkürzten krankheitsfreien Überlebens (DFS) oder des Gesamtüberleben (OS) in der Gesamtkohorte beobachtet werden (p = 0,169 und p = 0,921). Zusammenzufassend lässt sich sagen, dass die ITG α5β1-Expression mit einem aggressiveren Mammakarzinom assoziiert ist und an metastatischen Prozessen beteiligt sein könnte. Jedoch konnte in keiner der Patientinen mit metastasiertem Brustkrebs eine ITG α5β1-Expression auf CTCs nachgewiesen werden.

3. Die Expression von VCAM-1 wurde bereits mit dem Rückfall einer Tumorerkrankung sowie der Tumorzellendormanz in Verbindung gebracht. Zusätzliche Studien haben gezeigt, dass die Hemmung von VCAM-1 die Bildung von Metastasen im Knochenmark und anderen Organen unterdrücken kann. In dieser Studie wurde die VCAM-1-Expression auf CTCs von 149 metastatischen Bruskrebspatientinnen untersucht. Die erzielten Ergebnisse wurden anschließend mit klinischen Parametern verglichen. In 19 von 149 Proben konnten pan-Keratin-postive CTCs nachgewiesen werden. Innerhalb dieser Gruppe zeigten 11 Patientinnen (58,0%) ausschließlich VCAM-1-postive CTCs während 4 Patientinnen (21,0%) eine heterogene Expression aufwiesen. CTCs von 4 weiteren Patientinnen (21,0%) zeigten hingegen keine Signale für die Expression von VCAM-1. Bei der Auswertung der klinischen Daten konnten keine Korrelation zu klinisch-pathologischen Parametern ausfindig gemacht werden.

Zusammenfassend zeigen die Ergebnisse dieser Studie, dass Jagged1, ITG α5β1 und VCAM-1 möglicherweise bei der Bildung von Metastasen eine wichtige Rolle spielen und dadurch ein Potential als klinische Biomarker haben könnten. Jedoch müssten weitere Untersuchungen im Hinblick auf die Wirkungsmechanismen in Tumorzellen und in der Krebstherapie durchgeführt werden.

ABSTRACT

Breast cancer (BC) is the most frequently diagnosed cancer in women, accounting for 23% of cancer-related diagnoses each year. Metastasis detected at the late stages of disease remains the leading cause of death among BC patients, as limited treatment options are available for these patients. Circulating tumor cells (CTCs) are cells that have detached from primary tumor and are present in blood circulation. Numerous studies have detected CTCs in breast malignancies and showed a close correlation between the presence of CTCs and poor prognosis. Proteins such as Jagged1, integrin alpha5beta1 (ITG α5β1) and Vascular Cell Adhesion Molecule-1 (VCAM-1) have been associated with breast cancer formation and metastasis. Therefore, this study aimed to investigate the expression of these proteins in CTCs, DTCs and/or primary tumors and its clinical relevance in BC patients.

1. Notch pathway is activated in certain tumors, such as BC cells, and seems to be involved in the formation of osteolytic bone metastases (BM) in animal models. Notch ligand Jagged1 is a clinically and functionally important mediator of BM by activating the Notch pathway in bone normal cells. It is reported to be involved in the formation of BM and, therefore, associated with worse prognosis of breast tumor patients. Therefore, in this study, Jagged1 expression was assessed in CTCs of BC patients and compared to clinico-pathological parameters and patients' outcome. CTCs were enriched from peripheral blood of 100 metastatic patients with progressive disease and detected by pan-keratin/Jagged1/CD45 immunofluorescent staining. 21 metastatic BC patients with progressive disease were positive for CTCs, and 85.7 % of the CTCs also expressed Jagged1. Presence of Jagged1(+) CTCs was significantly associated with shorter progression-free survival in patients treated with bisphosphonates (P=0.013). To conclude, Jagged1 could become an interesting target to monitor bisphosphonate- based therapy response particularly in metastatic patients resistant to this kind of therapy.

2. ITG α5β1 is reported to be expressed in tumor cells and tumor neovessels, facilitating tumor invasion and proliferation. Its overexpression has been associated with worse prognosis and metastatic disease of cancer patients. In this study, ITG α5β1 expression was evaluated by immunohistochemistry on tissue microarrays comprising tumor samples of 411 BC patients and compared to clinico-pathological parameters and patients' outcome. Additionally, CTCs isolated from peripheral blood of 31 metastatic BC patients and DTCs from the bone marrow of 4 unmatched metastatic BC patients were analysed for ITG α5β1 expression using immunocytochemistry techniques. Not expressed, weak, moderate and strong ITG α5β1 expression was detected in 85 (31.7%), 53 (19.8%), 53 (19.8%) and 50 (18.7%) of 268 informative tumors, respectively. ITG α5β1 expression in primary tumors was significantly associated with M status (p=0.046), micro- and macrometastasis (p=0.007), ALDH1 expression (p=0.023), Jagged1 expression (p=0.025) and presence of DTCs in bone marrow (p=0.035). The expression was neither correlated to shorter disease-free (DFS) and overall survival (OS) in the total patient cohort (p=0.169 and p=0.921 respectively) nor in any other subset of patients. To sum up,

ITG α5β1 expression characterizes more aggressive breast carcinoma and might be involved in tumor cell dissemination and metastatic progression. However, none of the patients with metastatic BC presented CTCs or DTCs positive for ITG α5β1.

3. VCAM-1 has been associated with early relapse and the activation from tumor cell dormancy. Additionally, blocking of VCAM-1 effectively inhibits metastases formation in the bone marrow or other organs. VCAM-1 expression was assessed in CTCs found in peripheral blood of metastatic BC patients under therapy and correlated to clinical parameters and patients' outcome. Blood samples from 149 metastatic BC patients were screened for CTCs and VCAM-1. Nineteen of 149 metastatic BC patients were positive for CTCs. Within this group, 11 patients (58.0%) exclusively had VCAM-1(+)CTCs and 4 patients (21.0%) showed a heterogeneous expression of VCAM-1 in CTCs, whereas 4 patients (21.0%) had only VCAM-1(-) CTCs. VCAM-1 expression in CTCs did not correlate to primary tumor's characteristics and also did not indicate any specific site of metastases.

In conclusion, these results imply that Jagged1, ITG α5β1 and VCAM-1 might play an important role in monitoring breast cancer metastatic disease and have a potential to serve as clinical biomarkers. However, further investigation has to be conducted with respect to the mechanisms of action in tumor cells and cancer therapy.

1. INTRODUCTION- BREAST CANCER METASTASIS

1.1 Breast Cancer

Breast cancer is the most common type of cancer among women worldwide, with a high death rate for young patients aged between 20 and 59. According to a research which took place in Europe in 2014, 232.670 women with breast cancer were recorded that corresponds to the 29% of overall new cancer cases. Moreover, 40,000 (15% of cancer deaths) breast deaths were estimated the same year rendering breast cancer the first leading cause of cancer deaths in Europe for women. The same year in the United States, 89.300 (15% of overall cancer deaths) breast deaths were estimated rendering breast the second cause of cancer deaths after lung cancer (in the United States) for females [1,2].

Approximately, the 90% of cancer patients decease because of tumor growths in distant sites, i.e metastatic spread of the primary tumor [3,4]. Particularly in breast cancer however, it is estimated that the 67% of women die of other reasons such as cerebrovascular diseases, various heart diseases and dementia, than restricted, no progressive disease [5].

1.1.1 Breast cancer classification

Breast cancer is a heterogeneous disease, which means that genetic and phenotypic features may differ among patients (inter-tumor heterogeneity) and among cancer cells within a tumor mass (intra-tumor heterogeneity). Therefore, breast cancer patients are necessary to be encountered individually by validating classification aspects as a means of early diagnosis, as prognostic and predictive factors and finally as a determinant of a personalized therapy. Breast cancer can be classified according to histopathological differences, stage (TNM classification), molecular subtype, pathologic grade, DNA profile and other classification approaches [6,7].

Histopathology of breast cancer

The considerable majority of breast tumors are derived from the epithelial tissue of the mammary gland composed of lobules (glands where milk production occurs) or ducts (small tubes which transfer milk to the nipple) dispersed with fibro adipose connective tissue (Figure 1.1A). Histopathologically, breast cancer is broadly classified into *in situ* carcinoma (ISC) and invasive or infiltrating carcinoma (IC). ISC cancer cells proliferate within the epithelium without passing the basal membrane, i.e. invading the surrounding tissue, unlike IC which invades the surrounding tissue [4, 6, 8].

ISC is subclassified into ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS) depending on the ductal or lobular cell abnormal proliferation respectively (Table 1.1). DCIS is further classified into comedo carcinoma including aggressive characteristics and non-comedo carcinoma including less aggressive characteristics. Non-comedo DCIS includes solid, cribriform, papillary and micropapillary DCIS based on the pattern that proliferated cells line the ducts.

IC is subclassified into invasive ductal carcinoma (IDC), which is the dominant subtype (70%-80%) of IC, invasive lobular carcinoma (ILC) and invasive inflammatory carcinoma (IIC). Invasive ductal carcinoma is further classified into mucinous (colloid), tubular, medullary, papillary and cribriform invasive ductal carcinoma and invasive lobular carcinoma into classic, solid, alveolar and tubulolobular invasive lobular carcinoma depending on the pattern of ductal and lobular cell growth and invasion respectively. Invasive inflammatory carcinoma is an aggressive disease which mostly affects ducts; therefore, invasive inflammatory carcinoma could be included in invasive ductal carcinoma classification. Invasive inflammatory carcinoma cancer cells block lymphatic vessels in skin covering the breast causing an inflammatory-like picture of swelling and red colour [6, 9, 10].

Table 1.1 Breast cancer classification according to histopathological differences

in situ carcinoma (ISC)	ductal carcinoma *in situ* (DCIS)	comedo	-
		non-comedo	solid
			cribriform
			papillary
			micropapillary
	lobular carcinoma *in situ* (LCIS)	-	-
invasive carcinoma (IC)	invasive ductal carcinoma (IDC)	mucinous (colloid)	-
		tubular	
		medullary	
		papillary	
		cribriform	
	invasive lobular carcinoma (ILC)	classic	-
		solid	
		alveolar	
		tubulolobular	
	invasive inflammatory carcinoma (IIC)	-	-

Table 1.2 List of TNM classification in breast tumors

Breast tumor	Characteristics
Tx	tumor cannot be assessed
T0	no evidence of primary tumor
Tis (CIS)	carcinoma *in situ*
T1	tumor ≤ 2 cm in greatest dimension
T1mic	microinvasion ≤ 0.1 cm in greatest dimension
T1a	tumor between 0.1-0.5 cm in greatest dimension
T1b	tumor between 0.5-1 cm in greatest dimension
T1c	tumor between 1-2 cm in greatest dimension
T2	tumor between 2-5 cm in greatest dimension
T3	tumor > 5 cm in greatest dimension
T4	tumor of any size with direct extension to chest wall or skin
T4a	extension to chest wall, not including pectoralis muscle
T4b	edema or ulceration or satellite skin nodules confined to the same breast
T4c	T4a and T4b
T4d	inflammatory carcinoma

Staging of breast cancer

TNM classification for staging breast cancer stands for tumor size (T), palpable lymph nodes (N) and metastasis (M). The large size of a tumor, lymph node dissemination and metastasis are associated with bad prognosis [8, 9, 11,12]. Moreover, cancer cell detection in peripheral blood indicates bad prognosis of patient's breast cancer [13,14] and thus, it was included in the new edition of the TNM tumor classification as cM0(i+) in 2010 [15,16,17]. TNM classification in breast tumors, in regional lymph nodes and in distant sites is listed in Tables 1.2, 1.3 and 1.4 respectively. Moreover, the main stages of the breast cancer disease are concisely listed in Table 1.5 [8,9, 11].

Table 1.3 List of TNM classification in regional lymph nodes

Regional Lymph Nodes	Characteristics
Nx	regional lymph nodes cannot be assessed
N0	no regional lymph node metastasis
N1mic	micrometastases between 0.02-0.2 cm and/or more than 200 cells
N1	metastases in 1 to 3 axillary lymph nodes
N2	metastases in 4 to 9 axillary lymph nodes
N3	metastases in 10 or more axillary lymph nodes

Table 1.4 List of TNM classification in distant metastasis

Regional Lymph Nodes	Characteristics
Mx	distant metastasis cannot be assessed
M0	no distant metastasis
M1	distant metastasis
cM0(i+)	cancer cell detection in peripheral blood

Table 1.5 List of the main stages of breast cancer

Stage 0-IV	Characteristics
Stage 0	Carcinoma *in situ*
Stage I	Tumor ≤2cm, negative lymph nodes
Stage II	Tumor from 2-5cm and/or positive lymph nodes
Stage III	Tumor >5cm and/or positive lymph nodes Inflammatory breast cancer
Stage IV	Distant metastasis beyond ipsilateral lymph nodes

Molecular subtype classification

The multiple nature of breast cancer is due to differences in molecular expression among cancer cells. Molecular subtype classification is based on the expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor (HER2) and the cellular marker of proliferation Ki-67. Breast cancer cells presenting negative results for ER, PR and HER2 are of note as they show that tumor outgrowth is neither supported by the ER and PR hormones, nor by the overexpression of HER2 receptors. This specific feature is described as triple-negative breast cancer and it is characterized by non-response to hormonal therapy or therapies that target HER2 receptors [18]. Molecular subtype classification is recorded in the Table 1.6:

Table 1.6 Molecular subtype classification of breast cancer [6, 19, 20, 24]

Subtype	ER	PR	HER2	KI-67	Frequency	Grade
Luminal A	Positive	High	Negative	Low, ≤ 14%	50-60%	Low
Luminal B	Positive	Low	Negative or Positive	High or any	10-20%	Intermediate
Basal-like or triple-negative	Negative	Negative	Negative	High	10-20%	High
$HER2^{+}$	Negative	Negative	Positive	Any	10-15%	High
Claudin low	Negative	Negative	Negative	Any	12-14%	High

Grades of breast cancer

Normal breast cells can be differentiated and undergo alterations in order to obtain specific features and function as components of breast tissue. Cancer cells lose progressively the ability of differentiation and normal function. Pathologic grading includes well differentiated (low-grade), moderately differentiated (intermediate-grade) and poorly differentiated (high-grade) breast cancer focusing on cancer cell morphology such as nuclear size and shape variability and uncontrolled cell division compared to normal cells. Poorly differentiated or high grade breast cancers are characterized by bad prognosis [6, 9]. Low-grade breast cancer is characterized by positive expression of ER and PR receptors as well as low HER2 and Ki-67 expression which correspond to Luminal A and B breast cancers. In contrast, high-grade breast cancer is characterized by no expression of ER and PR receptors, high HER2 and Ki-67 expression and increased microvessels which correspond to triple negative, $HER2^{+}$ and Claudin law breast cancers subtypes (Table 1.5) [19,22,24].

Familial, hereditary and sporadic cancer

Finally, breast cancer can be also classified into familial, hereditary and sporadic. Familial is called the breast cancer which appears in high rates among family members without any detected gene mutation and represents the 10-20% of breast cancer cases [23]. Hereditary and sporadic classification depends on inherited or somatic mutations. Hereditary breast cancer is often diagnosed at early ages, and includes specific inherited gene mutations in genes such as *BRCA1, BRCA2, PTEN, TP53, CDH1, STK11, CHEK2, BRIP1, ATM, and PALB2* [19]. Hereditary breast cancer accounts for approximately 2-5% of breast cancer cases associated with more aggressive disease course and lower survival rates [22]. Sporadic breast cancer representing > 90% of all breast cases is considered to be caused by an interplay of both genetic and environmental factors, dietary agents, hormones and normal aging [19,24,25,26].

1.1.2 Risk factors of breast cancer

Several genetic, lifestyle and environmental factors have been associated with increased risk of breast cancer [27] and include inherited genetic risk factors, gender, age, early menarche, nulliparity, late menopause, hormone replacement therapy, benign breast disease, specific syndromes such as ataxia-telangiectasia and Cowden syndromes, alcohol, high-fat diet and variant environmental factors including ionizing radiation (Table 1.7) [6,8,19,24,28].

Table 1.7 Risk factors of breast cancer

High risk factors	Relative risk
family history: breast cancer in first degree relative when being young	>2
advanced in years	>10
early menarche	3
nulliparity	3
late menopause	2
hormone replacement therapy	1.35
benign breast disease	4-5
alcohol consumption	1.3
high-fat diet	1.5
environmental factors	3

1.1.3 Diagnosis of breast cancer

Diagnostic tools used for breast cancer identification include mammography, Magnetic Resonance Imaging (MRI), Molecular Breast Imaging (MBI), breast biopsy, HER2 detection and blood-based assay and are outlined in Table 1.8. Clinical breast examination, breast imaging and biopsy constitute the 'triple test' and are performed simultaneously. HER2 presence was associated with a more aggressive disease resulting in the development of HER2 detection assays in order to administrate anti-HER2 antibodies as targeted therapy. Moreover, additional breast cancer related mutations and biomarkers can be detected in bloodstream at DNA, RNA or protein level [6,8,12].

Finally, cancer cells which have evaded from breast tumor and entered blood circulation can be used as early predictors for breast cancer progression and expansion [29,30].

Table 1.8 Diagnostic assays of breast cancer

Diagnostic assays		Principle of assay	Specimen	Objective
Breast Cancer Imaging	Mammography	X-ray	Breast tissue	Breast abnormalities
	Magnetic Resonance Imaging (MRI)	Nuclear magnetic resonance	Breast tissue	Breast abnormalities
	Molecular Breast Imaging (MBI)	Gamma imaging	Breast tissue	Breast abnormalities
Others	Breast biopsy	Needle biopsy	Breast tissue	Malignant cells
	HER2 detection	Immunohistochemistry (IHC) FISH	Breast tissue & Blood serum	Breast cancer related proteins Chromosomes
	Blood-based assay	Blood collection Cancer cell isolation Immunoassays	Blood serum	Breast cancer related biomarkers Cancer cells Mutations

1.1.4 Breast cancer therapy

Breast cancer treatment includes local and systemic treatment depending on their target, i.e. a particular area of tumor or cancer cells throughout the body. Mastectomy, axillary lymph node surgical dissection and radiation therapy refer to local treatment whereas chemotherapy, hormone and targeted therapy to systemic treatment [31]. Radiation and systemic therapy can be used in adjuvant treatments after surgery, in neoadjuvant treatments before treatment and in advanced breast cancer patients [32]. A combination of treatment is often applied depending on the histopathology, stage, grade and molecular subtype of the tumor with a view to more efficient results. Conserving or total mastectomy [33] along with lymph node dissection [34] and radiation therapy [35] constitute the standard management for early breast cancer disease in order to achieve tumor local retreat and prevent recurrence or tumor cell dissemination out of the delimited local area of breast.

Targeted therapy for breast cancer

Tumor heterogeneity among patients reflects on high diversity in development and prognosis of breast disease. Moreover, cytoxicity in normal as well as in malignant cells due to the traditional cancer treatment is a major challenge in therapeutic research [36,37]. Therefore, personalized therapy based on targeted agents has widely become the scientific center of attention. HER2 overexpression in primary tumor is associated with more aggressive characteristics and poor prognosis. The intravenously administrated monoclonal antibodies Trastuzumab (Herceptin), Pertuzumab and Ado-trastuzumab emtansine as well as the orally administrated Lapatinib have been developed to target HER2 protein. [38,39,40,41]

Additional targeted therapeutics currently discussed in breast cancer treatment are the cyclin-dependent kinase (CDK) 4 and 6 inhibitors Palbociclib, as well as a mammalian target of rapamycin and Everolimus (mTOR) and AKT inhibitors which prevent cell growth and division in hormone receptor-positive breast cancers [42,43].

Bevacizumab is an anti-angiogenic agent, approved by Food and Drug Administration (FDA) and widely used in many cancer treatments. It is a monoclonal antibody against tumor angiogenesis and therefore tumor development by targeting the vascular endothelial growth factor (VEGF). Bevacizumab used in metastatic breast cancer has been proved to contribute to tumor remission and the increase women survival [44,45,46].

With respect to targeted therapy and immunotherapy, a new approach including breasts cancer vaccines is examined in patients with aggressive, metastatic and chemotherapy resistant patients in order to induce immunization and prevent tumor recurrence [37,47]. Immune checkpoint inhibitors such as the programmed cell death receptor ligand 1 (PDL1) constitute a group of fascinating new therapeutic targets resulting in tumor reduction of breast cancer patients [48,49].

1.2 Metastatic progress of breast cancer

Metastasis or metastatic disease is called the phenomenon that cancer cells escape from primary tumors and drain into blood or lymphatic circulation, thereby being relocated and found in distant organs. The majority of invasive carcinomas (>80%) arise in the epithelial side of the basement membrane (Figure 1.1B) [50]. Cancer cells with more aggressive characteristics can break the basement membrane and translocate into the stroma (Figure 1.1C and D). Activation of angiogenesis and blood microvessel formation is demanded to facilitate cell intravasation into blood circulation (Figure 1.1E). In breast, cancer cells are alternatively migrating via lymphatic vessels in the mammary gland to lymph nodes and further to bloodstream [4,51]. Circulating Tumor Cells (CTCs) are cancer cells that escape from an established tumor and enter blood circulation or lymph nodes. For these processes, cancer cells may undergo specific modifications in morphology, motility and gene expression called epithelial-mesenchymal transition (EMT). During EMT, they lose their polarity, cell-cell contact and obtain a fibroblast-like shape, which increase the motility and invasiveness of the cells. Moreover, proteins such as E-cadherin, Epithelial Cell Adhesion Molecule (EpCAM) and keratins are suppressed, while N-cadherin, intermediate filament vimentin and proteases are expressed. In other

words, cancer cells change from epithelial to mesenchymal properties acquiring epithelial-mesenchymal plasticity in order to escape epithelial side and enter mesenchyme blood or lymph tissue [52,53]. Of note, in addition to pure epithelial or mesenchymal phenotype (which correspond to only epithelial or only mesenchymal marker expression respectively, EMT includes an intermediate state of semi-epithelial (or semi-mesenchymal) phenotype. In this intermediate state, both epithelial and mesenchymal markers may be present at the same time describing two phases: the epithelial phase with minor mesenchymal characteristics or the mesenchymal phase with minor epithelial characteristics [54,55].

The expression of EMT hallmarks and mesenchymal markers have been associated with high grade breast cancer, HER2 overexpression and poor outcome indicating the metastatic hazard of cancer cells undergoing EMT [56,57,58].

CTCs, following the definite route of bloodstream, initially pass through the right heart ventricle (venous blood), thereafter through the lungs and the left heart ventricle entering into the general arterial circulation. During their transfer in the lugs, cancer cells may reside in pulmonary capillary beds and establish metastasis there. Alternatively, they may attempt to escape from the lungs either due to the difference size among cancerous cells and internal capillary diameter or due to their potential coat with platelets and further drain into blood circulation [51,59].

However, there are multiple hurdles to tumor invasion and expansion rending tumor cells less successful in the initiation of a metastatic formation [3]. Firstly, CTCs may undergo apoptosis due to a detachment from extracellular matrix (anoikis), which normally provide the cells with valuable factors for their survival [60]. Subsequently, during their passage through the bloodstream, CTCs encounter stress factors such as rapid re-oxidation, pH changes and nutrient deficiency which might lead to apoptosis [61]. Platelets seem to play an important role in cancer cell survival in bloodstream by forming a protective cover around them increasing the metastatic occurrence [50]. Immune system is an additional hurdle that has to be overcome although it seems to have a dual effect on tumor expansion. On the one hand it may trigger DNA damage and inhibit tumor growth and on the other hand, it may promote

angiogenesis and tumor growth by producing growth factors, cytokines, chemokines and matrix metalloproteinases [16]. Besides, the micro environment around the new established tumor may form hostile conditions affecting tumor proliferation and development [61,62].

