Tampere University

SATU LUHTALA

Novel Biomarkers in *HER2*-amplified Breast Cancer

Histopathological and Clinical Associations

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ACADEMIC DISSERTATION
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PunaMusta Oy – Yliopistopaino
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To my family

ABSTRACT

Approximately one fifth of breast carcinomas represents aggressively behaving HER2-positive (HER2+) subtype, which is typically characterized with amplified *HER2* growth factor receptor coding gene and HER2 protein over-expression. The aim of this retrospective doctoral study was to clarify the prevalence and clinicopathological significance of certain cell growth regulating factors, cyclin E1, HER3, NEDD4-1, NRDP1, and MCM2, in *HER2*-amplified primary breast cancers. These biological factors have been shown to contribute to breast carcinogenesis and are suggested to predict patients' survival and response to HER2-targeted personalized therapies in HER2+ breast cancer. However, earlier studies have reported contradictory results. This study focused on clarifying the prognostic utility of selected markers in relation to early onset of disease recurrence and short-term (9-week) adjuvant trastuzumab therapy during the 5-year follow-up period. Archival diagnostic breast cancer tissues were used to characterize biomarkers' prevalence using immunohistochemistry-based detection methods and digital image analysis.

Cyclin E1, HER3 and MCM2 proteins were frequently over-expressed in breast carcinomas, especially in its aggressively behaving subtypes. These proteins were highly expressed in breast cancers that were characterized with negative hormone receptor (ER, PR) status, poor differentiation, high cell proliferation activity (Ki-67 labelling index), large tumour size, basal-like phenotype, and axillary lymph node infiltration. Co-amplification of cyclin E1 coding gene, *CCNE1*, and *HER2* was shown only in 7 to 8% of studied breast carcinomas. Cyclin E1 and MCM2 proteins were not confirmed to predict breast cancer recurrence and patients' survival on adjuvant trastuzumab therapy. Instead, low HER3 expression was associated with two-fold recurrence risk in *HER2*-amplified breast cancers. In addition to HER3, also large tumour size and positive lymph node status were found to independently predict recurrence in this subtype. This study demonstrated that HER3 is frequently expressed in breast cancers. Approximately 75% of studied breast cancers were demonstrated to over-express total HER3, irrespective of HER2 status. NRDP1 and NEDD4-1 proteins have been hypothetized to control cellular HER3 receptor quantity since are involved in HER3 degradation via ubiquitination. This study did not find clinicopathologically meaningful correlations for NRDP1 and NEDD4-1 in *HER2*-amplified breast carcinomas. Conclusively, results achieved in this study can probably be applied in subcategorizing and in more accurate determination of recurrence risk of *HER2*-amplified breast cancers during the diagnostics.

TIIVISTELMÄ

Noin viidesosa rintasyövistä edustaa aggressiivista HER2-positiivista (HER2+) alatyyppiä, jossa tyyppilöydöksinä ovat HER2 kasvutekijäreseptoria koodaavan geenin monistuma ja HER2 proteiinin yli-ilmentyminen. Tässä retrospektiivisessä väitöskirjatutkimuksessa selvitettiin solun kasvun säätelyyn osallistuvien tekijöiden, sykliini E1:n, HER3:n, NEDD4-1:n, NRDP1:n ja MCM2:n esiintymistä ja kliinispatologista merkitystä *HER2*-geenimonistuneessa primaarisessa rintasyövässä. Näiden biologisten tekijöiden on osoitettu olevan keskeisiä rintasyövän patogeneesissä, ja niillä on epäilty olevan ennusteellista merkitystä HER2+ rintasyövässä ja kohdennetun HER2-täsmälääkehoidon vasteen arvioinnissa. Aiempien tutkimusten tulokset ovat kuitenkin ristiriitaisia. Tässä tutkimuksessa keskityttiin selvittämään näiden tekijöiden ennusteellista merkitystä taudin varhaisen uusiutumisen sekä lyhytkestoisen (9 viikkoa) trastutsumabi liitännäislääkehoidon suhteen 5-vuotisen seurantajakson aikana. Tutkimusaineistona käytettiin arkistoituja diagnoosivaiheen rintasyöpäkudosnäytteitä. Biomarkkereiden esiintymistä selvitettiin immunohistokemiaa ja digitaalista kuva-analyysiä hyödyntäen.