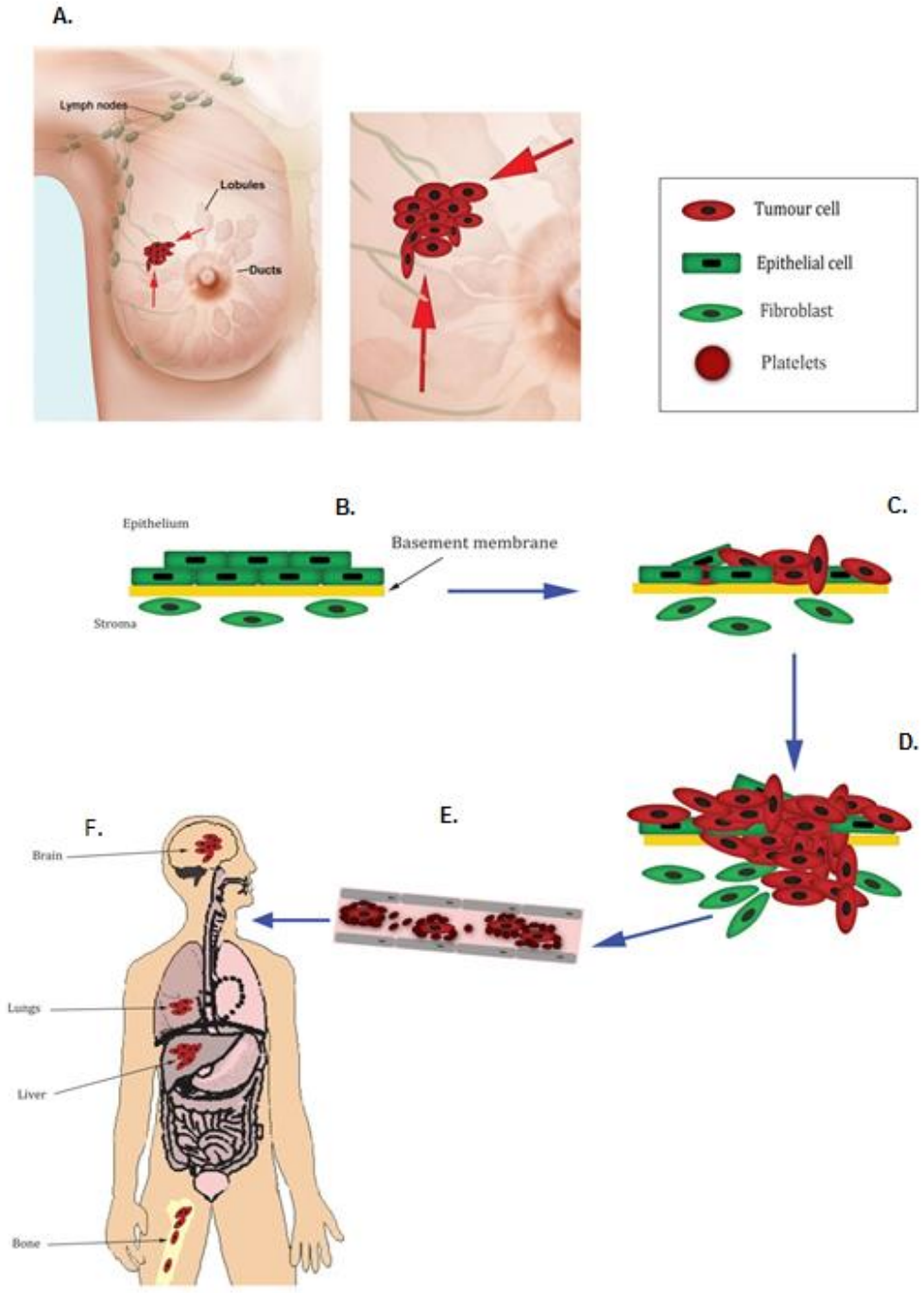

Figure 1.1 Breast Cancer Metastatic Progress: **A)** Breast tumors derived from epithelial tissue of mammary gland composed of lobules and ducts. **B)** No cancerous epithelium and stroma separated by basement membrane. **C)** Cancer cells with more aggressive characteristics break the basement membrane **D)** and translocate into the stroma **E)** and then into blood circulation. **F)** Tumor cells disseminated to remote sites [50].

According to ‘seed and soil’ hypothesis firstly described by Stephen Paget, although randomly distributed, cancer cells preferentially survive and grow in specific distant sites. This selectivity is authorized by interactions among the arrival tumor cells at potential metastatic sites, stromal cells and a variety of factors of tumor microenvironment, resulting in a formation of a pre-metastatic niche that it is able to home and facilitate cell proliferation and colonization. Previous studies have shown that tumor cells exposed to a non-cancerous microenvironment failed to proliferate and form metastasis [63].

To a large extent, metastasis outgrowh is of note determined by the anatomical layout of the circulatory system and blood vessel connection between primary and metastatic site. The cells which survive at the remote site, can undergo the reversed process from EMT, i.e. a mesenchymal-epithelial transition (MET), to form initially dormant minuscule tumor colonies (micrometastasis) which potentially develop into macroscopic metastasis (Figure 1.1F) [4].

1.3 ‘Liquid Biopsy’: Circulating Tumor Cells (CTCs) and Disseminated Tumor Cells (DTCs)

‘Liquid biopsy’ refers to a non-invasive clinical methodological approach that detects cancer cells as well as fragments of tumor DNA (cell free circulating tumor DNA, cfDNA or cell free ctDNA) shedded into peripheral blood or bone marrow. In addition to non-invasive advantage over tumor biopsy tissue, ‘liquid biopsy’ allows a real-time monitoring at different point of times of the disease by detecting potential genomic alternations or new molecular findings in the course of metastatic progression [15,16].

CTCs in many different tumor types seem to be significantly associated with metastatic progress [16,60,62,64,65,17], resistance to therapy and bad prognosis in both early (no metastatic) and late stage breast cancer [65]. Specifically in breast cancer, CTCs were found to be related to high grade disease (HER2 positive and triple negative primary tumors), early relapse and short overall survival [15,16,64].

CTC detection was recommended as an independent prognostic factor for early recurrence and death [13,14] resulting in CTCs inclusion in the new edition of the TNM tumor classification as cM0(i+) in 2010 [15].

These findings imply that CTCs, after further molecular characterization at protein or DNA level, might serve as biomarkers with high prognostic value in translational cancer research. Thus CTCs may predict the risk for disease progression, treatment determination based on personalized features and monitoring response to therapy [17,66].

The CTCs which survive are settled in distant organs, such as liver, bone and lungs may found tiny tumor colonies where equal proliferating rates to apoptotic rates are recorded remaining in a dormant state for a long course of time. These cells, called Disseminated Tumor Cells (DTC), might elude the dormant state and circulate towards distant sites or repatriate back to the primary site [64,67].

Besides CTCs, DTCs detected usually in bone marrow may be used as a 'liquid biopsy' in order to gain useful information about metastatic disease and treatment management. Bone marrow is a frequent recipient site of tumor cells in breast, prostate and lung cancer and it might contain a heterogeneous cell population originated from different primary tumors [16,68,69].

The presence of DTDs is also associated with early relapse and short overall survival in metastatic breast cancer patients. However, bone marrow aspiration is an invasive process, painful for the patient, and hence not approved in routine medical examination [68,70].

CTCs have been reported to survive several hours in blood circulation [71] with an approximate half-life 1 to 2.4 hours [72]. However, CTCs have been detected years after a surgery even at the time of death [73] indicating that latent DTCs harbored in micrometastases might replenish CTC supply for extended periods of time.

1.4 Technological advances in CTC enrichment

Analysis of CTCs derived from peripheral blood, might offer wealthy information about tumor progress having the additional advantage of non-invasive acquisition of patient material at several time points. However, CTC detection remains technologically challenging. CTCs are presented in low rates in peripheral blood amounting to one among 10^{6}-10^{8} Peripheral Blood Mononuclear Cells (PBMCs) meaning that 10ml of blood normally contain a range of 1-100 CTCs. Therefore, technologies of high sensitivity and specificity are necessary in order to isolate a high number of CTCs exempting PBMCs contamination (Figure 1.2, 1.3).

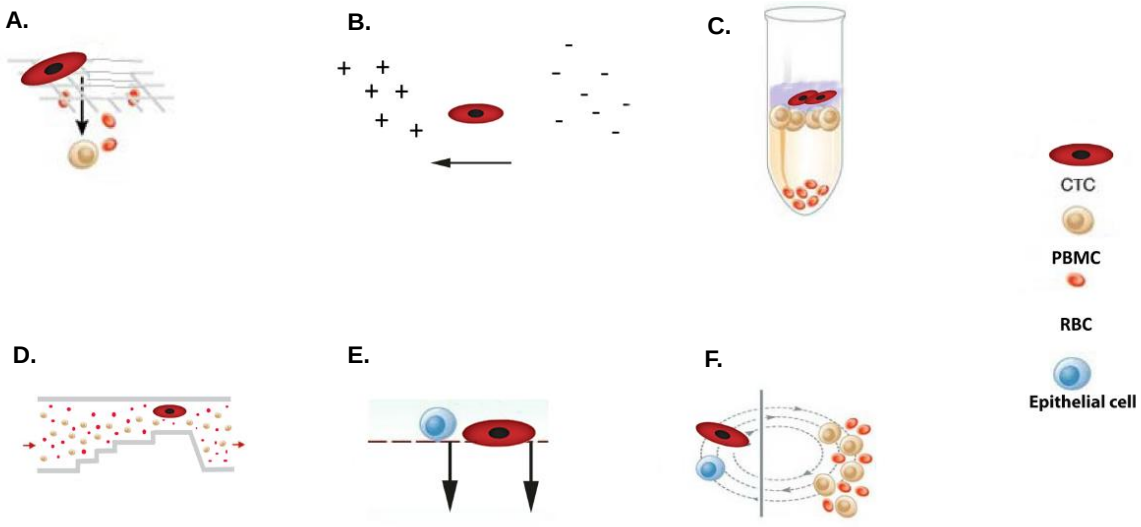

Figure 1.2 Technological advances in CTC enrichment based on physical properties: **A)** microfilters, **B)** dielectrophoretic field-flow fractionation (DEP-FFF) device, **C**) method of ficoll density gradient centrifugation, **D)** Parsortix system, **E)** method of invasive capacity, **F)** method of centrifugal forces [73].

There are more than forty different technological methodologies for CTC enrichment either based on physical properties or on biological properties. Regarding technologies based on physical properties, microfilters, microsieves or Parsortix system can be used to segregate CTCs from blood cells by retaining large CTCs (>5nm) and letting small blood cells (<5mn) go through. Dielectrophoretic field-flow fractionation (DEP-FFF) device is a representative example of isolating CTCs using different electric charges [74,75,76]. Other methodologies include ficoll density

gradient centrifugation which is described in next chapter, VitaAssay and DFF chip based on differences in density, invasive capacity and centrifugal forces respectively [76] (Figure 1.2).

Technologies based on biological properties reclaim the exclusive expression of epithelial cell surface markers in CTCs for positive selection and in PBMCs for negative selection (Figure 1.3). CTCs can be enriched using antibodies against Epithelial Cell Adhesion Molecule (EpCAM) or the combination of antibodies against EpCAM and keratins (positive enrichment) and further discriminated from PBMCs using antibodies against PBMC-cell surface Cluster of Differentiation (CD45) (negative enrichment). Immunomagnetic separation includes an additional methodology of CTC enrichment based on antibodies coating paramagnetic beads which bind to surface markers of a cell. A magnet captures and retains the beads together with the bound cells letting the non-binding cells to go through. Antibodies can be designed either against EpCAM for CTC positive selection among blood cells and their subsequent elution or against CD45 for PBMCs restraint and CTC transit through the magnet column [65,77]. Cell Search is an FDA cleared System used to monitor metastatic disease in breast, prostate and colorectal cancer patients. The nanowire CellCollector is coated with anti-EpCAM antibodies to captivate *in vivo* CTCs by applying for 30 minutes directly into the patient vein. The advantage of *in vivo* techniques for CTC isolation lies in the great amount of blood that flows over the wire (1500ml) and likely high amount of CTCs exposed to EpCAM antibodies [74]. However, some CTCs may downregulate epithelial markers due to EMT rendering technologies based on positive selection based on CK and EpCAM unable to detect them.

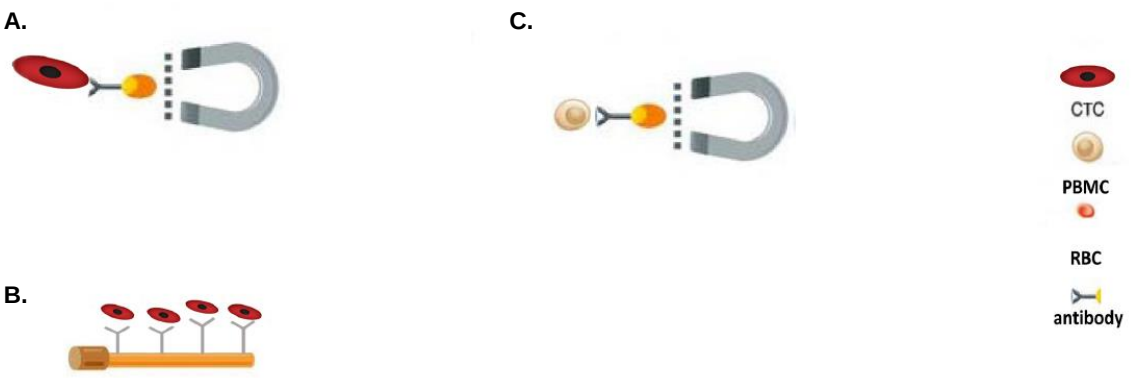

Figure 1.3 Technological advances in CTC enrichment based on biological properties: **A)** positive selection technologies (Cell Search and immunomagnetic separation) **B)** nanowires **C)** negative selection technologies (immunomagnetic separation) [73].

1.5 Metastatic breast cancer to the bone

The bone is the most frequent metastatic site from breast and prostate cancer. Bone metastasis presents high mortality rates amounting to 70% with a median survival less than six months in breast and prostate cancer patients. In addition to a fatal complication of the disease, it is associated with serious symptoms such as bone pain, bone fractures, hypercalcemia and nerve compression [78,79,80].

Bone is constructed by two different types of bone tissue: the cortical bone liable for body mechanical support and organ protection and the cancellous bone, also known as spongy or trabecular which is located at the edges of long bones, pelvic bones, ribs, skull, and the vertebrae in the spinal column. Cancellous pores contain red bone marrow, the bone cells (osteoblasts, osteocytes and osteoclasts) and they host the process of bone metabolism (Figure 1.4).

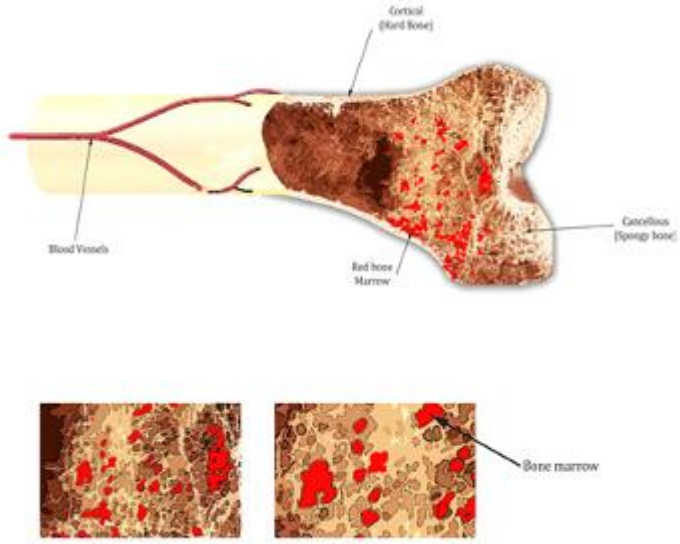

Figure 1.4 Cross- section of bone [81]

Normally, during the bone metabolic process, a characteristic remodeling of bone matrix occurs resulting in a replacement of the mature bone mass by a new one. Osteoclasts are involved in bone matrix break down (bone resorption) while osteoblasts provoke new bone construction (bone formation) maintaining bone homeostasis. There is a strong interaction and interdependence between osteoclasts and osteoblasts, mediated by Receptor Activator of Nuclear factor Kappa-B Ligand (RANKL) and Osteoprotegerin (OPG), both members of tumor necrosis factor (TNF) superfamily and produced by osteoblasts. RANKL binds to RANK receptor on the surface of osteoclast precursors stimulating osteoclast differentiation and maturation into functional osteoclasts. OPG competes RANK receptor in RANKL binding preventing osteoclast differentiation; hence bone resorption, and promoting osteoblast constructing activity and balance maintenance between the two processes (Figure 1.5).

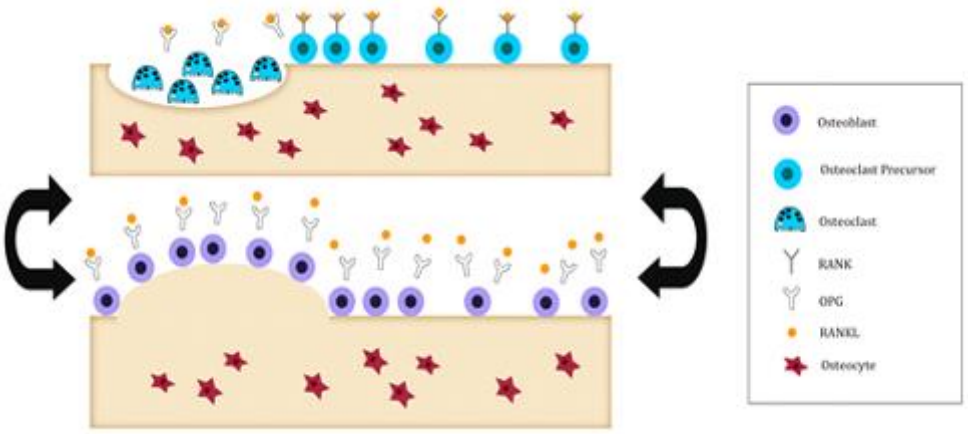

Figure 1.5 Bone metabolic process [77]

Four types of bone metastatic tumors have been recorded: osteolytic, osteoblastic, osteoporotic and a mixed type of osteolytic and osteoblastic tumor. Osteolytic tumors are characterized by high differentiated osteoclast pool leading to bone demineralization and bone degradation. Metastatic breast cancer causes usually osteolytic type of lesions. On the contrary, osteoblastic tumors are characterized by uncontrolled osteoblast activity and bone formation, a predominant form in e.g. metastatic prostate disease. Osteoporotic tumors are identified by faded-look bone without any destruction or increased density of bone. Sometimes, osteolytic and osteoblastic lesions co-exist in the same patient [79].

1.6 Therapeutic approaches against bone metastasis

Systemic and local treatments are two main approaches of treatment for bone metastases. Depending on the site and the extent of tumor, chemotherapy, hormone therapy, targeted therapy, immunotherapy, anti-angiogenic therapy, therapeutic radioisotopes and bisphosphonates can be used as systemic therapy for extended tumors, radiation therapy and surgery as local treatment for localized tumors or both systemic and local treatments can be prescribed [79].

The periosteal layer and intramedullary bone of bone tissue are highly innervated, hence pain-sensitive. Bone resorption induced by osteoclast activity generates an acidic environment activating acid-sensitive pain neurons. Therefore, one of the major and severe symptoms of bone metastatic disease is agonizing pain not eased by conventional pain suppressive drugs. Molecular compounds inhibiting factors associated with bone turnover considerably reduce bone pain in addition to anti-bone resorbing activity.

Therapeutic agents directing against bone cells and proteins such as osteoclasts, osteoblasts and tumor microenvironment proteins such as TGFβ and bone matrix proteins involved in bone resorption process have been designed and used in bone metastatic treatment [82].

Bisphosphonates

Bisphosphonate pharmaceutical compositions constitute an FDA approved clinical therapeutic bloc against bone degradation by attaching to apatite crystals which constitute the mineral areas of bone and directly affecting osteoclasts. They are used in treatment of hypercalcemia, osteoporosis and Paget’s disease besides metastatic bone disease [83].

In cancer, bisphosphonate therapy is widely used in multiple myeloma and bone metastatic breast, lung and prostate cancer patients, significantly reducing the risk of bone metastasis [79].

When bone surface containing bisphosphonates is dissolved by osteoclast resorbing activity, bisphosphonates are released affecting osteoclast survival and osteoclastogenesis. [84]. However, osteoblast-induced bone remodeling is prevented due to the natural prohibitive role of bisphosphonates for mineralization likewise synthetic pyrophosphate analog [85].

Potential adverse side effects after bisphosphonate medication include anemia, gastrointestinal symptoms, fatigue, fever, weakness, arthralgia, myalgia, hypocalcemia and osteonecrosis [86].

Denosumab

Denosumab, a fully human monoclonal antibody, represents the first RANKL inhibitor approved by FDA and it is used to treat osteoporosis, bone metastases and multiple myeloma. Moreover, it is widely administrated to postmenopausal women with risk of osteoporosis. Mimicking OPG mechanism of action, denosumab inhibits RANKL-osteoclast RANK receptor binding, hence indirectly prevents osteoclast precursor development and bone turnover [86,87].

Non osteoclast-targeting therapeutics

Multiple therapeutic agents target, besides osteoclasts, proteins that are involved in bone resorption, as well as osteoblasts. These agents include the recombinant OPG structure AMGN-0007 [88], the vitamin K analogue plumbagin; inhibitor of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κB) [89], EGFR kinase inhibitor Gefinitib [90], antibodies against PTHrP [91], and TGF-β signaling inhibitors such as BMP-7 [92]. In addition to beneficial effect on the anti- osteoclastogenetic process, targeting factors that reduce skeletal tumor burden is under investigation. Representative examples are the inhibitor of cathepsin K (odanacatib) which is produced by cancer cells [93], C-X-C chemokine receptor type 4 (CXCR4) blockade

by antibodies or CTCE-9908 synthetic peptidic antagonist [94] and PSK 1404 antagonist of integrin αvβ3 [95]. Moreover, DKK-1, sclerostin proteins as well as activin A cytokin might serve as novel therapeutic targets aiming at bone formation impulse in osteolytic bone metastatic disease [94,96].

Brian Ell and colleagues have proved that specific miRNAs such as miR-141 and miR-219 blockade the process of osteoclastogenesis by targeting osteoclast genes while soluble intracellular adhesion molecule (sICAM1) increase it through mediated NFκB signaling mediated by β2 integrin. These findings certainly render miRNAs potential therapeutic targets [97].

Finally, endothelin 1 antagonists inhibit osteoblast proliferation and differentiation in osteoblastic lesions of bone metastases [98].

1.7 Specific proteins potentially involved in metastatic breast cancer to the bone

Markers involved in bone metastatic processes may provide an insight into ongoing rates of bone metabolism and tumor-bone interactions. For example, Notch pathway directly mediates osteoclast differentiation, while members of transforming growth factor- β (TGF-β) superfamily such as activin A, the bone morphogenetic protein (BMP) antagonist noggin and the Wnt protein antagonist dickkopf-1 (DKK-1) prevent osteoblast differentiation in metastatic breast cancer [94]. Breast cancer cells often secrete molecules such as parathyroid hormone-related peptide (PTHrP), interleukin-6, interleukin-11 and EGF-like growth factors which indirectly react through RANKL or OPG on the bone microenvironment to promote osteoclast maturation and bone turnover. Moreover, cytokines such as macrophage colony stimulating factor (M-CSF) and prostaglandin promote osteoclast differentiation through RANKL activity. In addition to the interaction within cancer cells and bone cells, bone matrix lysis induces growth factors release, such as TGF-β and insulin-like growth factor-I, which feedback cancer cells by activating further growth. This is reported as the ‘vicious cycle’ of bone metastasis [99].

Different clinical studies demonstrated that overexpression of many of the genes or proteins mentioned above are associated with bone metastatic disease and correlate to poor prognosis [100,101,102], while the concealment of tumor-suppressor genes or protein inhibition enhanced osteolytic lesions in breast mouse models [102,103]. In this context, proteins potentially involved in bone metastatic disease are currently under investigation with a view to being reclaimed as prognostic tools and determining the kind of the treatment.

1.7.1 The role of Jagged1 protein in metastatic breast cancer

Jagged1 is a cell surface ligand expressed by mammalian cells which interacts with cell surface notch receptors to drive Notch signaling pathway. Notch receptors include four different transmembrane proteins: Notch1, Notch2, Notch3 and Notch4 that interact with five ligands: delta-like family ligands (DLL1, DLL3, and DLL4) and Jagged ligands (Jagged1 and Jagged2). After ligand binding, the notch receptor undergoes cleavage and its intracellular domain translocates to the nucleus to regulate transcriptional processes such as cell-cell interaction, multiple cell differentiation processes, cell proliferation and cell death [104].

Jag1 gene is located on chromosome 20 at the cytogenetic location 20p12.2 and includes 26 exons over 36kb. Loss of *Jag1* function results in an autosomal dominant disorder, the Alagille syndrome. *Jag1* produces a protein of 1218 amino acids. Jagged1 type I transmembrane protein and comprises a relatively large extracellular domain, an intramembrane domain and a smaller intracellular domain. Extracellular domain contains a 21- amino acid, N-terminal C2 phospholipid identification domain, followed by a 40 amino-acid Delta/Serrate/Lag-2 (DSL) domain, 16 Epidermal Growth Factors (EGF) repeats and a Cystein-Rich Domain (CRD). DSL domain is involved in Jagged1 binding to notch receptor and to cluster of differentiation 46 (CD46) which play a role in T cell regulation. C2 domain interaction with phospholipid bilayers of a neighboring cell seems to further mediate Notch signaling activation and EGF repeats are indispensable for enhancing the affinity of Jagged1 to Notch receptors [105]. Jagged1 protein can be cleaved into its extracellular and C-terminal intracellular domain by ADAM17 and γ secretase respectively. Extracellular soluble

Jagged1 binds to neighboring cells inducing Notch signaling pathway while the intracellular domain is responsible for inherent Jagged1 transformation through PDZ motif [106].

In adults, Jagged1 is highly expressed in heart, placenta, pancreas and prostate tissues, whereas lower expression is found in lung, liver, kidney, thymus, testis tissues and leukocytes [105].

Several studies have shown that Jagged1 expression is the most leading conductor of aberrant Notch pathway activation among Delta-like family and Jagged ligands and that it may be involved in tumor angiogenesis [107,108], cell growth, cancer stem cell self-renewal [109,110,111], EMT induction [112], metastatic progress and resistance to therapy [102].

In breast cancer, *in vivo* and ex vivo experiments demonstrated that high Jagged1 expression is associated with poor prognosis [113,114,115], recurrence and death [116] and thus it has predictive value [113,115]. In addition, it significantly correlated to high-grade breast cancers [109]. Furthermore, Jagged1 expression in breast primary tumors was correlated to higher tumor grade, vascular invasion, overexpression of HER2, high Ki-67 and ALDH1 positivity. Finally, the follow up of these patients showed that strong Jagged1 expression is correlated to lymph node positivity, metastatic progress and higher number of DTCs in bone marrow aspirates [115].

Sethi and colleagues proved that tumor cells present Jagged1 positively correlate to bone metastasis in patients with breast cancer, while when Jagged1 expression is knockdown, bone metastatic competence is reduced [102]. Besides, functional assay indicate that Jagged1 knockdown can inhibit tumor cell growth in mice [117]. Finally, Sethi and colleagues proposed two mechanisms of Jagged1 metastatic propensity, firstly through interleukin-6 release from osteoblasts promoting cell growth and secondly by stimulating osteoclast differentiation and subsequent bone turnover (Figure 1.6) [102].

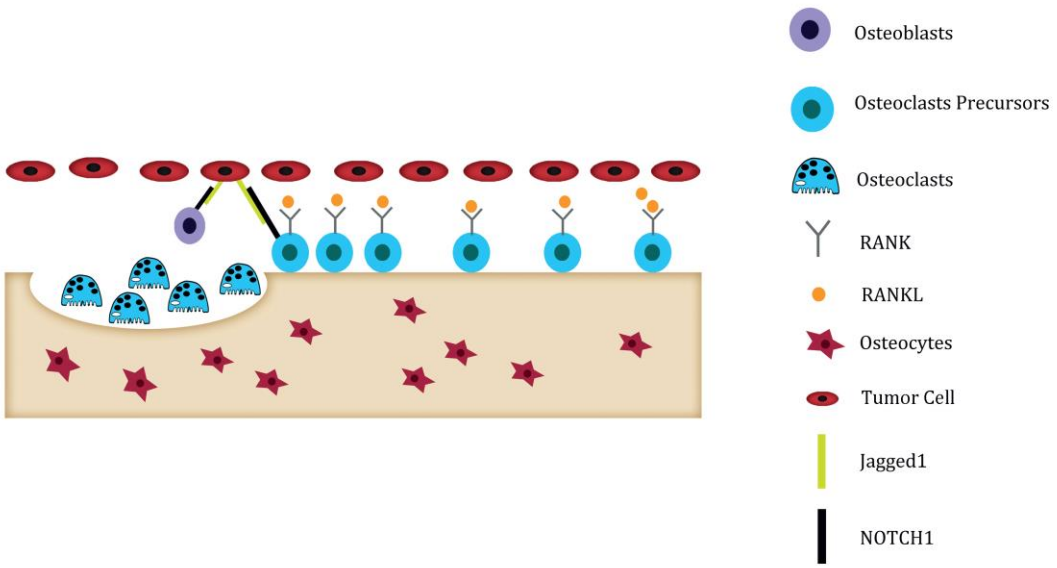

Figure 1.6 Role of Jagged1 protein in metastatic breast cancer [77]

Taking into consideration the synergistic role of Jagged1 expression in bone metastatic disease, Jagged1 inhibition might present a novel therapeutic approach for breast cancer patients with disease in progress. Gamma secretase inhibitors have been preclinically recognized to prevent the interaction between breast tumor cell with bone cell by blocking the activation of Notch signaling pathway.

1.7.2 The role of integrin alpha5 beta1 (ITG α5β1) protein in metastatic breast cancer

Integrins are heterodimeric transmembrane cell proteins which bind to extracellular matrix through extracellular matrix proteins such as collagens, fibronectin, and laminins resulting in regulation of cell cycle, cell shape, and motility. A distinct integrin molecule consists of two noncovalent associated transmembrane glycoprotein subunits called α and β. Each subunit is divided into an N-terminal large extracellular tracellular and transmembrane domains of subunit α which are retained together by a

disulfide bond. The extracellular domain of subunit α includes four divalent-cation-binding sites while the extracellular part of the β subunit one divalent-cation-binding site and a repeating cysteine-rich region. There are 18 types of α subunits and 8 types of β subunits whose combination forms 24 different αβ heterodimers human integrin heterodimers [118,119].

Integrins mediate cell attachment to the extracellular matrix by low affinity binding to their ligands via extracellular divalent cations including Ca^{2+} or Mg^{2+}, depending on the integrin. After the binding of an integrin to its ligand in the matrix, the intracellular part of the β subunit binds to several intracellular anchor proteins such as talin, α-actinin, and filamin. These anchor proteins in turn bind directly to other anchor proteins such as actin or vinculin leading to integrin clustering and cell adhesion to the matrix. Besides cell- extracellular matrix adhesion, integrins may also function as cell-cell adhesion molecules; for example in blood cells serving cell binding to other cells. Integrin clustering also activates intracellular signaling pathways through cytoplasmic protein interaction with the integrin intracellular domain or contiguous phosphorylations to positively or negatively mediate integrin binding to their ligands [118,120,121].

In cancer, integrins expressed on epithelial cells of solid tumors regulate cell attachment with the basement membrane and they potentially facilitate tumor survival and motility [118,120,121]. Integrins may be particularly involved in metastatic disease by mediating dissemination-related mechanisms including matrix-degrading prompt by proteases, survival signalling, invasion and proliferation. It has been indicated that integrin expression in tumor cells is correlated to disease progression in various cancers. Besides expression on tumor cells, integrins are expressed in tumor microenvironment components such as endothelial cells, fibroblasts, pericytes, bone marrow-derived cells, inflammatory cells and platelets functioning further tumor expansion mechanisms including angiogenesis, desmoplasia and immune response [118,121].

Integrin alpha5 beta1 (ITG α5β1) belongs to the integrin family and it is a receptor for fibronectin ligand [122]. ITG α5β1 is considered to present high expression levels in

tumor tissue compared to normal tissue [118] and its expression is associated with angiogenesis [123], EMT [124], and tumor invasion [125]. Expression of β1 integrin in breast tissue is significantly correlated to poor survival in invasive breast cancer [126] while β1 blockade seems to inhibit cell growth of resistant to HER2- targeted agents cells [127] and it seems to reduce migrating properties of bone metastatic breast cancer cells *in vitro* [119]. Moreover, *in vivo* studies have demonstrated that tumor growth in bone microenvironments was decreased when β1 integrin was knocked down [119]. However, ITG α5β1 contribution to cancer is controversial due to previous studies demonstrating that ITG α5β1 can suppress transformation under some conditions [128,129].

1.7.3 The role of VCAM-1 protein in metastatic breast cancer

Vascular cell adhesion molecule 1 (VCAM-1) is a transmembrane sialoglycoprotein 110kDa, which belongs to immunoglobulin gene family. VCAM-1 long extracellular domain contains mainly seven immunoglobulin-like domains [128] and its shorter intracellular domain interacts with Erzin protein strengthening VCAM-1 binding to actin cytoskeleton [129]. Another VCAM-1 isoform includes six immunoglobulin-like extracellular domains.

VCAM-1 is in principle expressed in epithelia, mesothelium, endothelium, myoblast, dendritic cells and bone marrow stromal cells, [130] mediating intercellular adhesion and interaction [131]. Its expression is induced by inflammation related cytokines such as TNF-α, interleukin-6, and TGF- β1 [132]. Moreover, VCAM-1 selectively binds to α4 integrins such as α4β1 or α4β7 structurally exposed on the surface of hematopoietic cells facilitating immune system cell passage and retention into inflammatory sites [130,133]. It has been reported that VCAM-1 expression in leukocytes and vascular endothelial cells seems to be involved in immune response by regulating leukocyte transendothelial trafficking and homing into tissues [132]. In addition to its transmembrane form, a secreted form of VCAM-1 protein has been shown to determine T- cell and monocyte directions as well as endothelial cell stimulation to promote angiogenesis in case of inflammation. VCAM-1 undergoes

proteolytic cleavage by neutrophil-derived serine proteases (such as neutrophil elastase and cathepsin G) or metalloproteases and it is abstracted from the cell surface [130].

Aberrant expression of VCAM-1 in tumor cells appears to be associated with tumor progression and metastasis via malignant cell interactions with tumor microenvironment and cell adhesion to vascular endothelial cells promoting angiogenesis [132,134,135,136,137,138,139]. Particularly, in bone marrow, Lu and colleagues proposed that VCAM-1 expression in cancer cells is linked to osteoclast progenitor differentiation into mature osteoclasts. In addition, growth factors released by bone matrix lysis feedback to cancer cells activating the 'vicious cycle' of osteolytic bone metastasis [140] (Figure 1.7). Excluding lung cancer little is known so far about the mechanism through which VCAM-1 protein interacts with tumor microenvironment in other cancers [131].

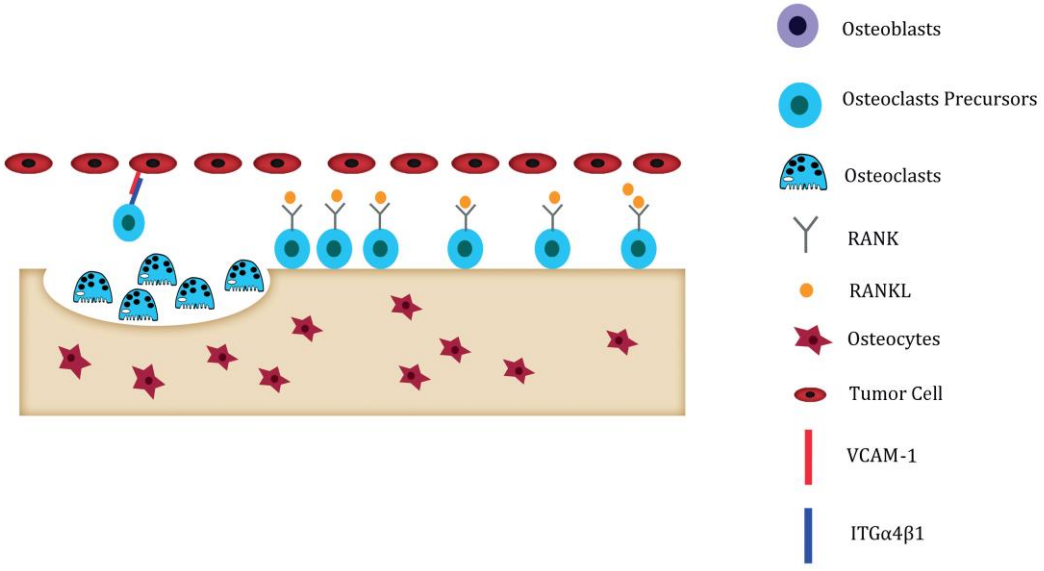

Figure 1.7 Role of VCAM-1 protein in metastatic breast cancer [77]

Studies on breast cancer mouse models have proved that VCAM-1 presence in malignant cells is involved in tumor growth, migration and EMT mediated by TGF-β1 and intereukin-6. It is of note correlated to advanced breast cancer disease and chemoresistance to doxorubicin, while VCAM-1 knockdown led to reduction of cell proliferation and bone metastatic formation [130,140]. Clinical studies on the serum of patients with breast carcinoma indicate that a high soluble VCAM-1 level is correlated to decreased survival and it seems to be a significant prognostic factor in patients with breast cancer [141].

Expression of VCAM-1 in breast cancer cells and its association with advanced and progressive disease recommend new agent design inhibiting VCAM-1 protein and monitoring the disease development. Natalizumab, an FDA approved monoclonal antibody against integrin α4 subunit was designed to prevent integring α4β dimeric formation, hence ITG α4β1 binding to VCAM-1. Although natalizumab is currently administrated in multiple sclerosis and inflammatory bowel disease, future insertion in metastatic breast cancer treatment is recommended by Comi [131,141].

Bisacurone, an additional drug compound possessing anti-inflammatory, anti-oxidant, and anti-metastatic properties, was found to suppress VCAM-1 expression in cancer cells indicating its potentially favorable use as a particular inhibitor of VCAM-1 positive tumor development [142].

AIM OF THE STUDY

This study aimed to investigate the expression of three different proteins: Jagged1, integrin alpha5beta1 (ITG α5β1) and Vascular Cell Adhesion Molecule-1 (VCAM-1) in primary tumors, CTCs and/or DTCs and their clinical relevance in breast cancer patients. The expression of these proteins has been associated with various tumor characteristics; however, their expression in CTCs and DTCs has not been studied so far. Finally, the results were discussed in terms of the potential use of the proteins as liquid biomarkers in metastatic breast cancer.

2. MATERIAL

2.1 Clinical material

The clinical material used in this study is listed in the Table 2.1

Table 2.1 List of the used clinical material treated at UKE

Nr	Type of tissue	Type of specimen	Number of specimen	Origin
1	Breast Cancer	TMA	411	UKE, Hamburg
2	Breast Cancer LNM	TMA	411	UKE, Hamburg
3	Breast Cancer stromal tissue	TMA	411	UKE, Hamburg
4	Breast Cancer stromal tissue LNM	TMA	411	UKE, Hamburg
5	Metastatic Breast Cancer peripheral blood	EDTA- blood	170	UKE, Hamburg
6	Health donors peripheral blood	EDTA- blood	-	UKE, Hamburg

2.1.1 Tissue MicroArray (TMA)

Four-hundred-eleven breast cancer patients treated at University Medical Center Hamburg-Eppendorf (Universitätsklinikum Hamburg-Eppendorf (UKE), Germany) during 1999-2006 were included in this study. The variable clinico-pathological and molecular parameters are documented in the Table 2.2. Last follow-up was completed in August 2014. The median follow-up was 117 months (SD ±50 months, range 2-262 months).

Table 2.2 Clinico-pathological and molecular parameters of breast cancer patients treated at UKE

Clinico-pathologic parameters	Status	Total cohort n	%
Age (years)	median=58		
	range	28-85	
	<median	206	50.2
	>=median	204	49.8
	total	410	
menopause	premenopause	105	26.3
	perimeopause	18	4,5
	postmenopause	277	69.3
	total	400	
T status	T1	217	53.2
	T2	156	38.2
	T3	21	5.1
	T4	14	3.4
	total	408	
N status	N0	255	62.5
	N1-3	153	37.5
	total	408	
M status	M0	386	96.5
	M1	14	3.5
	total	400	
Metastatic relapse	no	335	83.5
	yes	66	16.5
	total	401	
BM status	DTCs (-)	297	74.3
	DTCs (+)	103	25.8
	total	400	
TNM staging	IA	162	40.7
	IIA	117	29.4
	IIB	65	16.3
	IIIA	21	5.3
	IIIB	12	3
	IIIC	7	1.8
	IV	14	3.5
	total	398	
Grade	well differentiated (G1)	32	8
	moderately differentiated (G2)	221	55.3
	poorly differentiated (G3)	147	36.8
	total	400	

Clinico-pathologic parameters	Status	Total cohort n	%
Vascular invasion	**no**	342	91.2
	yes	33	8.8
	total	375	
Recurrence	**no**	296	76.7
	yes	90	23.3
	total	386	
Death	**no**	341	84.6
	yes	62	15.4
	total	403	
HR	**negative**	71	17.3
	positive	340	82.7
	total	411	
Her2 overexpression	**negative**	349	94.1
	positive	22	5.9
	total	371	
Molecular subtype	**luminal A**	136	38.1
	luminal B	159	44.5
	TNBC	61	17.1
	Her2-postive	1	0.3
	total	357	
Ki-67	**neg**	166	45.4
	pos	200	54.6
	total	366	
ALDH1	**neg**	268	90.5
	pos	28	9.5
	total	296	

2.1.2 Peripheral blood- Metastatic breast cancer patients

One-hundred-seventy metastatic breast cancer patients with disease in progression including patients with metastasis to bone, lung, lymph, liver and/or brain were enrolled in this study. The clinico-pathological and molecular parameters are documented in the Table 2.3. All patients were treated at University Medical Center Hamburg-Eppendorf (Hamburg, Germany) between 2012 and 2014. Blood samples (4-14ml each) were collected into EDTA-tubes after patients' informed consent (local ethics board (Ethik-Komission der Ärztekammer Hamburg) allowance nr PV3779).

Table 2.3 Clinico-pathological and molecular parameters of metastatic breast cancer patients treated at UKE

Clinico-pathologic parameters	Status	Total cohort n	%
Age	**median=66**		
(years)	**range**	41-83	
	<median	51	52.0
	>=median	47	48.0
	total	98	
T status	**T1**	31	39.2
	T2	29	36.7
	T3	6	7.6
	T4	13	16.5
	total	79	
N status	**N0**	26	33.8
	N1-3	51	66.2
	total	77	
M status	**M0**	33	60.0
	M1	22	40.0
	total	55	
Grade	**well differentiated (G1)**	5	7.5
	moderately differentiated (G2)	32	47.8
	poorly differentiated (G3)	30	44.8
	total	67	
HR	**negative**	16	16.3
	positive	82	83.7
	total	98	
Her2	**negative**	60	68.2
	positive	28	31.8
	total	88	
Metastatic	**no**	69	69.0
Progression	**yes**	31	31.0
	total	100	
Death	**No**	85	85.0
	Yes	15	15.0
	total	100	
Metastasis locus	**brain**	3	3.1
	bone	65	67.7
	liver	7	7.3
	lung	13	13.5
	no	7	7.3
	others	1	1.0
	total	96	
Therapy	**bisphosponates**	37	44.0
	denosumab	9	10.7
	osteoclast inhibitor	1	1.2
	other chemotherapy	37	44.0
	total	84	

Last follow-up was completed in November 2014. The median observation time was 11 months (SD ±8 months, range 1-25 months).

2.1.3 Peripheral blood - Healthy donors

Healthy donors with no cancer disease were enrolled in this study. Blood samples (7.5 ml each) were collected into EDTA-tubes after patients' informed consent (local ethics board (Ethik-Komission der Ärztekammer Hamburg) allowance nr PV3779) at Department of Transfusion Medicine, University Medical Center Hamburg-Eppendorf (Hamburg, Germany) between 2012 and 2015.

2.2 Ficoll density gradient centrifugation material

Ficoll density gradient centrifugation solutions are described in Table 2.4.

Table 2.4 List of ficoll density gradient centrifugation solutions

Nr	Name of solution	Storage	Company
1	Ficoll-Paque™ PLUS (density1.077±0.001 g/ml)	at 2-8°C	Amersham Biosciences, Germany
1	Human Lyse Buffer (10X)	at 2-8°C	R&D Systems, USA
3	Dulbecco's Phoshate Buffered Saline (DPBS) (1X)	at RT	Life Tecnologies, USA

2.3 Cell culture material

Cell culture media, solutions and breast cancer cell line cultures are described beneath in Tables 2.5 and 2.6.

Table 2.5 List of cell culture media and solutions

Nr	Name of medium/solution	Working Solutions	Storage	Company
1	Dulbecco's Modified Eagle's sterile filtered Medium (DMEM)	4.5 g/L Glucose Sodium Pyruvate 3.7 g/L $NaHCO_3$	at 2-8°C	Pan-Biotech, Germany
2	Roswell Park Memorial Institute sterile filtered Medium (RPMI)1640	2.0 g/L $NaHCO_3$	at 2-8°C	Pan-Biotech, Germany
3	Trypsin	0.05% Trypsin EDTA (1X)	at -20°C	Life Tecnologies, USA
4	Fetal Calf serum (FCS)	-	at -20°C	Gibco Life Technologies, USA
5	Penicillin Streptomycin	[+]10,000 Units/mL Penicillin [+]10,000 µg/mL Streptomycin	at -20°C	Life Tecnologies, USA
6	L-Glutamine	L-Glutamine 200mM (100X)	at -20°C	Life Tecnologies, USA
7	Dimethyl sulfoxide (DMSO)	-	at RT	Serva, Germany
8	Dulbecco's Phoshate Buffered Saline (DPBS) (1X)	-	at RT	Life Tecnologies, USA

Table 2.6 List of breast cancer cell line culture conditions

Nr	cell line	Type of tumor	Medium and conditions
1	SKBR3	adenocarcinoma	• DMEM • 10 % FCS • 1 % Penicillin-Streptomycin Mix • 200mM L-Glutamine at 37°C in 10% CO_2
2	MDA-MB-468	adenocarcinoma	
3	MDA-MB-231	adenocarcinoma	
4	MDA-MB-231-SCP2	adenocarcinoma	
5	MDA-MB-231-SCP6	adenocarcinoma	
6	MCF10A	fibrocystic	
7	MCF7	adenocarcinoma	
8	T47D	ductal carcinoma	
9	MDA-MB-231-BO2	adenocarcinoma	• RPMI • 10 % FCS • 1 % Penicillin-Streptomycin Mix • 200mM L-Glutamine at 37°C in 5% CO_2

2.4 Immunohistochemistry (IHC) and immunofluorescent (IF) staining material

Antibodies, immunohistochemistry and immunofluorescent staining solutions are described beneath in Tables 2.7 and 2.8 respectively.

Table 2.7 List of antibodies

Nr	Antibody/Target	Type	Clone	Host	Method	Dilution	Company
1	polyclonal anti-human CD399/JAG1	primary antibody	110-125	rabbit	IF	1:100	Acris Antibodies, Germany
2	monoclonal anti-human integrin alpha5beta1	primary antibody	JBS5	mouse	(a) IHC	(a)1:500	Millipore, USA
					(b) IF	(b)1:200	
3	monoclonal anti-human VCAM-1	primary antibody	6G9	mouse	IF	1:100	Novus Biologicals, USA
4	monoclonal anti-human Pan-Keratin directly labelled with Alexa Fluor 488	primary antibody	AE1/AE3	mouse	IF	1:700	eBioscience, Germany
5	monoclonal anti-human Pan-Keratin directly labelled with Alexa Fluor 488	primary antibody	C11	mouse	IF	1:300	Cell Signalling Technology, USA
6	monoclonal anti-human CD45 directly labelled with Alexa Fluor 647	primary antibody	HI30	mouse	IF	1:50	Biolegend, USA
7	monoclonal anti-human CD45	primary antibody	RP2/18 & RP2/22	mouse	IHC	1:100	Novocastra, Leica Biosystems
8	polyclonal anti-rabbit Alexa Fluor 555-conjugated	secondary antibody	-	goat	IF	1:200	Life Technologies, USA
9	polyclonal anti-mouse Alexa Fluor 555-conjugated	secondary antibody	-	goat	IF	1:200	Life Technologies, USA
10	biotinylated Rabbit/Mouse (Detection Kit Peroxidase/DAB, Rabbit/Mouse)	secondary antibody	-	-	IHC	-	Dako, Denmark
11	polyclonal anti-mouse HRP-conjugated	secondary antibody	-	rabbit	combined IF & IHC	1:100	Dako, Denmark

Table 2.8 List of IHC and IF staining solutions

Nr	Method	Name of solution	Storage	Working solution	Company
1	IHC	Citrate Buffer (pH 6.0)	at 2-8°C	-	Biogenex, USA
2	IHC	Streptavidin conjugated to HRP in DPBS	at 2-8°C	-	Dako, Denmark
3	IHC	HRP-substrate buffer	at 2-8°C	-	Dako, Denmark
4	IHC	Hematoxylin	at RT	-	Merck, Germany
5	IF & HRP/DAB	Peroxidase-Blocking Solution	at 2-8°C	-	Dako, Denmark
6	IF & HRP/DAB	DAB	at 2-8°C	1:50 DAB in HRP-substrate buffer	Dako, Denmark
7	IF	Fixation Solution B (EPIMET Epithelial Cell Detection Kit)	at RT	135µl Fixation Solution B in 10 mL DPBS (1X)	Micromet, Germany
8	IF	Paraformaldehyde (PFA)	at -20°C	2% PFA in DPBS (1X)	Sigma Technologies, USA
9	IF	Tween 20	at RT	0.1% Tween 20 in DPBS (1X)	Promega, USA
10	IF	Triton X-100	at RT	0.1% Triton X-100 in DPBS (1X)	Sigma Life Science, USA
11	IF	AB Serum	at 2-8°C	10% AB Serum in DPBS (1X)	Bio-RAD Medical Diagnostics GmbH, Germany
12	IF	Vectashield Mounting Medium with DAPI	at 2-8°C	-	Vector Laboratories, USA
13	IF & IHC	Dulbecco's Phoshate Buffered Saline (DPBS) (1X)	at RT	-	Life Tecnologies, USA

2.5 Equipment- Apparatus

Apparatus constitute equipment are described beneath in Table 2.9

Table 2.9 List of apparatus

Nr	Name of apparatus	use	Company
1	Multiguge 3_{S-R}	Ficoll density gradient centrigugation	Heraeus,Germany
2	Megafuge 40R	Ficoll density gradient centrigugation	Thermo Scientific, USA
3	Rotofix 32	Cell spin down	Hettich, Germany
4	Axiostar plus light microscope	Cell counting	Zeiss, Germany
5	Discovery C15 light and fluorescence microscopy	IHC visualising	Leica, Germany
6	Axioplan 2 light and fluorescence microscopy	IF visualising	Zeiss, Germany
7	Hera cell 150	Cell incubation	Thermo Scientific, USA
8	Waterbath	Solution incubation	GFL,Germany

.

3. METHODS

3.1 Ficoll density gradient centrifugation - Method description

Each blood samples (4-14ml) was processed within a maximum of 24 h after collection. Peripheral blood mononuclear cells (PBMCs) were enriched using the Ficoll-Paque gradient centrifugation. The blood was mixed with DPBS (1X) (total volume 30ml), carefully layered on 20 mL Ficoll-Hypaque and centrifuged for 30 minutes at 1400 rpm (acceleration=1, deceleration=1) (Picture 3.1.A). The PBMCs fraction, preferably containing CTCs, was collected and re-suspended in DPBS (1X) (total volume 50ml) and centrifuged for 10 minutes (Picture 3.1.B) at 1400 rpm (acceleration=9, deceleration=9). The red cell pellet was incubated in 1 mL of Lyse Buffer (10X) for 3 minutes in order to purify PBMCs from remained red blood cells (RBCs), re-suspended in DPBS (1X) (total volume 50ml) and centrifuged again for 10 minutes at 1400 rpm (acceleration=9, deceleration=9). White cell pellet was re-suspended in 5ml of DPBS (1X) and spun down in centrifugation pocket for 3 minutes at 1200 rpm to prepare the microscopic slides (each one containing 500000 cells). The excess volume of DPBS (1X) was discarded. The slides were left overnight to air-dry at room temperature and were used within 24 hours for further analysis. For long-term storage, slides were wrapped in aluminium foil and stored at -80°C (Picture 3.1.C).

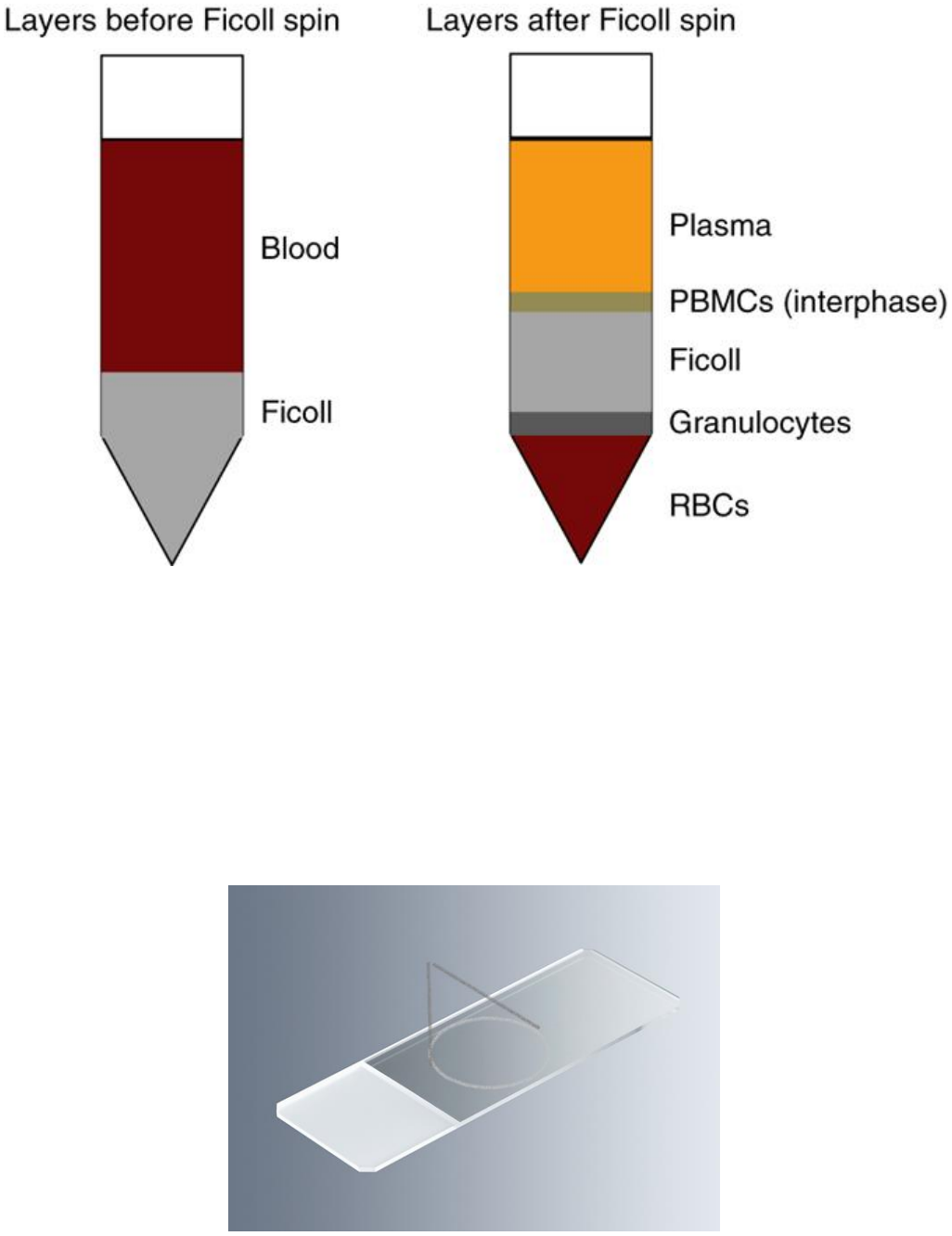

Figure 3.1 Ficoll density gradient centrifugation: **A)** Ficoll-blood layers depiction before centrifugation and **B)** Ficoll-blood layers depiction after centrifugation **C)** Microscopic slide after cell spin down

3.2 Preparation of cell line material

Each cell line was cultured in an appropriate medium at 37°C as described in Table 2.6. The passages were taken in every 2-3 days depending on a cell line and confluence status. The cryo-preservation vials were prepared in a standard way with the usage of a proper medium and 10% DMSO.

Cultured cells were spiked into the serum of enriched PBMCs from the peripheral blood of healthy donors after ficoll density gradient centrifugation (rate: 1 tumor cell in 20 PBMCs) and followed by centrifugation for 3 minutes at 1200 rpm in order to spin down the cells onto a slide surface. The excess volume of DPBS (1X) was discarded. The slides were left overnight to air-dry at room temperature and used the following day for further analysis. If not, they were wrapped in an aluminum foil and stored at -80°C.

3.3 Integrin alpha5 beta1 (ITG α5β1) immunohistochemical staining (IHC)

Deparaffinized TMA sections were treated for 5 minutes in citrate buffer (pH 6.0, Biogenex, USA) at 120°C at a steamer and incubated for 16 hours at 4°C with mouse monoclonal anti-human ITG α5β1antibody diluted 1:500 in Dako REAL^{TM} Antibody Diluent. The staining was envisioned by DAKO ChemMate Detection Kit Peroxidase/DAB, Rabbit/Mouse. Particularly, slides were incubated for 10 minutes with biotinylated secondary anti- rabbit and anti-mouse antibodies and 5 minutes with peroxidase blocking solution. Subsequently, sections were incubated with streptavidin conjugated to HRP for 10 minutes and with DAB chromogen solution diluted 1:50 in HRP substrate buffer. All specimens were counterstained with hematoxylin.

3.4 Immunofluorescent staining (IF)

In general, all immunoflorescent stainings were performed on 3 slides containing 500000 cells each. If patient's slides were negative for CTCs another set of 3 slides was used for subsequent analysis to prove the result.

3.4.1 Jagged1/Keratin/CD45 immunofluorescent staining

The slides were fixed for 10 minutes with the Fixation Solution B for Epithelial Cell Detection Kit (135 µL diluted in 10 mL of DPBS (1X)) and permeabilized for 5 minutes in 0.1% Tween 20 in DPBS (1X). Subsequently, the cells were incubated with AB blocking serum, diluted 1:10 in DPBS (1X) for 20 minutes.

Slides were incubated for 45 minutes with an anti-human Jagged1 polyclonal rabbit antibody in AB blocking serum diluted 1:10 in DPBS (1X). As secondary antibody, an anti-rabbit Alexa 455–conjugated antibody diluted 1:200 in AB blocking serum was used for 45 minutes. Subsequently, cells were incubated with the anti-human pan-keratin antibody AE1/AE3 and C11 both directly labeled with Alexa 488 diluted 1:700 and 1:300 respectively in AB blocking serum for 45 minutes. Cells were further incubated with the anti-human CD45 directly labelled with Alexa 647 diluted 1:50 in AB blocking serum. All specimens were counterstained with DAPI VectaShield Medium and covered with coverslips.

3.4.2 ITG α5β1/Keratin/CD45 immunofluorescent staining

The cells were fixed for 10 minutes with 2% PFA in DPBS (1X) and subsequently incubated with AB blocking serum, diluted 1:10 in DPBS (1X) for 20 minutes.

Slides were incubated for 45 minutes with an anti-human monoclonal ITG α5β1 mouse antibody in AB blocking serum diluted 1:10 in DPBS (1X). As secondary antibody, an anti-mouse Alexa 455–conjugated antibody diluted 1:200 in AB blocking serum was used for 45 minutes. Subsequently, cells were incubated with the anti-human pan-keratin antibody AE1/AE3 and C11 both directly labeled with Alexa 488 diluted 1:700 and 1:300 respectively in AB blocking serum for 45 minutes. Cells were further incubated with the anti-human CD45 directly labelled with Alexa 647 diluted 1:50 in AB blocking serum. All specimens were counterstained with DAPI VectaShield Medium and covered with coverslips.

3.4.3 ITG α5β1 immunofluorescent staining in combination with HRP/DAB for CD45 detection

The cells were fixed for 10 minutes with 2% PFA in DPBS (1X) and subsequently incubated with Peroxidase-Blocking Solution for 5 minutes and AB blocking serum, diluted 1:10 in DPBS (1X) for 20 minutes.

Slides were incubated for 45 minutes with the anti-human monoclonal CD45 mouse antibody (NCL-LCA-RP, Novocastra) in AB blocking serum diluted 1:10 in DPBS (1X). As secondary antibody, an anti-mouse HRP–conjugated antibody diluted 1:100 in AB blocking serum was used for 30 minutes. Slides were further incubated for 10 minutes with DAB. Subsequently, cells were incubated with an anti-human monoclonal ITG α5β1 mouse antibody and a secondary anti-mouse Alexa 455–conjugated antibody diluted 1:200 both in

AB blocking serum for 45 minutes. Cells were further incubated with the anti-human pan-keratin antibody AE1/AE3 and C11 both directly labeled with Alexa 488 diluted 1:700 and 1:300 respectively in AB blocking serum for 45 minutes. All specimens were counterstained with DAPI VectaShield Medium and covered with coverslips.

3.4.4 VCAM-1 /Keratin/CD45 immunofluorescent staining

The slides were fixed for 10 minutes with the Fixation Solution B for Epithelial Cell Detection Kit (135 µL diluted in 10 mL of DPBS (1X)) and permeabilized for 5 minutes in 0.1% Tween 20 in DPBS (1X). Subsequently, the cells were incubated with AB blocking serum, diluted 1:10 in DPBS (1X) for 20 minutes.

Slides were incubated for 45 minutes with an anti-human VCAM-1 monoclonal mouse antibody in AB blocking serum diluted 1:10 in DPBS (1X). As secondary antibody, an anti-mouse Alexa 455–conjugated antibody diluted 1:200 in AB blocking serum was used for 45 minutes. Subsequently, cells were incubated with the anti-human pan-keratin antibody AE1/AE3 and C11 both directly labeled with Alexa 488 diluted 1:700 and 1:300 respectively in AB blocking serum for 45 minutes. Cells were further incubated with the anti-human CD45 directly labelled with Alexa 647 diluted 1:50 in AB blocking serum. All specimens were counterstained with DAPI VectaShield Medium and covered with coverslips.

4. RESULTS

4.1 Jagged1 expression in breast cancer

Jagged1 expression was examined in seven breast tumor cell lines (Table 2.6) in the background of PBMCs to optimize and validate an immunofluorescent staining protocol for detection of putative CTC and analysis of Jagged1 expression in those cells. All tumor cells were identified as positive for the epithelial marker-tumor specific Keratin (K^+), positive for the nuclear counterstain DAPI ($DAPI^+$), negative for the PBMC- specific antigen CD45 ($CD45^-$) and positive for Jagged1 ($Jagged1^+$).

Furthermore, CTCs were enriched from peripheral blood of metastatic patients with progressive disease and evaluated by immunofluorescent staining. Jagged1 expression was compared to clinico-pathological parameters and patients' outcome.

4.1.1 Jagged1 expression in breast tumor cell lines

All tested 'standard' breast cancer cell lines were found positive for Jagged1 in a dot-like pattern in cytoplasm and nucleus (Figure 4.1 and 4.2). Jagged1 expression was stronger in MDA-MB-231-SCP2 cells with high affinity to bone compared to MDA-MB-231-SCP6 with low affinity to bone (Figure 4.2, A. and B.). When MDA-MB-231-SCP2 cell lines were transfected with shRNA silencing Jagged1 (MDA-MB-231-SCP2 TR shRNA), Jagged1 expression was weaker in comparison to MDA-MB-231-SCP2 (Figure 4.2, A. and C.). The majority of leukocytes and thrombocytes were positive for Jagged1 expression in all analyzed patients.

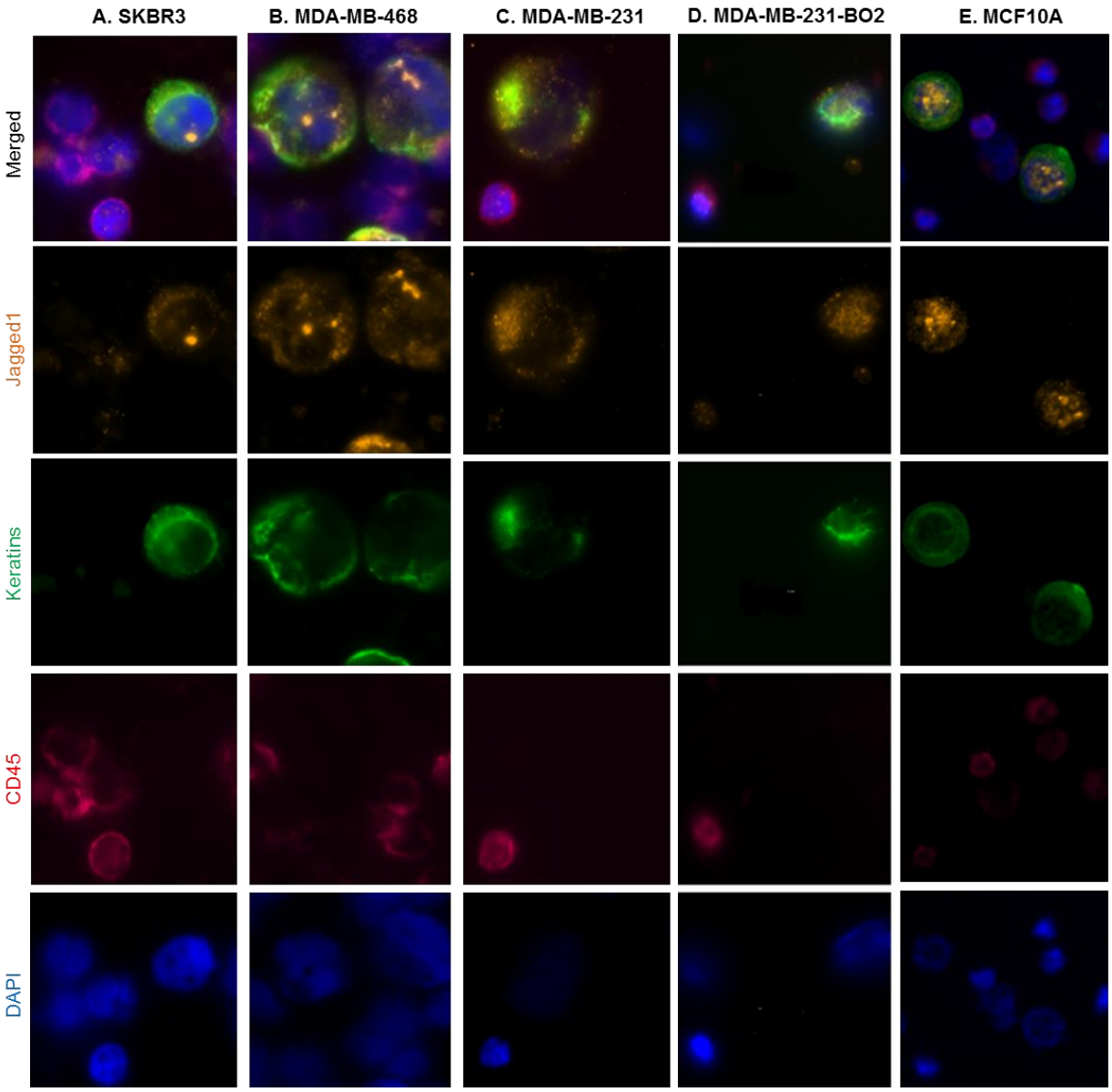

Figure 4.1 Representative images of Jagged-positive breast tumor cells **A**. SKBR3 (630x magnification), **B**. MDA-MB-468 (630x magnification), **C**. MDA-MB-231 (630x magnification), **D**. MDA-MB-231-BO2 (630x magnification) and **E.** MCF10A (400x magnification) in the background of leukocytes, stained with Jagged1 (orange) / keratins (green) /CD45 (red) counterstained with DAPI (blue) to visualize cells' nuclei. Tumor cells were defined as $K^+/DAPI^+/CD45^-/Jagged1^+$.

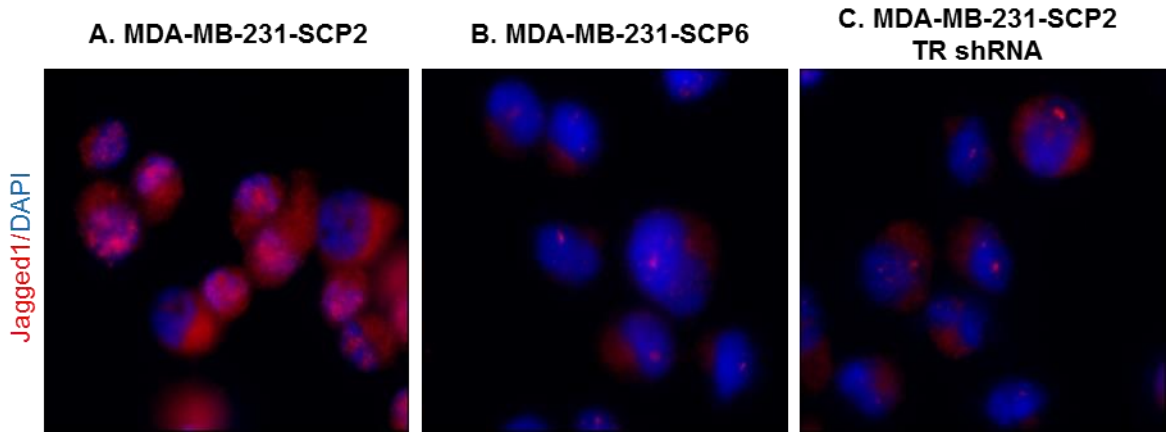

Figure 4.2 Representative images of Jagged-positive breast tumor cells **A**. MDA-MB-231-SCP2, **B**. MDA-MB-231-SCP6 and **C.** MDA-MB-231-SCP2 cell lines TR shRNA. Jagged1 expression was weaker after silencing Jagged1 in MDA-MB-231-SCP2 cells (630x magnification).

4.1.2 Expression of Jagged1 in Circulating Tumor Cells (CTCs) of metastatic breast cancer patients

Jagged1 expression was evaluated in CTCs of 100 metastatic patients with progressive disease including patients with metastasis to bone, lung, lymph, liver and/or brain (Table 2.3). CTCs were detected by $K^+/DAPI^+/CD45^-$ immunofluorescent staining (Chapter 3.4.1) in the background of PBMCs ($K^-/DAPI^+/CD45^+$) and further assessed for Jagged1 expression.

Twenty-one patients were detected with CTCs with a range from 1-101 cells per 1500000 PBMCs. Three patients (14.3%) presented with CTCs only negative for Jagged1 (Jagged1(-)CTCs) (range from 1-4 cells per 1500000 PBMCs), 15 (71,4%) presented with only Jagged1(+)CTCs (range from 1-12 cells per 1500000 PBMCs) while 3 (14.3%) patients displayed heterogeneous expression of Jagged1 in their CTCs (range from 1-17 Jagged1(-) cells and 1-84 Jagged1(+) cells per 1500000 PBMCs). When present, Jagged1 was expressed moderately or strongly in all CTCs in a dot-like pattern in cytoplasm and nucleus (Figure 4.3). The majority of leukocytes were positive for Jagged1 expression in all analyzed patients.

The presence of total CTCs and Jagged1(+)CTCs did not correlate to primary tumor's characteristics and also did not indicate any specific site of metastases. Both CTCs and Jagged1(+)CTCs were significantly associated with shorter progression-free survival (PFS) in patients treated with bisphosphonates (p=0.026 and p=0.013 respectively) (Figure 4.4 B. and E). Although not statistically significant, Jagged1(+)CTCs seemed to predict shorter progression-free survival in the total cohort of patients and shorter overall survival in patients with bone metastasis (p=0.078 and p=0.082 respectively) (Figure 4.4, D. and F).

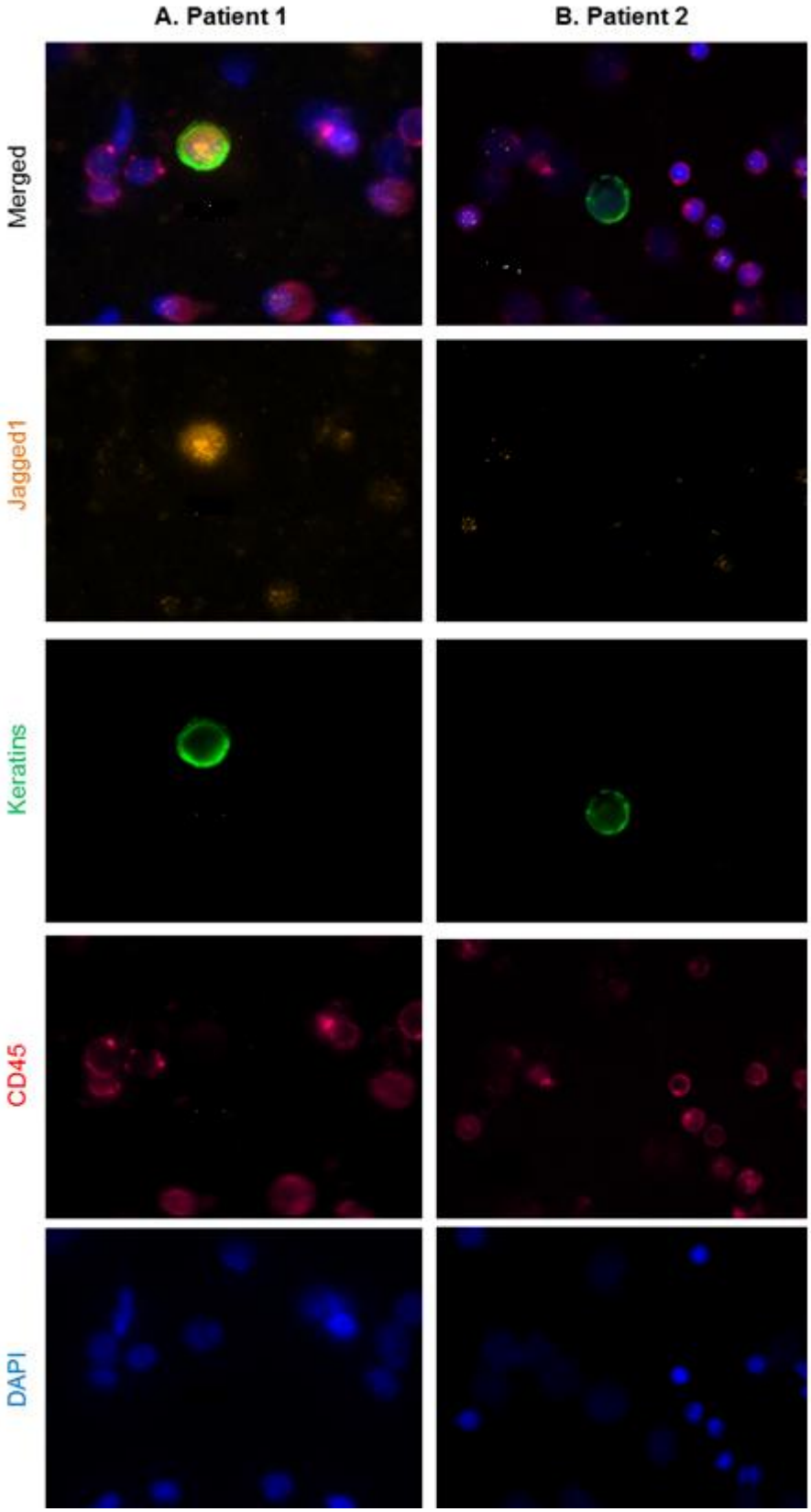

Figure 4.3 Representative images of **A**. a Jagged-positive CTC (100x magnification) and **B**. a Jagged-negative CTC (400x magnification) in the background of leukocytes, stained with Jagged1 (orange) / keratins (green) /CD45 (red) counterstained with DAPI (blue) to visualize cells' nuclei. Tumor cells were defined as K^{+}/$DAPI^{+}$/$CD45^{-}$/$Jagged1^{+/-}$.

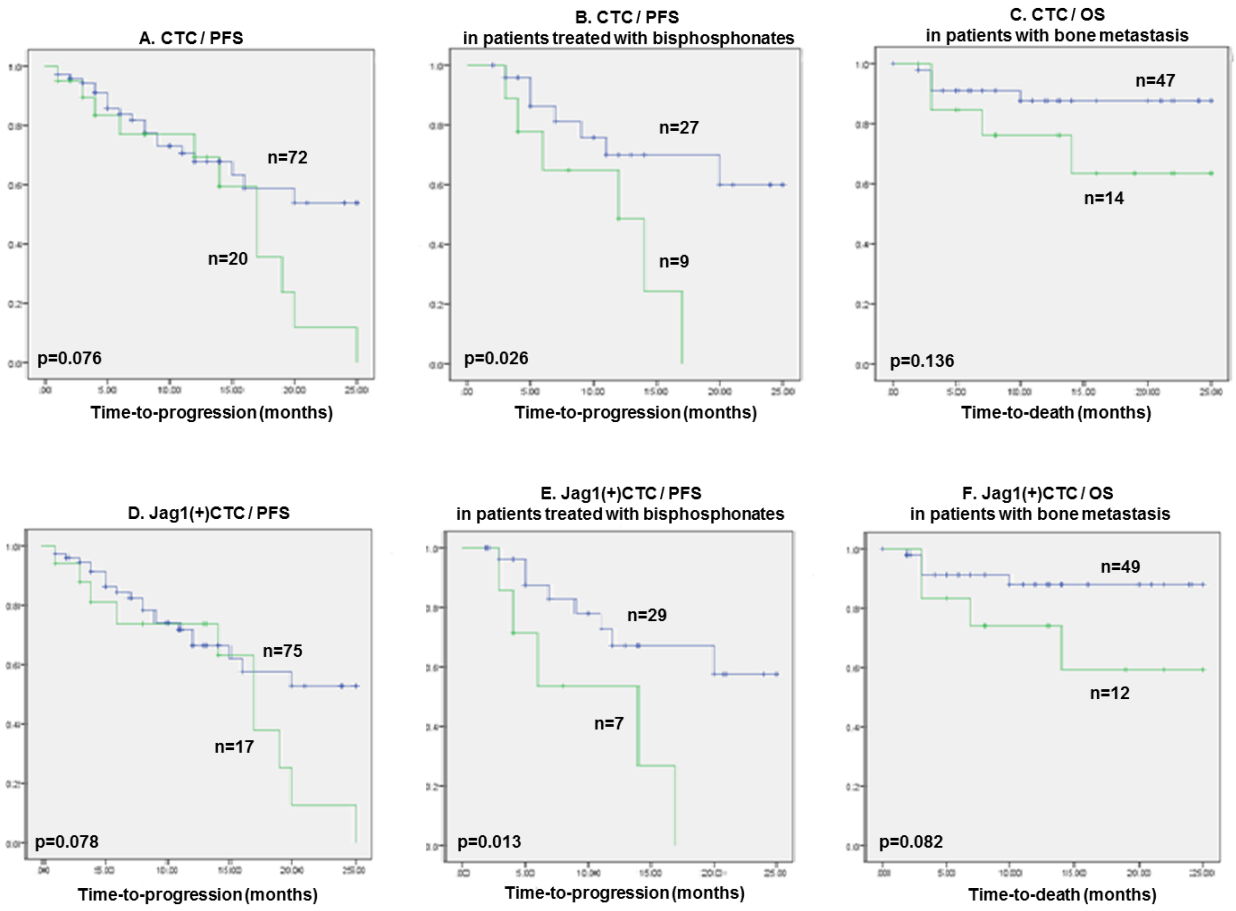

Figure 4.4 Kaplan-Meier curves. **A.** Association of CTC number with PFS in total cohort of patients, **B.** Association of CTC number with PFS in patients treated with bisphosphonates, **C.** Association of CTC number with OS in patients with bone metastasis, **D.** Association of Jagged1(+)CTCs with PFS in total cohort of patients, **E.** Association of Jagged1(+)CTCs with PFS in patients treated with bisphosphonates, **F.** Association of Jagged1(+)CTCs with OS in patients with bone metastasis

4.2 Integrin alpha5 beta1 (ITG α5β1) expression in breast cancer patients

Integrin alpha5 beta 1 expression was initially examined in seven breast tumor cell lines (Table 2.6) in the background of PBMCs to set up and validate an immunofluorescent protocol for putative CTC detection and ITG α5β1 analysis. All tumor cells were identified as K^+/$DAPI^+$/$CD45^-$/ ITG $\alpha5\beta1^+$ or K^+/$DAPI^+$/$CD45^-$/ITG $\alpha5\beta1^-$.

ITG α5β1 expression was examined in primary tumors and of breast cancer patients using an immunohistochemical method. Furthermore, CTCs and DTDs were enriched from peripheral blood and bone marrow respectively of metastatic patients with progressive disease and evaluated by immunofluorescent staining. ITG α5β1 expression was finally compared to clinico-pathological parameters and patients' outcome.

4.2.1 Expression of ITG α5β1 in breast tumor cell lines

MDA-MB-231, MDA-MB-231-SCP2, MDA-MB-231-SCP6 and MCF10A breast tumor cell lines were found positive for ITG α5β1 in a dot-like pattern in cytoplasm and nucleus, and in plasma membrane. Weak expression of integrin ITG α5β1 was observed in MDA-MB-231-BO2 cells (Figure 4.5). No ITG α5β1 expression was observed in SKBR3 and MDA-MB-468 cell lines (Figure 4.6). The majority of leukocytes and thrombocytes were positive for integrin alpha5 beta1expression.

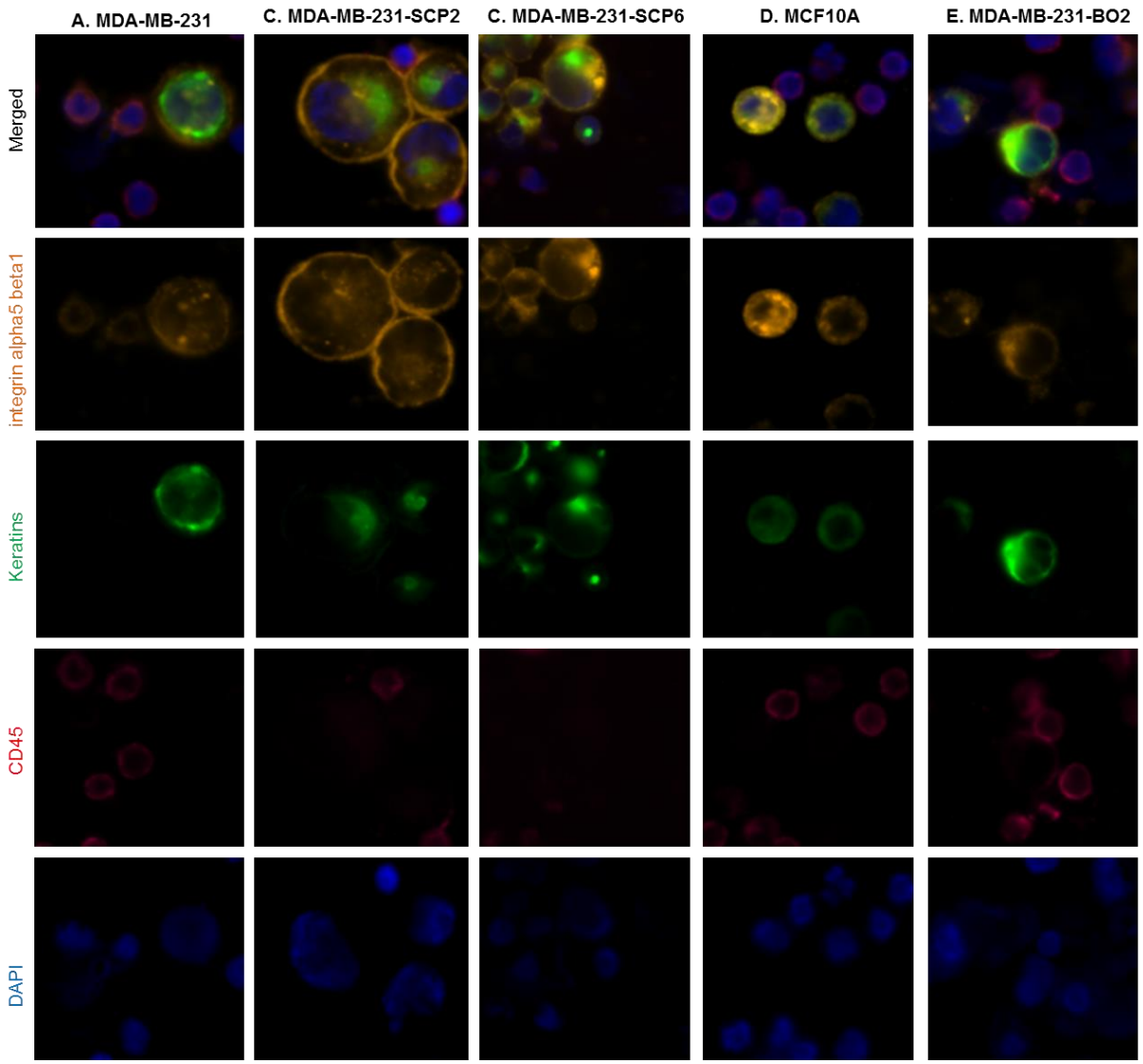

Figure 4.5 Representative images of ITG α5β1-positive breast tumor cells **A**. MDA-MB-231, **B**. MDA-MB-231-SCP2, **C**. MDA-MB-231-SCP6, **D**. MCF10A and **E**. MDA-MB-231-BO2 in the background of leukocytes and stained with integrin alpha5 beta1 (orange) / keratins (green) /CD45 (red) counterstained with DAPI (blue) to visualize cells' nuclei. Tumor cells were defined as K^+/$DAPI^+$/$CD45^-$/ integrin ITG $\alpha5\beta1^+$ (400x magnification).

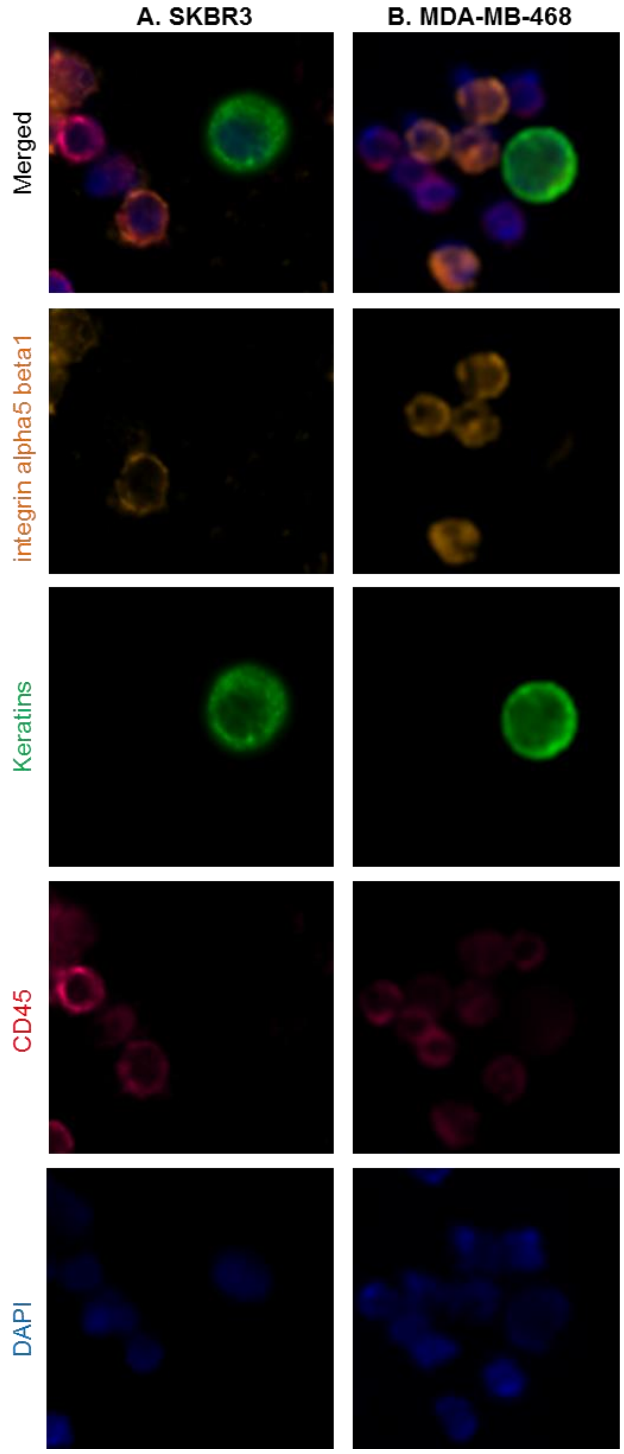

Figure 4.6 Representative images of ITG α5β1–negative breast tumor cells **A**. SKBR3 (400x magnification) and **B**. MDA-MB-468 (200x magnification) in the background of leukocytes, stained with integrin alpha5 beta1 (orange) / keratins (green) /CD45 (red) counterstained with DAPI (blue) to visualize cells' nuclei. Tumor cells were defined as K^+/$DAPI^+$/$CD45^-$/ ITG $\alpha5\beta1^-$.

4.2.2 Expression of ITG α5β1 in primary tumors (TMAs) of breast cancer patients

ITG α5β1 expression was evaluated by immunohistochemistry in 268 informative tumor tissues and 76 LNMs. In primary breast cancers, negative, weak, moderate and strong ITG α5β1 expression was detected in 85 (31.7%), 53 (19.8%), 80 (29.9%) and 50 (18.7%) patients respectively. The percentage of ITG α5β1 -positive cells ranged from 1 to 100 with a median of 50%. One-hundred- sixty two (60.4%) patients were positive for integrin ITG α5β1 expression, when index score and its median calculated from all samples were used as a classificator and cut-off, respectively. Subcellular localization of the staining and percentage of marker-positive tumor cells were documented. No, membranous and cytoplasmic expression was detected in 85 (31.7%), 23 (8.6%) and 160 (59.7%) of the primary breast cancers, respectively.

In LNMs, 26 (34.2%), 21 (27.6%), 16 (21.1%), 13 (17.1%) of the samples were characterized by negative, weak, moderate and strong ITG α5β1 expression, respectively. The percentage of ITG α5β1 -positive cells ranged from 5 to 100 with a median of 50%. Fourteen (18.4%) cases were positive for ITG α5β1 expression in LNMs according to the index score. No, membranous and cytoplasmic expression was detected in 26 (34.2%), 7 (9.2%) and 43 (56.6%) of the LNMs, respectively.

In primary tumors, ITG α5β1 expression correlated to M1 status (p=0.046), presence of DTCs in bone marrow aspirates (p=0.035) as well as micro- and/or macrometastasis (p=0.007). It was also significantly associated with ALDH1 and Jagged1 expression (p=0.023 and p=0.025 respectively) (Table 4.1).

ITG α5β1 expression did not correlate to shorter disease-free (DFS) and overall survival (OS) in total cohort (p=0.169 and p=0.921 respectively) or other subsets (Figure 4.7).

Table 4.1 Correlations of ITG α5β1 expression in primary tumors to clinical parameters

Clinico-pathologic parameters	Status	Integrin alpha5 beta1				p-value
		Neg		Pos		
		n	%	n	%	
Age	**median=58**					
(years)	**range**					
	<median	56	53.3	88	54.3	
	>=median	49	46.7	74	45.7	
	total					0.874
menopause	**premenopause**	27	26	48	30.1	
	perimeopause	8	7.7	4	2.5	
	postmenopause	69	66.3	107	67.3	
	total					0.129
T status	**T1**	51	48.6	90	55.6	
	T2	41	39.0	61	37.7	
	T3	6	5.7	8	4.9	
	T4	7	6.7	3	1.9	
	total					0.200
N status	**N0**	60	57.7	97	59.9	
	N1-3	44	42.3	65	40.1	
	total					0.724
M status	**M0**	101	99	155	96.3	
	M1	1	1.0	6	3.7	
	total					0.178
M status final	**M0**	99	97.1	146	90.7	
	M1	3	2.9	15	9.3	
	total					**0.046**
metastasis locus	**bone**	1	14.3	9	50	
	brain	1	14.3	2	11.1	
	visceral	4	57.1	4	22.2	
	others	1	14.3	3	16.7	
	total					0.310
TNM	**IA**	38	37.3	66	41	
staging	**IIA**	26	25.5	48	29.8	
	IIB	20	19.6	28	17.4	
	IIIA	8	7.8	7	4.3	
	IIIB	7	6.9	2	1.2	
	IIIC	2	2	4	2.5	
	IV	1	1	6	3.7	
	total					0.136
Grade	**well differentiated (G1)**	8	8,0	9	5.6	
	moderately differentiated (G2)	57	57.0	92	57.5	
	poorly differentiated (G3)	35	35.0	59	36.9	
	total					0.743

Clinico-pathologic parameters	Status	Integrin alpha5 beta1				
		Neg		Pos		p-value
		n	%	n	%	
Recurrence	**No**	89	89,0	130	82.3	
	Yes	11	11.0	28	17.7	
	total					0.142
Death	**No**	90	91.8	146	91.3	
	Yes	8	8.2	14	8.8	
	total					0.870
BM status	**DTCs (-)**	84	82.4	113	71.1	
	DTCs (+)	18	17.6	46	28.9	
	total					**0.039**
Micro- and	**No**	84	82.4	107	67.3	
macrometastasis	**Yes**	18	17.6	52	32.7	
	total					**0.007**
HR	**negative**	21	19.8	26	16.0	
	positive	85	80.2	136	84.0	
	total					0.428
Her2	**negative**	69	97.8	134	93.1	
	positive	2	2.2	10	6.9	
	total					0.111
Molecular	**HR-pos**	72	80	111	77.1	
subtype I	**Her2-postive**	2	2.2	10	6.9	
	Basal-like	16	17.8	23	16.0	
	total					0.276
Molecular	**luminal A**	36	43.4		36.4	
subtype II	**luminal B**	31	37.3		46.9	
	TNBC	16	19.3		16.1	
	Her2-postive	0	0		0.7	
	total					0.449
Ki-67	**neg**	42	50.6	68	45.3	
cut-off:10%	**Pos**	41	49.4	82	54.7	
	total					0.440
ALDH1	**neg**	85	97.7	102	89.5	
expression	**pos**	2	2.3	12	10.5	
	total					**0.023**
Jag1	**neg**	63	67.7	58	52,3	
expression	**pos**	30	32.3	53	47.7	
	total					**0.025**

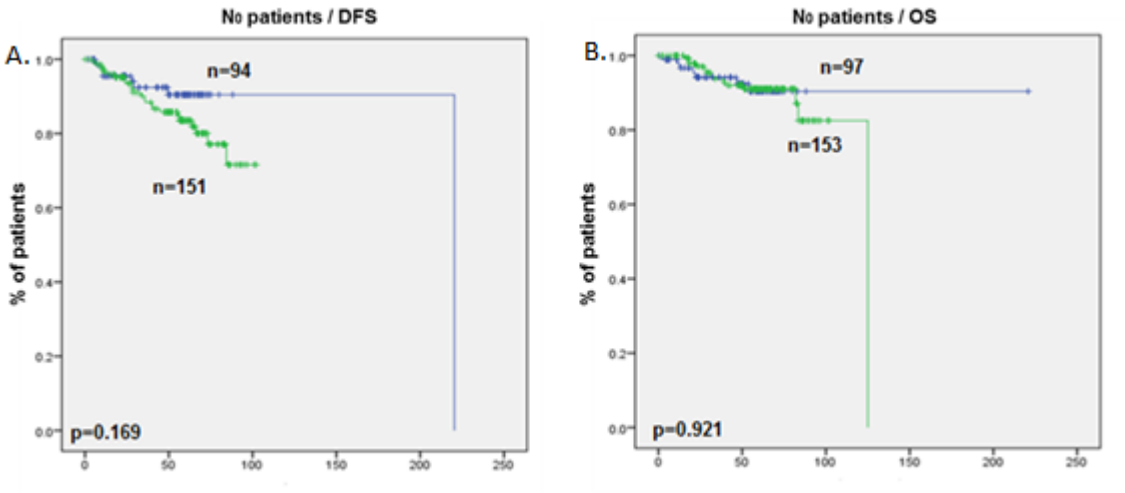

Figure 4.7 Kaplan-Meier curves. ITG α5β1 correlations to A) DFS and B) OS in patients with lymph node-negative breast cancer

4.2.3 Expression of ITG α5β1 in Circulating Tumor Cells (CTCs) and Disseminated Tumor Cells (DTCs) of metastatic breast cancer patients

ITG α5β1 expression was evaluated in CTCs of 31 patients included in the cohort of metastatic breast cancer patients with progressive disease. CTCs were detected by $K^+/DAPI^+/CD45^-$ immunofluorescent staining in the background of PBMCs ($K^-/DAPI^+/CD45^+$) and further assessed for ITG α5β1 expression.

Nine patients were positive for CTCs (range from 1-40 cells per 1500000 PBMCs) but none of patients presented ITG α5β1(+)CTCs. The majority of leukocytes were positive for integrin ITG α5β1 expression in all analyzed patients (Figure 4.8 A.).

EMT occurring during tumor cell dissemination might be followed by epithelial-specific antigen downregulation such as keratins and EpCAM [73]. Therefore, CTCs having undergone EMT might restrain keratin expression. In order to identify potential presence of $K^-/DAPI^+/CD45^-/ITG\ \alpha5\beta1^+$ tumor cell phenotype, an additional immunocytochemistry (ICC) protocol was established based on HRP/DAB staining of CD45. Therefore, CD45 and DAPI staining was combined with morphologic identification to identify tumor out of the leukocyte background. All morphologically tumor-like and CD45-negative cells of seven patients presented negative ITG α5β1 expression (Figure 4.9).

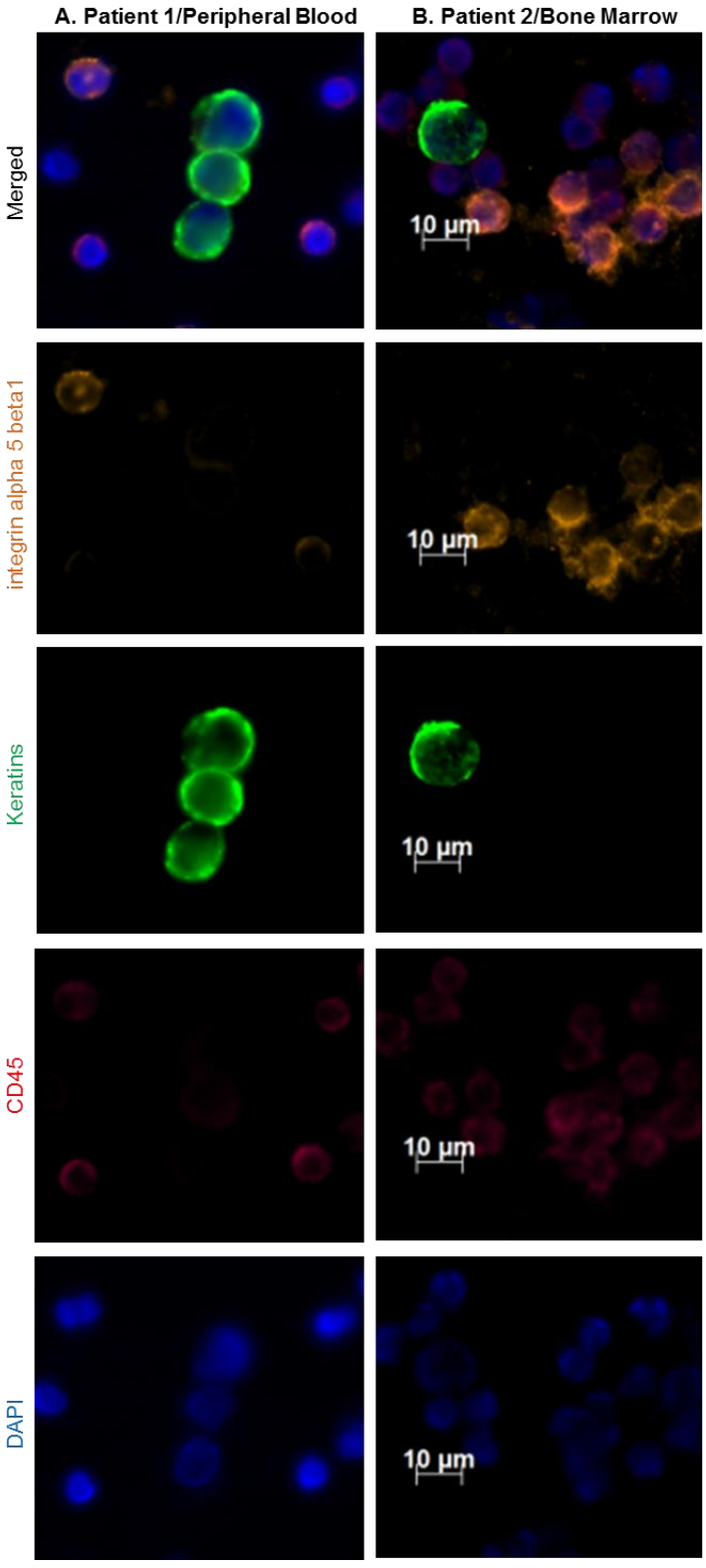

Figure 4.8 Representative images of **A**. integrin alpha5 beta1-negative CTCs and **B**. an integrin alpha5 beta1-negative DTC in the background of HSC, stained with integrin alpha5 beta1 (orange) / keratins (green) /CD45 (red) counterstained with DAPI (blue) to visualize cells' nuclei. Tumor cells were defined as K^+/$DAPI^+$/$CD45^-$/ integrin alpha5 beta1 (400x magnification).

Disseminated tumor cells (DTCs) enriched from bone marrow of unmatched 4 metastatic patients were detected by $K^+/DAPI^+/CD45^-$ immunofluorescent staining in the background of hematopoietic stem cells (HSC) ($K^-/DAPI^+/CD45^+$). DTCs were further assessed for ITG α5β1 expression.

Three patients were positive for DTCs (range from 1-12 cells per 1500000 HSC) but none of patients presented with integrin ITG α5β1(+)DTCs. The majority of HSC were positive for ITG α5β1 expression in all analyzed patients (Figure 4.8 B.).

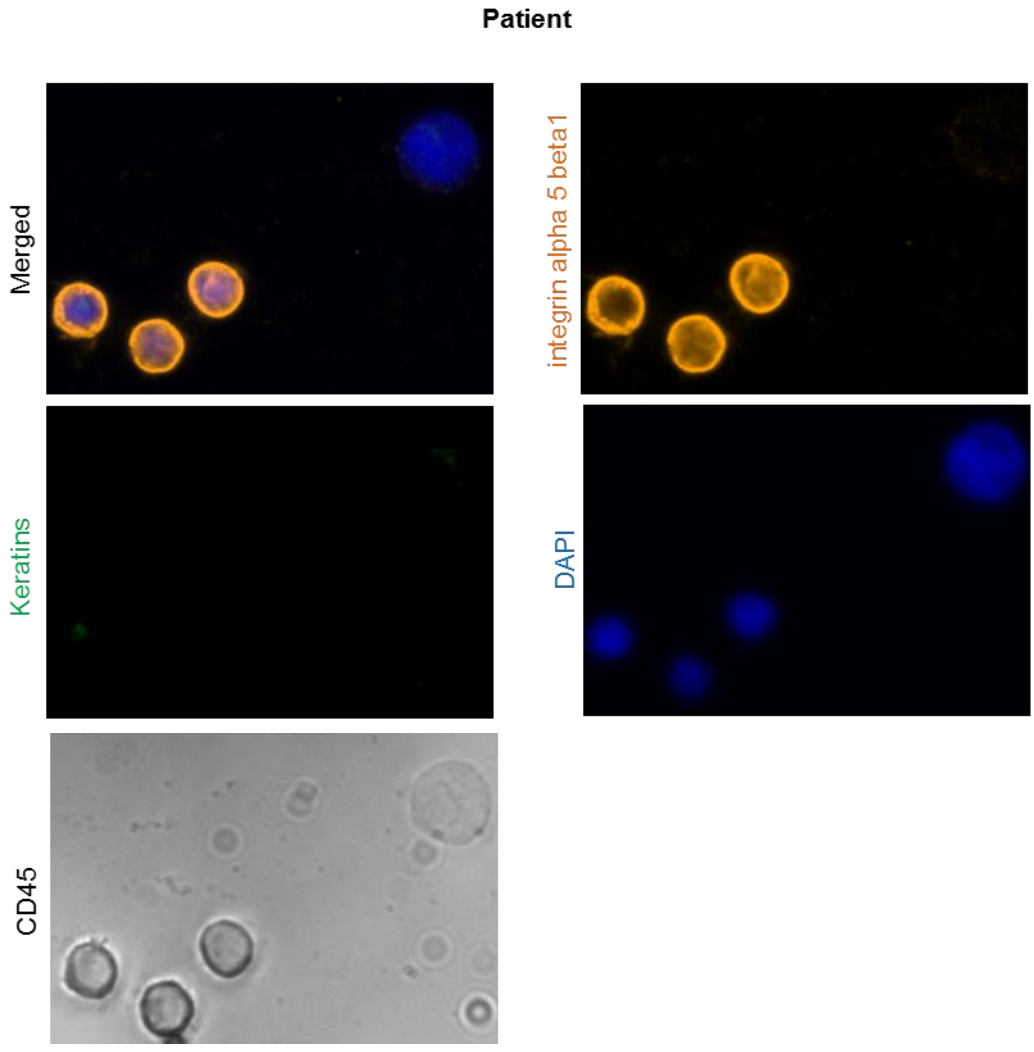

Figure 4.9 Representative image of an integrin alpha5 beta1-negative CTC in the background of PBMCs, stained with integrin alpha5 beta1 (orange) / keratins (green) /CD45 (grey) counterstained with DAPI (blue) to visualize cells' nuclei. CTC was defined as $K^-/DAPI^+/CD45^-$/ integrin alpha5 beta1.

4.3 VCAM-1 expression in breast cancer patients

VCAM-1 expression was initially examined in seven breast tumor cell lines (Table 2.6) in the background of PBMCs to set up and validate an immunofluorescent protocol for putative CTC detection and VCAM-1 analysis. All tumor cells were identified as K^+/$DAPI^+$/$CD45^-$/ $VCAM\text{-}1^+$.

VCAM-1 expression was examined in CTCs enriched from peripheral blood of metastatic patients with progressive disease and evaluated by immunofluorescent staining. VCAM-1 expression was finally compared to clinico-pathological parameters and patients' outcome.

4.3.1 Expression of VCAM-1 in breast tumor cell lines

All tested breast tumor cell lines were found positive for VCAM-1 in a dot-like pattern in cytoplasm, nucleus and plasma membrane. Leukocytes also expressed VCAM-1 (Figure 4.10). The majority of leukocytes were positive for Jagged1 expression in all analyzed patients.

4.3.2 Expression of VCAM-1 in Circulating Tumor Cells (CTCs) of metastatic breast cancer patients

VCAM-1 expression was evaluated in CTCs of 149 metastatic breast cancer patients. CTCs were detected by K^+/$DAPI^+$/$CD45^-$ immunofluorescent staining in the background of PBMCs (K^-/$DAPI^+$/$CD45^+$) and further assessed for VCAM-1.

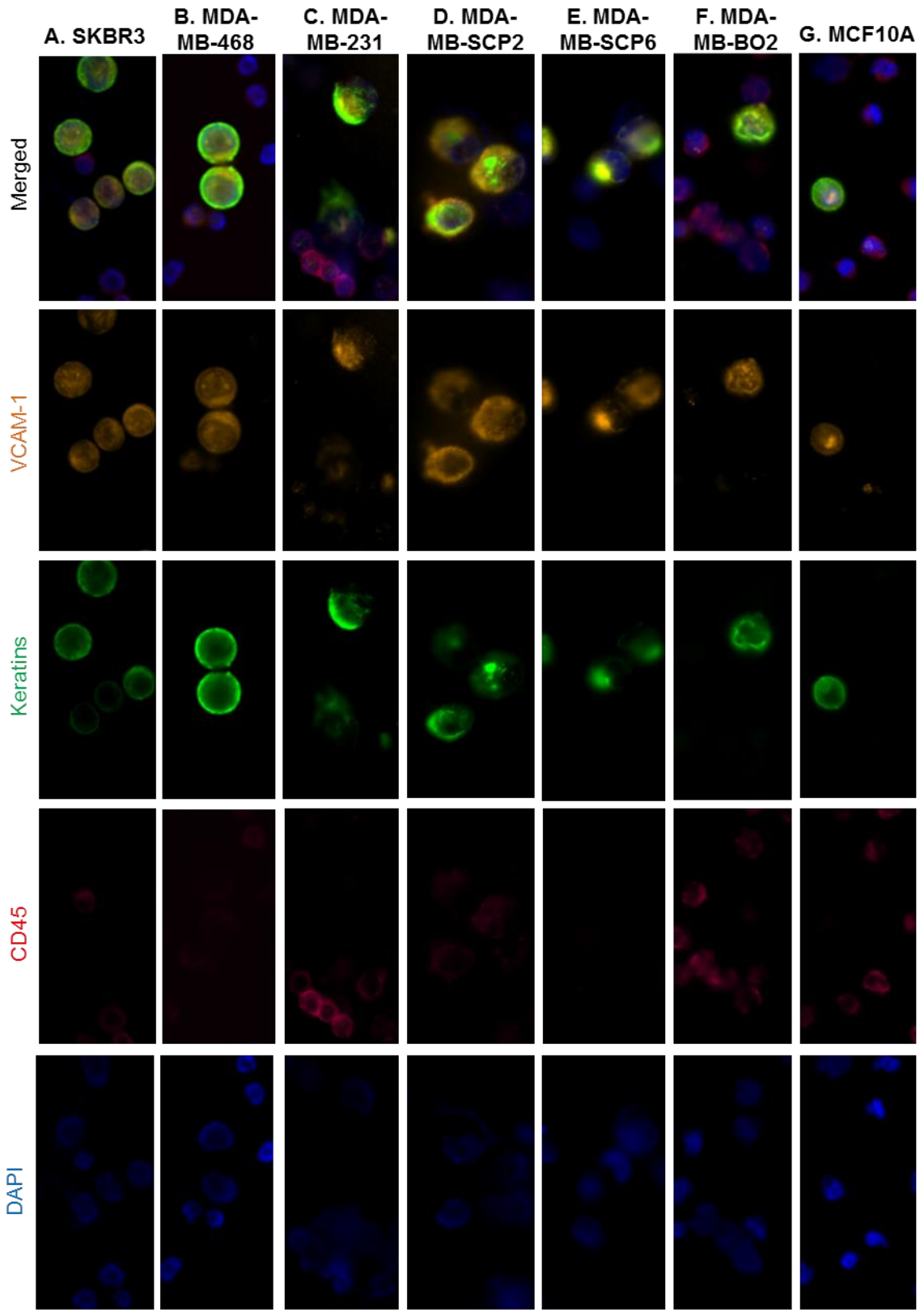

Figure 4.10 Representative images of VCAM-1 -positive breast tumor cells **A**. SKBR3, **B**. MDA-MB-468 and **C**. MDA-MB-231, **D**. MDA-MB-231-SCP2, **E**. MDA-MB-231-SCP6, **F**. MDA-MB-231-BO2 and **G**. MCF10A in the background of leukocytes, stained with VCAM-1 (orange) / keratins (green) /CD45 (red) counterstained with DAPI (blue) to visualize cells' nuclei. Tumor cells were defined as K^+/$DAPI^+$/$CD45^-$/ $VCAM\text{-}1^+$(400x magnification).

Nineteen patients were detected with CTCs with a range from 1-87 cells per 1500000 PBMCs. Four patients (21.0%) presented with CTCs only negative for VCAM-1(VCAM-1(-)CTCs) (1 cell per 1500000 PBMCs), 11 (58.0%) presented with only VCAM-1 (+)CTCs (range from 1-87 cells per 1500000 PBMCs) while 4 (21.0%) patients displayed heterogeneous expression of VCAM-1 (range from 1-8 VCAM-1(-) cells and 1-85 VCAM-1(+) cells per 1500000 PBMCs) in their CTCs. When present, VCAM-1 was expressed moderately or strongly in all CTCs in a dot-like pattern in cytoplasm and plasma membrane (Figure 4.11). The majority of leukocytes were positive for VCAM-1 expression in all analyzed patients. Microscope analysis was performed with 400x magnification.

VCAM-1 expression in CTCs did not correlate to primary tumor's characteristics and also did not indicate any specific site of metastases (Table 4.2).

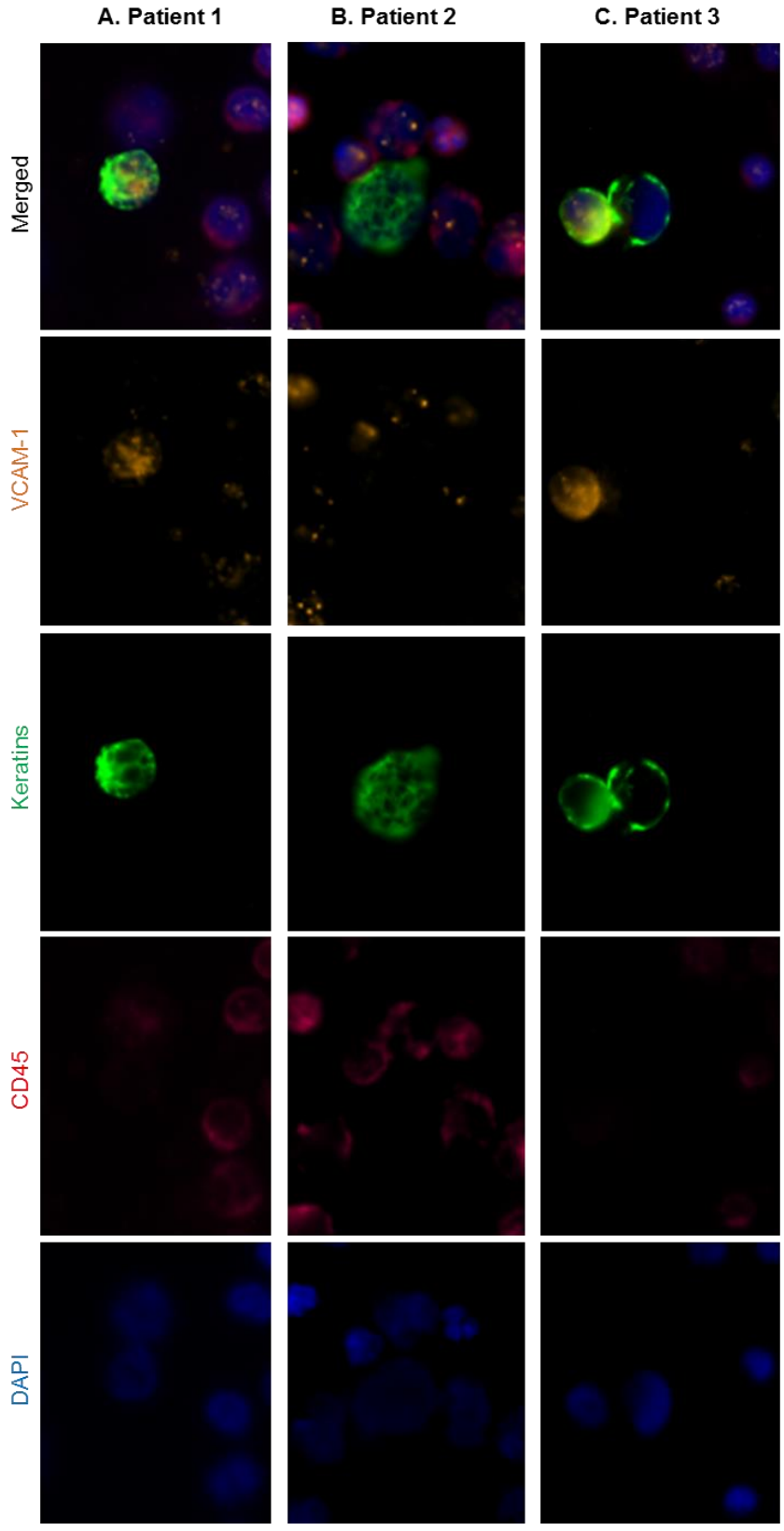

Figure 4.11 Representative images of patients with **A**. a VCAM-1-positive CTC **B**. a VCAM-1-negative CTC and **C.** two CTCs of an heterogeneous patient with a VCAM-1-positive and a VCAM-1-negative CTC in the background of leukocytes, stained with VCAM-1 (orange) / keratins (green) /CD45 (red) counterstained with DAPI (blue) to visualize cells' nuclei. Tumor cells were defined as K^{+}/$DAPI^{+}$/$CD45^{-}$/$VCAM\text{-}1^{+/-}$ (400x magnification).

Table 4.2 Correlations of VCAM-1 expression in CTCs to clinical parameters

Clinico-pathologic parameters	Status	VCAM-1				p-value
		neg		pos		
		n	%	n	%	
T status	**T1**	24	38.7	1	12.5	
	T2	23	37.1	5	62.5	
	T3	4	6.5	1	12.5	
	T4	11	17.7	1	12.5	
	total	62	100	8	100	0.394
N status	**N0**	21	37.5	1	11.1	
	N1	24	42.9	7	77.8	
	N2	4	7.1	1	11.1	
	N3	7	12.5	0	0.0	
	total	56	100	9	100	0.182
M status	**M0**	24	58.5	5	71.4	
	M1	17	41.5	2	28.6	
	total	41	100.0	7	100	0,519
Grade	**well differentiated (G1)**	5	9.3	1	11.1	
	moderately differentiated (G2)	28	51.9	4	44.4	
	poorly differentiated (G3)	21	38.9	4	44.4	
	total	54	100.0	9	100.0	0.918
ER	**negative**	13	17.6	4	40.0	
	1	58	78.4	6	60.0	
	12	3	4.1	0	0.0	
	total	74	100.0	10	100.0	0.225
PgR	**negative**	18	24.3	4	40.0	
	1	53	71.6	6	60.0	
	2	1	1.4	0	0.0	
	12	2	2.7	0	0.0	
	total	74	100.0	10	100.0	0.705
Her2	**negative**	45	68.2	5	71.4	
	positive	21	31.8	2	28.6	
	total	66	100	7	100	0.860
Metastasis locus	**bone negative**	21	27.3	5	45,5	
	positive	56	72.7	6	54.5	
	total	77	100	1	100	0.216
	brain negative	73	94.8	10	90.9	
	positive	4	5.2	1	9.1	
	total	77	100	11	100	0.602
	liver negative	45	58.4	5	45.5	
	positive	32	41.6	6	54.5	
	total	77	100	11	100	0.416
	lung negative	46	59.7	8	72.2	
	positive	31	40.3	3	27.3	
	total	77	100	11	100	0.408
	lymph negative	48	62.3	5	45.5	
	positive	29	37.7	6	54.5	
	total	77	100	11	100	0.285
	others negative	51	66.2	26	33.8	
	positive	26	33.8	3	27.3	
	total	77	100.0	11	100	0.668

5. DISCUSSION

Breast cancer is the most common type of cancer among women worldwide, with a high death rate for young patients aged between 20 and 59 [1]. Approximately, the 90% of cancer patients decease because of metastatic spread of the primary tumor and tumor growth in distant sites [3,4]. CTCs can escape from an established tumor, undergo EMT and enter blood circulation or lymphatic vessels. After wandering in the circulatory system CTCs arrive at specific metastatic sites, with bone as one of the main metastatic sites of breast cancer. Those cells which are able to extravasaste and survive at the remote sites, can undergo MET and form micrometastasis which can potentially develop into macroscopic metastasis (Figure 1.1F) [3,4,143]. Detection of CTCs has been reported in numerous studies on breast cancer patients and the presence of CTCs has been associated with poor prognosis [15,16,17,60,62,64,65,88,89].

Angiogenesis plays an important role in metastatic progress. As far as a normal body is concerned, angiogenesis is a normal process necessary for development of new blood vessels needed for growth, development and wound healing. The main angiogenic factors that regulate angiogenic processes unbder both normal conditions and during cancer progression include Fibroblast Growth Factors (aFGF and bFGF), Transforming Growth Factors (TGF-α and TGF-β), Platelet-Derived Growth Factor (PDGF), angiogenin, Vascular Permeability Factor (VPF), Tumor Necrosis Factors (TNF-α and TNF- β) and Vascular Endothelial Growth Factor (VEGF). In contrast to normal cells, tumor cells under hypoxia conditions induce a chaotic vascular network that can promote the invasive behavior of tumor cells by helping to escape from the tumor mass [19]. Indeed, tumors with rich vascular supplies have been associated with metastatic progress and poor prognosis in breast cancer patients, a fact which justifies the vascular invasion hypothesis, i.e. angiogenesis vital role in tumor dissemination [22,26,144].

Cancer stem cells (CSCs) represent a small subpopulation of total cancer cell number and by definition share similar self-renewal capacity as normal stem cells giving rise to multipotent progenitor cells. CSCs are thought to originate from normal stem cells that have undergone malignant alternations. The aggressive breast cancer molecular subtypes Claudin low and triple negative in contrast to luminal A and B subtype, are characterized by high expression of stemness features such as $CD44^{+}/CD24^{low}$ and aldehyde dehydrogenase 1 positive ($ALDH1^{+}$) expression [18,145]. CSC approach might be quite promising in terms of a treatment perspective by targeting small CSC subpopulation, which initiates cell proliferation and tumor development [146].

Analysis of CTCs that have detached from primary tumor and are present in blood circulation, might offer wealthy information about tumor progression. CTCs have the additional advantage of non-invasive acquisition of patient material at several time points. However, CTC detection remains technologically challenging. CTCs are presented in low rates in peripheral blood amounting to one among 10^{6}-10^{8} PBMCs meaning that 10ml of blood normally contain between 1 and 100 CTCs. Therefore, technologies of high sensitivity and specificity are necessary in order to isolate a high number of CTCs exempting PBMCs contamination (Figure 1.2, 1.3).

Currently, there are more than forty different technological methodologies for CTC enrichment either based on physical properties or on biological properties [74,75,76]. In the current study, CTCs were enriched using ficoll density gradient centrifugation, which is based on differences in density of the molecules. This method has the advantage that CTCs enrichment is unbiased and does thus not depend on the expression of specific surface markers. Ficoll density gradient centrifugation methodology is described in detail in chapter 9.

Besides CTCs, DTCs can be frequently detected in bone marrow may be used as a 'liquid biopsy' in order to gain useful information about metastatic disease and treatment management. DTCs in contrast to CTCs represent tumor cells that has passed an additional step in the metastatic cascade i.e. extravasation from the blood vessels. The presence of DTDs is also associated with early relapse and short overall survival in breast cancer patients [16,68,69,70]. However, bone marrow aspiration is an invasive process, painful for the patient, and hence not approved in routine medical examination.

In this study three different proteins were investigated for their potential use as liquid biomarkers in metastatic breast cancer. The expression of all of these proteins has been associated with various tumor characteristics, however, their expression on CTCs and DTCs has not been studied so far. Notch pathway has been shown to be activated in breast cancer cells that form osteolytic bone metastases in animal models. Notch ligand Jagged1 is reported to be involved in the formation of osteolytic bone metastases and, therefore, hypothetically associated with worse prognosis of breast tumor patients [104,105,106,107,108,109,110,111,112,113,114,115,116,117]. ITG α5β1 is expressed on tumor cells and tumor neovessels facilitating tumor invasion and proliferation [118,119,120,121,122,123,124,125,126, 127,128,129]. VCAM-1 has been associated with early relapse and the activation from tumor cell dormancy. Additionally, blocking of VCAM-1 effectively inhibits metastases formation into the bone marrow or other organs [128,129,130,131,132,133,134,135,136,137,138,139,140,141,142].

5.1 The role of Jagged1 protein in metastatic breast cancer

The activation of Notch pathway in breast cancer cells has been associated with the formation of osteolytic bone metastases. Notch ligand Jagged1 expression plays a major role in aberrant Notch pathway activation and has been associated with several malignant processes including tumor angiogenesis, cell growth, cancer stem cell self-renewal, EMT induction, metastatic progress and resistance to therapy [107,108,109,110,111,112]. In breast cancer, Jagged1 expression in primary tumors is associated with poor prognosis, tumor recurrence and death. In addition, the expression significantly correlated to higher tumor grade, vascular invasion, overexpression of HER2, high Ki-67 and ALDH1 positivity. Finally, the follow up of a group of patients showed that strong Jagged1 expression is correlated to lymph node positivity, metastatic progress and higher number of DTCs in bone marrow aspirates. Finally, it is demonstrated that Jagged1 expression has a positive predictive value of DFS [109,113,114,115,116].

In the current study, we focused on the clinical relevance of Jagged1 expression in tumor progression in response to therapy. In this context, CTCs derived from peripheral blood of breast cancer patients with progressive disease were investigated, for the first time, for Jagged1 expression. These results were correlated to patients' outcomes in order to determine Jagged1 association with disease progression in relation to bone modulating treatment.

According to previous studies of primary tumors, Jagged1 expression describes more aggressive breast carcinoma and potential involvement in tumor cell dissemination and metastatic progression. In particular, Jagged1 protein, present in tumor cells, is significantly correlated to vascular invasion, lymph node and DTC positivity, which implies an increased propensity to

spread through the blood and/or lymph circulation and outgrow into overt metastasis at distant sites [147].

According to the current data, all tested ‘standard’ breast cancer cell lines were found positive for Jagged1, which is consent with previous results pointing out that Jagged1 protein is highly expressed in patients with metastatic disease [113,146,148]. Moreover, Jagged1 expression was stronger in MDA-MB-231-SCP2 cells with high affinity for bones compared to MDA-MB-231-SCP6 with low affinity for bones (Figure 5.1 and 5.2), which supports the association between Jagged1 and bone metastasis [101].

In patients, Jagged1 expression on CTCs was investigated in the peripheral blood of 100 metastatic patients with progressive disease. 21 metastatic BC patients with progressive disease were positive for CTCs, and 85.7 % of the CTCs also expressed Jagged1. It is noteworthy that Jagged1 expressed on CTCs indicated shorter progression-free survival (PFS) in metastatic breast cancer patients treated with bisphosphonates but no other bone-modulating drugs. Bisphosphonates constitute an FDA approved clinical therapeutic bloc against bone degradation. The mechanism of this action includes the attachment of bisphosphonates to the apatite crystals which constitute the mineral areas of bone and directly affecting osteoclasts [83]. In vivo, bisphosphonates significantly reduce the risk of bone metastasis [78]. Based on our results we can hypothesize that Jagged1 protein status in tumor cells characterizes resistance of metastatic breast cancer patients to bisphosphonate-based therapy due to therapy insufficiency to overcome increased osteoclast activity in bone lesions. Therefore, Jagged1 expression in CTCs might serve as a biomarker of bisphosphonate treatment failure. These results may also recommend that bisphosphonate treatment combined with Jagged1 targeting agents (such as Jagged1 neutralizing antibodies) may achieve better therapeutic response in bone metastatic patients with Jagged1-positive CTCs.

The fact that no other bone-modulating drug than bisphosphonates indicated shorter PFS in patients with tumor cells positive for Jagged1 could be biased by the low number of cases treated with drugs other than bisphosphonates (only 10 cases treated with other drugs). Clearly larger studies need to be conducted in order to investigate the potential association between Jagged1 expression and bone-modulating drugs such as the FDA approved denosumab, the monoclonal antibody against RANKL.

Jagged1 expression on CTCs was associated neither with shorter PFS in the total cohort of metastatic breast cancer patients nor with overall survival (OS) in the cohort of bone- specific metastatic breast cancer patients. In addition, the number and presence of CTCs was also unrelated to both shorter PFS of the total cohort, and OS of patients with bone- specific metastatic breast cancer. However, CTC counts in patients treated with bisphosphonates showed shorter PFS. These rather surprising results may be explained by the low number of CTCs detected in the peripheral blood of the investigated cohort. The used method has been shown to be less sensitive although highly specific compared to other systems and thus a significantly larger study population would have been needed [73,74,75].

In conclusion, Jagged1 could become a fascinating future target to monitor bisphosphonate- based therapy particularly in patients resistant to this kind of therapy. In addition to the potential application of Jagged1 as a clinical biomarker and a therapeutic target to eliminate cancer cell dissemination to bone [147], Jagged1 targeting agents in combination with bisphosphonates could bring off better therapeutic results in breast cancer patients with metastatic progression. Still other studies using perhaps more sensitive CTC detection methods should be conducted in order to assess it exact clinical potential.

5.2 Integrin alpha5 beta1 (ITG α5β1) expression in breast cancer

Integrins are commonly expressed on epithelial cells of solid tumors and mediate cell attachment with the basement membrane [118,120] as well as the dissemination-related mechanisms in order to facilitate tumor survival, invasion and expansion to distant organs [118,120,121]. Integrin expression in tumor cells is correlated to disease progression in various cancers. In addition to the expression on tumor cells, integrins are expressed in tumor microenvironment components such as endothelial cells, fibroblasts, pericytes, bone marrow-derived cells, inflammatory cells and platelets regulating further tumor expansion mechanisms including angiogenesis and immune response [118,121]. ITG α5β1 is reported to be expressed in tumor cells and tumor neovessels facilitating tumor invasion and proliferation [118]. ITG α5β1 expression is particularly associated with angiogenesis [123], EMT [124], and tumor invasion [125]. Its overexpression is also associated with bad prognosis and metastatic disease of cancer patients [126]. Blockade of β1 subunit seems to inhibit cell growth of HER2- resistant breast cancer cells [127] and it seems to reduce migrating properties of bone metastatic breast cancer cells *in vitro* [119]. Moreover, *in vivo* studies have shown that tumor growth in bone microenvironments was decreased when β1 integrin was knocked down [119]. However, ITG α5β1 contribution to metastatic progress is controversial due to previous studies demonstrating that ITG α5β1 can suppress transformation under some conditions [128,129].

In the present study, we focus on the clinical relevance of ITG α5β1 expression in breast cancer. We have assessed ITG α5β1 expression in two independent clinical sample cohorts. We first analyzed its expression in primary tumors to investigate its clinical relevance and potential utility as a prognostic marker to predict cancer progression. Additionally, CTCs derived from peripheral blood of breast cancer patients with progressive disease were investigated, for the first time, for ITG α5β1 expression. These results

followed correlations to patients' outcomes in order to associate ITG α5β1 with disease progression.

Interestingly, ITG α5β1 was shown to be associated with metastatic progression and more aggressive conduct in primary breast carcinomas but no ITG α5β1 was observed in CTCs and DTDs of metastatic breast cancer patients. The presence of ITG α5β1 protein in tumor cells of primary tumor lesions indicated a high risk of micro- and/or macrometastasis in the examined breast cancer patients. Furthermore, it correlated with M1 status and high numbers of DTCs in bone marrow aspirates at the time of diagnosis.

ITG α5β1 expression in tumors was characterized by a simultaneous high ALDH1 and Jagged1 expression. It might be assumed that ITG α5β1positive tumors with features of cancer stem and progenitor cells (ALDH1 expression) have the highest probability to establish overt metastases at distant sites. However, this issue merits further investigation. Furthermore, during the normal development of central nervous system, progenitor cells are activated in neural microenvironment through ITG β1 subunit activating the Notch pathway [149]. However, a direct relationship between ITG α5β1 and Jagged1 positivity is unclear; hence a potential ITG β1 involvement in Jagged1 and the Notch pathway regulation has to be further examined.

ITG α5β1protein was expressed in 60.4% of primary breast tumors, while the group of Yao found that the ITG β1 subunit was expressed in 79% of patients with invasive breast cancer who had a median follow-up of 8.4 years [125].

Regarding the ITG α5β1 analysis on CTCs and DTCs, none of patients presented tumor cells positive for ITG α5β1, despite the fact that the majority of leukocytes highly expressed this protein in all analyzed patients. Tumor cells are thought to undergo EMT through downregulation of epithelial-specific

antigens including keratins in order to escape from the primary tumor and invade into blood circulation [149,150]. Previous studies indicate that loss of keratin expression in tumor cells seems to amount up to 20% of the cell population [51]. Specifically, Pecot and colleagues identified two different CTC populations in patients with breast, ovarian and colorectal cancer: cells expressing keratins but lacking the PBMC-specific antigen CD45 and cells lacking both keratins and CD45 [151]. Therefore, based on this study, we focused on these cells that lack both keratins and CD45 proteins and we further analyzed them for ITG α5β1. However, all cells with this phenotype were found ITG α5β1 negative.

Taking into consideration these results, two hypotheses could be proposed. Firstly, ITG α5β1 seems to be in particular expressed in tumor cells of epithelial mammary tissue but absent from disseminating tumor cells detected in blood circulation and/or bone marrow. Apparent downregulation of ITG α5β1 might be explained by integrin normal process of internalization and externalization cycle within the cell. When an adherence between cells or between a cell and the surrounding matrix is achieved, integrins are released from cell surface into endocytic cycle of cytoplasm and regenerated back onto the surface [115,121]. Consequently, ITG α5β1 internalization might function as a part of biological alterations occurring in tumor cells in order to get detached from the epithelium or/ and effectively spread to distant sites. Certainly, the mechanism of the integrin endocytic cycle in the context of the tumor cell dissemination has to be further investigated.

Secondly, the analyzed cohort includes breast cancer patients with progressive disease that were receiving chemotherapy or/and hormonotherapy as a systemic treatment. Chemotherapy drug administration is characterized by indisputable cytotoxic effects on a wide spectrum of cells besides cancer cells which might affect protein function in these cells. Therefore, tumor cells circulating in peripheral blood might be affected by the therapeutic agents and suppress protein expression such as ITG α5β1.

To conclude, ITG α5β1 seems to be associated with the metastatic progression of breast cancer. Therefore, it could become an interesting indicator for more aggressive disease and serve as a potential therapeutic target. However, further investigation has to be conducted with respect to the complicated mechanism of ITG α5β1 action in tumor cells of the breast epithelium and disseminating tumor cells.

5.3 The role of VCAM-1 protein in metastatic breast cancer

VCAM-1 expression in tumor cells appears to be linked to metastatic disease via interactions between tumor cells and tumor microenvironment. In addition, it seems to be related to cell adhesion to vascular endothelial cells promoting angiogenesis. Particularly, in bone marrow, aberrant expression of VCAM-1 in cancer cells is associated with osteoclast progenitor differentiation into mature osteoclasts. Moreover, growth factors released by bone matrix lysis feedback to cancer cells activating the 'vicious cycle' of osteolytic bone metastasis (Figure 1.7) [132,134,135,136,137,138,139,140].

The presence of VCAM-1 in malignant cells of breast cancer mouse models seems to be involved in tumor growth, migration, activation from tumor cell dormancy and EMT mediated by TGF-β1 and intereukin-6. It is significantly correlated to advanced breast cancer disease and chemoresistance to doxorubicin. Additionally, blocking of VCAM-1 effectively inhibits metastases formation into the bone marrow or other organs [130,140]. Clinical studies on the serum of patients with breast carcinoma indicate that a high soluble VCAM-1 level is correlated to early relapse, decreased survival and it seems to be a significant prognostic factor in patients with breast cancer [141].

In this particular study, we focused on the clinical relevance of VCAM-1 expression to tumor progression and response to therapy. In this context, CTCs derived from peripheral blood of breast cancer patients with progressive disease were investigated for VCAM-1 expression. These results were accompanied by correlations to patients' outcomes in order to associate VCAM-1 protein with disease progression.

In this patient cohort 19 metastatic had cytokeratin positive CTCs. Within this group, 58% patients had exclusively VCAM-1 positive CTCs and 21% patients

showed a heterogeneous expression of VCAM-1 on the CTCs, and 21% patients had no VCAM-1 detectable expression on the CTCs. VCAM-1 expression in CTCs did not correlate to primary tumor's characteristics and it also did not indicate any specific site of metastases. Moreover, according to the current findings, VCAM-1 protein was not associated with chemoresistance to doxorubicin unlike a previous *in vitro* study [129]. This study was clearly hampered by the very low number of identified CTCs and thus the clinical relevance of the different patterns of VCAM-1 expression on these cells was hard to assess. Clearly, similar to the results of jagged 1, new studies using other CTC detection methods need to be performed in the future.

To sum up, 'liquid biopsy' together with primary tumor evaluation followed by CTC or/and DTC characterization is a promising clinical approach which could offer useful information about tumor progression, tumor expansion to metastatic sites as well as therapeutic drug monitoring. In addition to this, attention should be given to thrombocytes playing a crucial role in tumor survival and dissemination through blood circulation. Thrombocytes are potentially involved in tumor cell migration by escorting them into safe sites within distant tissues [60]. During the present analysis, thrombocytes expressing Jagged1, VCAM-1 and ITG α5β1 were observed accompanying CTCs which comes to confirm the idea that these proteins are potentially involved in the thrombocyte activity to protect and regulate tumor cell dissemination.

In conclusion, the results of this study imply that Jagged1, ITG α5β1 and VCAM-1 proteins are associated with progress of breast cancer disease and tumor expansion in distant organs. Particularly, the expression of Jagged1 protein in tumor cells seems to characterize resistance of metastatic breast cancer patients to bisphosphonate-based therapy. Therefore, Jagged1 expression in CTCs could become a biomarker of monitoring breast cancer metastatic disease particularly with respect to bisphosphonate treatment. ITG

α5β1 might serve as an interesting indicator for more aggressive disease and become a potential therapeutic target. Moreover, the low number of CTCs presented in the investigated cohort seems to be insufficient for the evaluation of VCAM-1 clinical relevance to tumor progression. Further investigation into Jagged1, ITG α5β1 and VCAM-1 proteins has to be conducted with respect to the mechanisms of action in tumor cells and cancer therapy.

REFERENCES

[1] Siegel R, Ma J, Zou Z, Jemal A. Cancer Statistics. CA Cancer J Clin. 2014;64(1):9-29

[2] Malvezzi M, Bertuccio P, Levi F, La Vecchia C, Negri E. European cancer mortality predictions for the year 2014. Ann Oncol. 2014;25(8):1650-6

[3] Valastyan S, Weinberg RA. Tumor Metastasis: Molecular insights and evolving paradigms. Cell. 2011;147(2):275-92

[4] Weinberg RA. (2007). The biology of cancer (pp. 641-722). 2nd ed. New York, USA: Garland Science

[5] Cuzick J. Statistical controversies in clinical research: long-term follow-up of clinical trials in cancer. Ann Oncol. 2015;26(12):2363-6

[6] Nounou MI, ElAmrawy F, Ahmed N, Abdelraouf K, Goda S, Syed-Sha-Qhattal H. Breast Cancer: Conventional Diagnosis and Treatment Modalities and Recent Patents and Technologies. Breast Cancer (Auckl) 2015;9(Suppl 2):17-34

[7] Skibinski A, Kuperwasser C. The origin of breast tumor heterogeneity. Oncogene. 2015;34(42):5309-16

[8] Vogelstein B, Kinzler KW. (2002). The Genetic Basis of Human Cancer. 2nd ed. New York, USA: McGraw-Hill Professional

[9] Fabbri A, Carcangiu ML, Carbone A. (2008). Histological Classification of Breast Cancer. In E. Bombardieri, L. Gianni, G. Bonadonna (Eds.), Breast Cancer: Nuclear Medicine in Diagnosis and Therapeutic Options (pp. 3-14). Heidelberg, Germany: Springer-Verlag Berlin Heidelberg

[10] Dawood S, Cristofanilli M. Inflammatory Breast Cancer: What Progress Have We Made? Oncology. 2011; 25(3):264-70, 273

[11] Singletary SE, Allred C, Ashley P, Bassett LW, Berry D, Bland KI, Borgen PI, Clark G, Edge SB, Hayes DF, Hughes LL, Hutter RV, Morrow M, Page DL, Recht A, Theriault RL, Thor A, Weaver DL, Wieand HS, Greene FL. Revision of the American Joint Committee on Cancer Staging System for Breast Cancer. J Clin Oncol. 2002 ;20(17):3628-36

[12] Wells BL, Horm JW. Stage at diagnosis in breast cancer: race and socioeconomic factors. Am J Public Health. 1992 October; 82(10): 1383–1385

[13] Brooks MD, Burness ML, Wicha MS. Therapeutic implications of cellular heterogeneity and plasticity in breast cancer. Cell Stem Cell. 2015;17(3):260-71

[14] Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thürlimann B, Senn HJ. Strategies for subtypes—dealing with the diversity of breast cancer: highlights

of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. Ann Oncol. 2011;22(8):1736-47

[15] Ribelles N, Perez-Villa L, Jerez JM, Pajares B, Vicioso L, Jimenez B, de Luque V, Franco L, Gallego E, Marquez A, Alvarez M, Sanchez-Muñoz A, Perez-Rivas L, Alba E. Pattern of recurrence of early breast cancer is different according to intrinsic subtype and proliferation index. Breast Cancer Res. 2013 ;15(5):R98

[16] Pantel K, Alix-Panabières C. Real-time liquid biopsy in cancer patients: fact or fiction? Cancer Res. 2013;73(21):6384-8

[17] Lianidou ES, Mavroudis D, Georgoulias V. Clinical challenges in the molecular characterization of circulating tumour cells in breast cancer. Br J Cancer. 2013;108(12):2426-32

[18] Eroles P, Bosch A, Pérez-Fidalgo JA, Lluch A. Molecular biology in breast cancer: Intrinsic subtypes and signaling pathways. Cancer Treat Rev. 2012;38(6):698-707

[19] Malcolm RA. (2007). The cancer handbook. 2nd ed. Chichester, England:John Wiley & Sons

[20] Lianidou ES, Melegos DN, Diamandis EP. BRCA1 Tumor Suppressor Gene Product Shares Immunoreactive Epitopes with a Protein Present in Seminal Plasma. Clin Biochem 1997;30(5):425-32

[21] Jackson JG, Lozano G. The mutant p53 mouse as a pre-clinical model. Oncogene. 2013 ;32(37):4325-30

[22] Shiovitz S, Korde LA. Genetics of breast cancer: a topic in evolution. Ann Oncol. 2015;26(7):1291-9

[23] Evans DG, Fentiman IS, McPherson K, Asbury D, Ponder BA, Howell A. Familial breast cancer. BMJ.1994;308(6922):183-187

[24] Sotiriou C, Neo SY, McShane LM, Korn EL, Long PM, Jazaeri A, Martiat P, Fox SB, Harris AL, Liu ET. Breast cancer classification and prognosis based on gene expression profiles from a population-based study. Proc Natl Acad Sci U S A. 2003;100(18):10393-8

[25] Fox SB, Generali DG, Harris AL. Breast tumour angiogenesis. Breast Cancer Res. 2007;9(6):216

[26] Leek RD. The prognostic role of angiogenesis in breast cancer..Anticancer Res. 2001;21(6B):4325-31

[27] Pantel K, Brakenhoff RH, Brandt B. Detection, clinical relevance and specific biological properties of disseminating tumour cells. Nat Rev Cancer. 2008;8(5):329-40

[28] Voutsadakis IA. The network of pluripotency, epithelial–mesenchymal transition, and prognosis of breast cancer. Breast Cancer 2015;7:303-19

[29] Lawson DA, Bhakta NR, Kessenbrock K, Prummel KD, Yu Y, Takai K, Zhou A3, Eyob H, Balakrishnan S, Wang CY, Yaswen P, Goga A, Werb Z. Single-cell analysis reveals a stem-cell program in human metastatic breast cancer cells. Nature 2015;526(7571):131-5

[30] Morimoto K, Kim SJ, Tanei T, Shimazu K, Tanji Y, Taguchi T, Tamaki Y, Terada N, Noguchi S. Stem cell marker aldehyde dehydrogenase 1-positive breast cancers are characterized by negative estrogen receptor, positive human epidermal growth factor receptor type 2, and high Ki67 expression. Cancer Sci. 2009;100(6):1062-8

[31] Zheng R, Wang J, Wu Q, Wang Z, Ou Y, Ma L, Wang M, Wang J, Yang Y Expression of ALDH1 and TGFβ2 in benign and malignant breast tumors and their prognostic implications. Int J Clin Exp Pathol. 2014;7(7):4173-83

[32] Wu S, Xue W, Huang X, Yu X, Luo M, Huang Y, Liu Y, Bi Z, Qiu X, Bai S. Distinct prognostic values of ALDH1 isoenzymes in breast cancer. Tumour Biol. 2015;36(4):2421-6

[33] Zhong Y, Lin Y, Shen S, Zhou Y, Mao F, Guan J, Sun Q. Expression of ALDH1 in breast invasive ductal carcinoma: an independent predictor of early tumor relapse. Cancer Cell Int. 2013;13(1):60

[34] Reya T, Morrison SJ, Clarke MF, Weissman IL Stem cells, cancer, and cancer stem cells. Nature. 2001;414(6859):105-11

[35] Weinberg RA. (2007). The biology of cancer (pp.25-56). 2nd ed. New York, USA: Garland Science

[36] Galmarini CM, Mackey JR, Dumontet C Nucleoside analogues and nucleobases in cancer treatment. Lancet Oncol. 2002;3(7):415-24

[37] Degardin M, Bonneterre J, Hecquet B, Pion JM, Adenis A, Horner D, Demaille A Vinorelbine (Navelbine) as a salvage treatment for advanced breast cancer.M. Ann Oncol;5(5):423-6

[38] Kaye SB. New antimetabolites in cancer chemotherapy and their clinical impact. Br J Cancer. 1998;78 Suppl 3:1-7

[39] Pivot X, Gligorov J, Müller V, Curigliano G, Knoop A, Verma S, Jenkins V, Scotto N, Osborne S, Fallowfield L. Patients' preferences for subcutaneous trastuzumab versus conventional intravenous infusion for the adjuvant treatment of HER2-positive early breast cancer: final analysis of 488 patients in the international, randomized, two-cohort PrefHer study. Ann Oncol. 2014;25(10):1979-87

[40] Grabinski N, Möllmann K, Milde-Langosch K, Müller V, Schumacher U, Brandt B, Pantel K, Jücker M. 5AKT3 regulates ErbB2, ErbB3 and estrogen receptor α expression and contributes to endocrine therapy resistance of ErbB2+ breast tumor cells from Balb-neuT mice. Cell Signal. 2014;26(5):1021-9

[41] Howell A. Pure oestrogen antagonists for the treatment of advanced breast cancer.Anthony. Endocr Relat Cancer. 2006;13(3):689-706

[42] Smith IE, Dowsett M. Aromatase Inhibitors in Breast Cancer. N Engl J Med. 2003;348(24):2431-42

[43] Emons G, Schally AV. The use of luteinizing hormone releasing hormone agonists and antagonists in gynaecological cancers. Hum Reprod. 1994;9(7):1364-79

[44] Garay JP, Park BH. Androgen receptor as a targeted therapy for breast cancer. Am J Cancer Res. 2012;2(4):434-45

[45] Azim HA Jr, de Azambuja E, Colozza M, Bines J, Piccart MJ. Long-term toxic effects of adjuvant chemotherapy in breast cancer. Ann Oncol. 2011;22(9):1939-47

[46] Curigliano G, Spitaleri G, Pietri E, Rescigno M, de Braud F, Cardillo A, Munzone E, Rocca A, Bonizzi G, Brichard V, Orlando L, Goldhirsch A. Breast cancer vaccines: a clinical reality or fairy tale? Ann Oncol. 2006;17(5):750-62

[47] Verma S, Miles D, Gianni L, Krop IE, Welslau M, Baselga J, Pegram M, Oh DY, Diéras V, Guardino E, Fang L, Lu MW, Olsen S, Blackwell K. Trastuzumab Emtansine for HER2-Positive Advanced Breast Cancer. N Engl J Med. 2012;367(19):1783-91

[48] Sabatier R, Finetti P, Mamessier E, Adelaide E, Chaffanet M, Ali HR, Viens P, Caldas C, Birnbaum D, Bertucci F. Prognostic and predictive value of PDL1 expression in breast cancer. Oncotarget. 2015;6(7):5449-64

[49] Mazel M, Jacot W, Pantel K, Bartkowiak K, Topart D, Cayrefourcq L, Rossille D, Maudelonde T, Fest T, Alix-Panabiéres C. Frequent expression of PD-L1 on circulating breast cancer cells. Mon Oncol. 2015;9(9):1773-82

[50] Barcellos-Hoff MH, Lyden D, Wang TC. The evolution of the cancer niche during multistage carcinogenesis. Nat Rev Cancer. 2013;13(7):511-8

[51] Bitting RL, Boominathan R, Rao C, Kemeny G, Foulk B, Garcia-Blanco MA, Connelly M, Armstrong AJ. Development of a method to isolate circulating tumor cells using mesenchymal-based capture. Methods. 2013;64(2):129-36

[52] Bednarz-Knoll N, Alix-Panabières C, Pantel K Plasticity of disseminating cancer cells in patients with epithelial malignancies. Cancer Metastasis Rev. 2012;31(3-4):673-87

[53] Sarrio D, Rodriguez-Pinilla SM, Hardisson D, Cano A, Moreno-Bueno G, Palacios J. Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. Cancer Res. 2008;68:989–997

[54] DiMeo TA, Anderson K, Phadke P, Fan C, Perou CM, Naber S, Kuperwasser C. A novel lung metastasis signature links Wnt signaling with cancer cell self-renewal and epithelial-mesenchymal transition in basal-like breast cancer. Cancer Res. 2009;69:5364–5373

[55] Carpenter RL, Paw I, Dewhirst MW, Lo HW. Akt phosphorylates and activates HSF-1 independent of heat shock, leading to Slug overexpression and epithelial-mesenchymal transition (EMT) of HER2-overexpressing breast cancer cells. Oncogene 2015;34(5):546-57

[56] Zisis C, Tsakiridis K, Kougioumtzi I, Zarogoulidis P, Darwiche K, Machairiotis N, Zaric B, Katsikogiannis N, Kesisis G, Stylianaki A, Li Z, Zarogoulidis K. The management of the advanced colorectal cancer: management of the pulmonary metastases. J Thorac Dis. 2013;5 Suppl 4:S383-8

[57] Buchheit CL, Weigel KJ, Schafer ZT. Cancer cell survival during detachment from ECM: multiple barriers to tumor progression. Nat Rev Cancer. 2014;14(9):632-41

[58] Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation.Douglas. Cell 2011;144(5):646-74

[59] Uppal A, Wightman SC, Ganai S, Weichselbaum RR, An G. Investigation of the essential role of platelet-tumor cell interactions in metastasis progression using an agent-based model. Theor Biol Med Model. 2014 Apr 12;11:17

[60] de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. Nat Rev Cancer. 2006 Jan;6(1):24-37

[61] Smith HA, Kang Y. The metastasis-promoting roles of tumor-associated immune cells. J Mol Med (Berl). 2013 ;91(4):411-29

[62] Lv Q, Gong L, Zhang T, Ye J, Chai L, Ni C, Mao Y. Prognostic value of circulating tumor cells in metastatic breast cancer: a systemic review and meta-analysis. Clin Transl Oncol. 2015

[63] Müller V, Stahmann N, Riethdorf S, Rau T, Zabel T, Goetz A, Jänicke F, Pantel K. Circulating tumor cells in breast cancer: correlation to bone marrow micrometastases, heterogeneous response to systemic therapy and low proliferative activity. Clin Cancer Res. 2005;11(10):3678-85

[64] Slade MJ, Payne R, Riethdorf S, Ward B, Zaidi SA, Stebbing J, Palmieri C, Sinnett HD, Kulinskaya E, Pitfield T, McCormack RT, Pantel K, Coombes RC Comparison of bone marrow, disseminated tumour cells and blood-circulating tumour cells in breast cancer patients after primary treatment. Br J Cancer. 2009;100(1):160-6

[65] Rack B, Schindlbeck C, Jückstock J, Andergassen U, Hepp P, Zwingers T, Friedl TW, Lorenz R, Tesch H, Fasching PA, Fehm T, Schneeweiss A, Lichtenegger W, Beckmann MW, Friese K, Pantel K, Janni W. Circulating Tumor Cells Predict Survival in Early Average-to-High Risk Breast Cancer Patients. J Natl Cancer Inst. 2014;106(5)

[66] Pantel K, Alix-Panabières C, Riethdorf S. Cancer micrometastases. Nat Rev Clin Oncol 2009;6(6):339-51

[67] Bidard FC, Vincent-Salomon A, Gomme S, Nos C, de Rycke Y, Thiery JP, Sigal-Zafrani B, Mignot L, Sastre-Garau X, Pierga JY. Disseminated tumor cells of breast cancer patients: a strong prognostic factor for distant and local relapse. Clin Cancer Res. 2008nou;14(11):3306-11

[68].Pantel K, Alix-Panabières C. Bone marrow as a reservoir for disseminated tumor cells: a special source for liquid biopsy in cancer patients. Bonekey Rep. 2014;3:584

[69] Marsden CG, Wright MJ, Carrier L, Moroz K, Rowan BG. Disseminated breast cancer cells aquire a highly malignant and aggressive metastatic phenotype during metastatic latency in the bone. PLoS One. 2012;7(11)

[70] Meng S, Tripathy D, Frenkel EP, Shete S, Naftalis EZ, Huth JF, Beitsch PD, Leitch M, Hoover S, Euhus D, Haley B, Morrison L, Fleming TP, Herlyn D, Terstappen LW, Fehm T, Tucker TF, Lane N, Wang J, Uhr JW. Circulating Tumor Cells in Patients with Breast Cancer Dormancy. Clin Cancer Res. 2004;10(24):8152-62

[71] Bednarz-Knoll N, Alix-Panabières C, Pantel K. Clinical relevance and biology of circulating tumor cells. Breast Cancer Res. 2011;13(6):228

[72] Hong MK, Macintyre G, Wedge DC, Van Loo P, Patel K, Lunke S, Alexandrov LB, Sloggett C, Cmero M, Marass F, Tsui D, Mangiola S, Lonie A, Naeem H, Sapre N, Phal PM, Kurganovs N, Chin X, Kerger M, Warren AY, Neal D, Gnanapragasam V, Rosenfeld N, Pedersen JS, Ryan A, Haviv I, Costello AJ, Corcoran NM, Hovens CM. Tracking the origins and drivers of subclonal metastatic expansion in prostate cancer. Nat Commun. 2015;6:6605

[73] Joosse SA, Gorges TM, Pantel K. Biology, detection, and clinical implications of circulating tumor cells. EMBO Mol Med. 2015;7(1):1-11

[74] Alix-Panabières C, Pantel K. Challenges in circulating tumour cell research. Nat Rev Cancer. 2014;14(9):623-31

[75] Alix-Panabières C, Pantel K. Technologies for detection of circulating tumor cells: facts and vision. Lab Chip. 2014;14(1):57-62

[76] Riethdorf S, Fritsche H, Müller V, Rau T, Schindlbeck C, Rack B, Janni W, Coith C, Beck K, Jänicke F, Jackson S, Gornet T, Cristofanilli M, Pantel K. Detection of circulating tumor cells in peripheral blood of patients with metastatic breast cancer: a validation study of the CellSearch system. Clin Cancer Res. 2007;13(3):920-8

[77] Weilbaecher KN, Guise TA, McCauley LK. Cancer to bone: a fatal attraction. Nat Rev Cancer. 2011;11(6):411-25

[78] Theriault RL, Theriault RL. Biology of bone metastases. Cancer Control. 2012;19(2):92-101

[79] Clézardin P. Therapeutic targets for bone metastases in breast cancer. P. Breast Cancer Res. 2011;13(2):207

[80] Witzel I, Oliveira-Ferrer L, Pantel K, Müller V, Wikman H. Breast cancer brain metastases: biology and new clinical perspectives. Breast Cancer Res. 2016;18(1):8

[81] Schmidt-Nielsen K. Scaling; Why is animal size so important?. Cambridge University Press. 1984; 69: 129–130

[82] Hiraga T. Targeted Agents in Preclinical and Early Clinical Development for the Treatment of Cancer Bone Metastases. Expert Opin Investig Drugs. 2016

[83] Maruotti N, Corrado A, Neve A, Cantatore FP. Bisphosphonates: effects on osteoblast. Eur J Clin Pharmacol 2012;68(7):1013-8

[84] Drake MT, Clarke BL, Khosla S. Bisphosphonates: mechanism of action and role in clinical practice. Mayo Clin Proc 2008; 83:1032

[85] Bellido T, Plotkin LI. Novel actions of bisphosphonates in bone: preservation of osteoblast and osteocyte viability. Bone. 2011;49(1):50-5

[86] Esposito M, Kang Y. Targeting tumor-stromal interactions in bone metastasis. Pharmacol Ther. 2014;141(2):222-33

[87] Kearns AE, Khosla S, Kostenuik PJ. Receptor Activator of Nuclear Factor κB Ligand and Osteoprotegerin Regulation of Bone Remodeling in Health and Disease. Endocr Rev. 2008;29(2):155-92

[88] Body JJ, Greipp P, Coleman RE, Facon T, Geurs F, Fermand JP, Harousseau JL, Lipton A, Mariette X, Williams CD, Nakanishi A, Holloway D, Martin SW, Dunstan CR, Bekker PJ. A phase I study of AMGN-0007, a recombinant osteoprotegerin construct, in patients with multiple myeloma or breast carcinoma related bone metastases. Cancer. 2003;97(3 Suppl):887-92

[89] Sung B, Oyajobi B, Aggarwal BBPlumbagin inhibits osteoclastogenesis and reduces human breast cancer-induced osteolytic bone metastasis in mice through suppression of RANKL signaling. Mol Cancer Ther. 2012;11(2):350-9

[90] Normanno N, De Luca A, Aldinucci D, Maiello MR, Mancino M, D'Antonio A, De Filippi R, Pinto A. Gefitinib inhibits the ability of human bone marrow stromal cells to induce osteoclast differentiation: implications for the pathogenesis and treatment of bone metastasis. Endocr Relat Cancer. 2005;12(2):471-82

[91] Gallwitz WE, Guise TA, Mundy GR. Guanosine nucleotides inhibit different syndromes of PTHrP excess caused by human cancers in vivo. J Clin Invest. 2002;110(10):1559-72

[92] Yin JJ, Selander K, Chirgwin JM, Dallas M, Grubbs BG, Wieser R, Massagué J, Mundy GR, Guise TA. TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. J Clin Invest. 1999 ;103(2):197-206

[93] Jensen AB, Wynne C, Ramirez G, He W, Song Y, Berd Y, Wang H, Mehta A, Lombardi A. The cathepsin K inhibitor odanacatib suppresses bone resorption in women with breast cancer and established bone metastases: results of a 4-week, double-blind, randomized, controlled trial. Clin Breast Cancer. 2010 ;10(6):452-8

[94] Mundy GR. Metastasis: Metastasis to bone: causes, consequences and therapeutic opportunities. Nat Rev Cancer. 2002;2(8):584-93

[95] Zhao Y, Bachelier R, Treilleux I, Pujuguet P, Peyruchaud O, Baron R, Clément-Lacroix P, Clézardin P. Tumor αvβ3 Integrin Is a Therapeutic Target for Breast Cancer Bone Metastases. Cancer Res 2007;67(12):5821-30

[96] Fulciniti M, Tassone P, Hideshima T, Vallet S, Nanjappa P, Ettenberg SA, Shen Z, Patel N, Tai YT, Chauhan D, Mitsiades C, Prabhala R, Raje N, Anderson KC, Stover DR, Munshi NC. Anti-DKK1 mAb (BHQ880) as a potential therapeutic agent for multiple myeloma. Blood. 2009;114(2):371-9

[97] Ell B, Mercatali L, Ibrahim T, Campbell N, Schwarzenbach H, Pantel K, Amadori D, Kang Y. Tumor-Induced Osteoclast miRNA Changes as Regulators and Biomarkers of Osteolytic Bone Metastasis. Cancer Cell 2013;24(4):542-56

[98] Yin JJ, Mohammad KS, Käkönen SM, Harris S, Wu-Wong JR, Wessale JL, Padley RJ, Garrett IR, Chirgwin JM, Guise TA. A causal role for endothelin-1 in the pathogenesis of osteoblastic bone metastases. Proc Natl Acad Sci U S A. 2003;100(19):10954-9

[99] Kang Y, Siegel PM, Shu W, Drobnjak M, Kakonen SM, Cordón-Cardo C, Guise TA, Massagué J. A multigenic program mediating breast cancer metastasis to bone. Cancer Cell 2003;3(6):537-49

[100] Rajski M, Vogel B, Baty F, Rochlitz C, Buess M. Global gene expression analysis of the interaction between cancer cells and osteoblasts to predict bone metastasis in breast cancer. PLoS One. 2012;7(1):e29743

[101] Sethi N, Dai X, Winter CG, Kang Y. Tumor-derived JAGGED1 promotes osteolytic bone metastasis of breast cancer by engaging notch signaling in bone cells. Cancer Cell. 2011;19(2):192-205

[102] Kang Y, He W, Tulley S, Gupta GP, Serganova I, Chen CR, Manova-Todorova K, Blasberg R, Gerald WL, Massagué J. Breast cancer bone metastasis mediated by the Smad tumor suppressor pathway. Proc Natl Acad Sci U S A. 2005;102(39):13909-14

[103] Kopan R, Ilagan MX. The Canonical Notch Signaling Pathway: Unfolding the Activation Mechanism. Cell 2009;137(2):216-33

[104] Grochowski CM, Loomes KM, Spinner NB. Jagged1 (JAG1): Structure, expression, and disease associations. Gene 2016;576(1 Pt 3):381-4

[105] Li D, Masiero M, Banham AH, Harris AL. The notch ligand JAGGED1 as a target for anti-tumor therapy. Front Oncol. 2014;4:254

[106] Ottone C, Krusche B, Whitby A, Clements M, Quadrato G, Pitulescu ME, Adams RH, Parrinello S. Direct cell-cell contact with the vascular niche maintains quiescent neural stem cells. Nat Cell Biol. 2014;16(11):1045-56

[107] Ghiabi P, Jiang J, Pasquier J, Maleki M, Abu-Kaoud N, Rafii S, Rafii A. Endothelial cells provide a notch-dependent pro-tumoral niche for enhancing breast cancer survival, stemness and pro-metastatic properties. PLoS One. 2014;9(11)

[108] Buckley NE, Nic An tSaoir CB, Blayney JK, Oram LC, Crawford NT, D'Costa ZC, Quinn JE, Kennedy RD, Harkin DP, Mullan PB. BRCA1 is a key regulator of breast differentiation through activation of Notch signaling with implications for anti-endocrine treatment of breast cancers. Nucleic Acids Res. 2013;41(18):8601-14

[109] Androutsellis-Theotokis A, Leker RR, Soldner F, Hoeppner DJ, Ravin R, Poser SW, Rueger MA, Bae SK, Kittappa R, McKay RD. Notch signalling regulates stem cell numbers in vitro and in vivo. Nature 2006;442(7104):823-6

[110] Karanu FN, Murdoch B, Gallacher L, Wu DM, Koremoto M, Sakano S, Bhatia M. The notch ligand jagged-1 represents a novel growth factor of human hematopoietic stem cells. J Exp Med. 2000;192(9):1365-72

[111] Leong KG, Niessen K, Kulic I, Raouf A, Eaves C, Pollet I, Karsan A. Jagged1-mediated Notch activation induces epithelial-to-mesenchymal transition through Slug-induced repression of E-cadherin. J Exp Med. 2007;204(12):2935-48

[112] Reedijk M, Odorcic S, Chang L, Zhang H, Miller N, McCready DR, Lockwood G, Egan SE. High-level coexpression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival. Cancer Res. 2005;65(18):8530-7

[113] Dickson BC, Mulligan AM, Zhang H, Lockwood G, O'Malley FP, Egan SE, Reedijk M. High-level JAG1 mRNA and protein predict poor outcome in breast cancer. Mod Pathol. 2007;20(6):685-93

[114] Shimizu M, Cohen B, Goldvasser P, Berman H, Virtanen C, Reedijk M. Plasminogen activator uPA is a direct transcriptional target of the JAG1-Notch receptor signaling pathway in breast cancer. Cancer Res. 2011;71(1):277-86

[115] Iwamoto DV, Calderwood DA. Regulation of integrin-mediated adhesions. Curr Opin Cell Biol. 2015;36:41-7

[116] Cohen B, Shimizu M, Izrailit J, Ng NF, Buchman Y, Pan JG, Dering J, Reedijk M. Cyclin D1 is a direct target of JAG1-mediated Notch signaling in breast cancer. Breast Cancer Res Treat. 2010;123(1):113-24

[117] Bednarz-Knoll N, Efstathiou A, Gotzhein F, Wikman H, Mueller V, Kang Y, Pantel K. Potential Involvement of Jagged1 in Metastatic Progression of Human Breast Carcinomas. Clin Chem. 2016;62(2):378-86

[118] Desgrosellier JS, Cheresh DA. Integrins in cancer: biological implications and therapeutic opportunities. Nat Rev Cancer 2010;10(1):9-22

[119] Varner JA, Emerson DA, Juliano RL. Integrin alpha 5 beta 1 expression negatively regulates cell growth: reversal by attachment to fibronectin. Mol Biol Cell. 1995;6(6):725-40

[120] Clezardin P. Recent insights into the role of integrins in cancer metastasis. Cell Mol Life Sci. 1998;54(6):541-8

[121] Schaffner F, Ray AM, Dontenwill M. Integrin α5β1, the Fibronectin Receptor, as a Pertinent Therapeutic Target in Solid Tumors. Cancers (Basel). 2013;5(1):27-47

[122] Avraamides CJ, Garmy-Susini B, Varner JA. Integrins in angiogenesis and lymphangiogenesis. Nat Rev Cancer. 2008;8(8):604-17

[123] Imanishi Y, Hu B, Jarzynka MJ, Guo P, Elishaev E, Bar-Joseph I, Cheng SY Angiopoietin-2 stimulates breast cancer metastasis through the alpha(5)beta(1) integrin-mediated pathway. Cancer Res. 2007;67(9):4254-63

[124] Jia Y, Zeng ZZ, Markwart SM, Rockwood KF, Ignatoski KM, Ethier SP, Livant DL. Integrin fibronectin receptors in matrix metalloproteinase-1-dependent invasion by breast cancer and mammary epithelial cells. Cancer Res. 2004;64(23):8674-81

[125] Yao ES, Zhang H, Chen YY, Lee B, Chew K, Moore D, Park C. Increased beta1 integrin is associated with decreased survival in invasive breast cancer. Cancer Res. 2007;67(2):659-64

[126] Huang C, Park CC, Hilsenbeck SG, Ward R, Rimawi MF, Wang YC, Shou J, Bissell MJ, Osborne CK, Schiff R. β1 integrin mediates an alternative survival pathway in breast cancer cells resistant to lapatinib. Breast Cancer Res. 2011;13(4)

[127] Thibaudeau L, Taubenberger AV, Theodoropoulos C, Holzapfel BM, Ramuz O, Straub M, Hutmacher DW. New mechanistic insights of integrin β1 in breast cancer bone colonization. Oncotarget. 2015;6(1):332-44

[128] Giancotti FG, Ruoslahti E. Elevated levels of the α5 β1 fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. Cell. 1990 Mar 9;60(5):849-59

[129] Wang PC, Weng CC, Hou YS, Jian SF, Fang KT, Hou MF, Cheng KH. Activation of VCAM-1 and its associated molecule CD44 leads to increased malignant potential of breast cancer cells. Int J Mol Sci. 2014;15(3):3560-79

[130] Chen Q, Massagué. J Molecular Pathways: VCAM-1 as a potential therapeutic target in metastasis. Clin Cancer Res. 2012;18(20):5520-5

[131] Hynes RO. Metastatic Cells Will Take Any Help They Can Get. Cancer Cell 2011;20(6):689-90

[132] Ding YB, Chen GY, Xia JG, Zang XW, Yang HY, Yang L. Association of VCAM-1 overexpression with oncogenesis, tumor angiogenesis and metastasis of gastric carcinoma. World J Gastroenterol. 2003;9(7):1409-14

[133] Chen Q, Zhang XH, Massagué J. Macrophage binding to receptor VCAM-1 transmits survival signals in breast cancer cells that invade the lungs. Cancer Cell. 2011;20(4):538-49

[134] Alexiou D, Karayiannakis AJ, Syrigos KN, Zbar A, Kremmyda A, Bramis I, Tsigris C. Serum levels of E-selectin, ICAM-1 and VCAM-1 in colorectal cancer patients: correlations with clinicopathological features, patient survival and tumour surgery. Eur J Cancer. 2001;37(18):2392-7

[135] Tichet M, Prod'Homme V, Fenouille N, Ambrosetti D, Mallavialle A, Cerezo M, Ohanna M, Audebert S, Rocchi S, Giacchero D, Boukari F, Allegra M, Chambard JC, Lacour JP, Michiels JF, Borg JP, Deckert M, Tartare-Deckert S. Tumour-derived SPARC drives vascular permeability and extravasation through endothelial VCAM1 signalling to promote metastasis. Nat Commun. 2015;6:6993

[136] Wang J, Ma R, Sharma A, He M, Xue J, Wu J, Dun B, Li G, Wang X, Ji M, She JX, Tang J. Inflammatory serum proteins are severely altered in metastatic gastric adenocarcinoma patients from the Chinese population. PLoS One. 2015;10(4)

[137] Andrew AS, Gui J, Hu T, Wyszynski A, Marsit CJ, Kelsey KT, Schned AR, Tanyos SA, Pendleton EM, Ekstrom RM, Li Z, Zens MS, Borsuk M, Moore JH, Karagas MR. Genetic polymorphisms modify bladder cancer recurrence and survival in a USA population-based prognostic study. BJU Int. 2015;115(2):238-47

[138] Huang J, Zhang J, Li H, Lu Z, Shan W, Mercado-Uribe I, Liu J. VCAM1 expression correlated with tumorigenesis and poor prognosis in high grade serous ovarian cancer. Am J Transl Res. 2013;5(3):336-46.

[139] Lu X, Mu E, Wei Y, Riethdorf S, Yang Q, Yuan M, Yan J, Hua Y, Tiede BJ, Lu X, Haffty BG, Pantel K, Massagué J, Kang Y. VCAM-1 promotes osteolytic expansion of indolent bone micrometastasis of breast cancer by engaging α4β1-positive osteoclast progenitors. Cancer Cell. 2011;20(6):701-14

[140] O'Hanlon DM, Fitzsimons H, Lynch J, Tormey S, Malone C, Given HF Soluble adhesion molecules (E-selectin, ICAM-1 and VCAM-1) in breast carcinoma. Eur J Cancer. 2002;38(17):2252-7

[141] Comi G. Treatment of multiple sclerosis: role of natalizumab. Neurol Sci 2009;30 Suppl 2:S155-8

[142] Sun DI, Nizamutdinova IT, Kim YM, Cai XF, Lee JJ, Kang SS, Kim YS, Kang KM, Chai GY, Chang KC, Kim HJ. Bisacurone inhibits adhesion of inflammatory monocytes or cancer cells to endothelial cells through down-regulation of VCAM-1 expression. Int Immunopharmacol. 2008;8(9):1272-81

[143] Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, Reuben JM,Doyle GV, Allard WJ, Terstappen LW, Hayes DF. Circulating Tumor Cells, DiseaseProgression, and Survival in Metastatic Breast Cancer.Massimo. N Engl J Med.2004;351(8):781-91

[144] Schneider BP, Miller KD. Angiogenesis of Breast Cancer. J Clin Oncol. 2005;23(8):1782-90

[145] Shih T, Lindley C. Bevacizumab: an angiogenesis inhibitor for the treatment of solid malignancies. Clin Ther. 2006;28(11):1779-802

[146] Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest. 2009;119(6):1420-8

[147] Bednarz-Knoll N, Efstathiou A, Gotzhein F, Wikman H, Mueller V, Kang Y, Pantel K. Potential Involvement of Jagged1 in Metastatic Progression of Human Breast Carcinomas. Clin Chem. 2016;62(2):378-86

[148] Reedijk M, Pinnaduwage D, Dickson BC, Mulligan AM, Zhang H, Bull SB, O'Malley FP, Egan SE, Andrulis IL. JAG1 expression is associated with a basal phenotype and recurrence in lymph node-negative breast cancer. Breast Cancer Res Treat 2008;111(3):439-48

[149] Joosse SA, Hannemann J, Spötter J, Bauche A, Andreas A, Müller V, Pantel K. Changes in keratin expression during metastatic progression of breast cancer: impact on the detection of circulating tumor cells. Clin Cancer Res. 2012;18(4):993-1003

[150] Effenberger KE, Borgen E, Eulenburg CZ, Bartkowiak K, Grosser A, Synnestvedt M, Kaaresen R, Brandt B, Nesland JM, Pantel K, Naume B. Detection and clinical relevance of early disseminated breast cancer cells depend on their cytokeratin expression pattern. Breast Cancer Res Treat. 2011;125(3):729-38

[151] Pecot CV, Bischoff FZ, Mayer JA, Wong KL, Pham T, Bottsford-Miller J, Stone RL, Lin YG, Jaladurgam P, Roh JW, Goodman BW, Merritt WM, Pircher TJ, Mikolajczyk SD, Nick AM, Celestino J, Eng C, Ellis LM, Deavers MT, Sood AK. A novel platform for detection of CK+ and CK- CTCs. Cancer Discov. 2011;1(7):580-6

ACKNOWLEDGEMENTS

I would like to give my special thanks to all the **breast cancer patients** that participated in this study.

I would like to thank **Prof. Dr. med. Klaus Pantel** for giving me an opportunity to work in the Department of Tumor Biology with this fascinating scientific topic, interesting discussions and excellent supervision.

Many thanks to **Prof. Dr. rer nat Peter Heisig** from Chemistry department of Hamburg for evaluating my dissertation.

I am very thankful to **Dr. Tobias Gorges** for his contribution to supervising my PhD projects, helpful comments and corrections, and interesting ideas.

I would like to thank **Dr. habil. Harriet Wikman** for her support in writing this dissertation with helpful comments and corrections.

I would like to show my appreciation to **Prof. Dr. med. Volkmar Müller** for supporting with clinical material. I express my gratitude to all the medical doctors for supporting the projects with clinical samples and excellent cooperation.

My thanks go also to **BONE-NET consortium**, including both professors and early stage researchers, for fascinating discussions during our meetings around Europe and sharing great knowledge about tumor biology, helpful comments and outstanding ideas.

I feel very thankful to **Prof, Dr. Evi Lianidou** for introducing me to cancer research by giving me the opportunity to work in the lab of Clinical Chemistry at the University of Athens and for motivating me to continue in this field.

I would like to say ‘thank you’ to all the people from Department of Tumor Biology, especially **Małgorzata Stoupiec** for her priceless technical support, **Małgorzata Stoupiec** and **Jola Kropidlowski** for psychological support, **Ola Węglarz** for a great time that we had working together and for starting a great friendship, **Susanne Hoppe**, **Cornelia Coith, Oliver Mauermann** and **Antje Andreas** for the excellent technical support.

My thanks go also to my parents -**Athanasios** and **Eleni**- and my sisters – **Katerina** and **Areti**- for absolutely everything, especially for believing in me, supporting me and standing always by me, my nephews -Thanos and Savvas- for making my day by calling me every morning, my boyfriend, **Andreas** for his enormous help and tolerance and **Penny Monogiou** for her invaluable friendship and support in all levels, in addition to her great help in the illustration of the thesis. Last but not least, I would like to thank all my friends that helped me a lot both in Hamburg (especially **Nikos, Aris, Stavroula, Constantin, Elena, Penny, Maria**) as well as to all my friends from longdistance that were supporting me and visiting me (in particular I would like to thank **Spiros, Eva, Eleni, Aris, Maria Papadaki**).

THANK YOU!

DECLARATION ON OATH

„I hereby declare on oath, that I have written the present dissertation by my own and have not used other than the acknowledged resources and aids. The submitted written version corresponds to the version on the electronic storage medium. I hereby declare that I have not previously applied or pursued for a doctorate (Ph.D. studies). “

Date, signature