Sykliini E1, HER3 ja MCM2 proteiinien yli-ilmentymisen todettiin olevan verrattain yleistä ja liittyvän aggressiivisesti käyttäytyvään rintasyöpätyyppiin. Näiden proteiinien esiintyminen oli voimakasta rintasyövissä, joille oli tyypillistä hormonireseptorien (ER, PR) puuttuminen, matala erilaistumisaste, korkea jakautumisaktiivisuus (Ki-67 indeksi), suuri kasvaimen koko, basaalinen tyyppi sekä metastasointi kainalon imusolmukkeisiin. Sykliini E1:tä koodaavan *CCNE1* geenin monistuma todettiin vain 7-8% *HER2*-geenimonistuneista rintasyövistä. Sykliini E1 ja MCM2 proteiineilla ei todettu olevan yhteyttä taudin uusiutumiseen eikä trastutsumabi liitännäislääkehoidon vasteeseen. HER3 proteiinin vähäisen esiintymisen todettiin sen sijaan liittyvän *HER2*-geenimonistuneen rintasyövän kaksinkertaiseen uusiutumisriskiin. HER3:n lisäksi suuri kasvaimen koko ja kainalolevinneisyys todettiin keskeisiksi lisääntynyttä uusiutumisriskiä itsenäisesti kuvaaviksi ennustetekijöiksi. Tämän tutkimuksen mukaan HER3 proteiinin esiintyminen oli erittäin yleistä; HER2 statuksesta riippumatta noin 75% tutkituista rintasyövistä yli-ilmensi HER3 proteiinia. NRDP1 ja NEDD4-1 proteiinien on arveltu vaikuttavan HER3 reseptorien esiintymiseen solussa koska ne osallistuvat HER3:n hajotuksen säätelyyn ubikitinaation kautta. Tässä tutkimuksessa näillä tekijöillä ei todettu olevan kliinispatologista merkitystä *HER2*-geenimonistuneessa rintasyövässä. Tässä väitöskirjatutkimuksessa saatua tietoa voidaan mahdollisesti hyödyntää luokittelussa ja arvioitaessa *HER2*-geenimonistuneiden rintasyöpien uusiutumisriskiä tarkemmin jo diagnoosihetkellä.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following Original Publications, which are referred to hereafter by the corresponding Roman numerals (I-IV).

I. **Luhtala S**, Staff S, Tanner M, Isola J. Cyclin E amplification, over-expression, and relapse-free survival in HER-2-positive primary breast cancer. *Tumour Biol.* 2016;37(7):9813-9823.

II. **Luhtala S**, Staff S, Barok M, Tanner M, Isola J. Comparison of Antibodies for Immunohistochemistry-based Detection of HER3 in Breast Cancer. *Appl Immunohistochem Mol Morphol.* 2018;26(3):212-219.

III. **Luhtala S**, Staff S, Kallioniemi A, Tanner M, Isola J. Clinicopathological and prognostic correlations of HER3 expression and its degradation regulators, NEDD4-1 and NRDP1, in primary breast cancer. *BMC Cancer.* 2018;18(1):1045.

IV. **Luhtala S**, Haapaniemi T, Staff S, Isola J. Fluoro-Chromogenic Labelling for Detection of MCM2 to Assess Proliferation Activity in HER2-amplified Breast Carcinomas. *Appl Immunohistochem Mol Morphol.* 24th October 2018 (doi: 10.1097/PAI.0000000000000716, Epub ahead of print).

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Additional unpublished data, indicated in the text, are presented.

ABBREVIATIONS

ADCC	antibody-dependent cell-mediated cytotoxicity
AUC	area under curve
BAC	bacterial artificial chromosome
BCSS	breast cancer-specific survival
BSA	bovine serum albumin
CCNE1	*cyclin E1*
Cdk	cyclin-dependent kinase family
Cdk2	cyclin-dependent kinase 2
CI	confidence interval
CISH	chromogenic *in situ* hybridization
CK	cytokeratin
CMG	a complex formed by Cdc45, MCM2-7, and GINS
CSC	cancer stem cell
DAB	3',3'-diaminobenzidine tetrahydrochloride
DCIS	ductal carcinoma *in situ*
DFS	disease-free survival
DIA	digital image analysis
DIG	digoxigenin
ECD	extracellular domain
EGFR	human epidermal growth factor receptor (HER1)
EL	full-length cyclin E1
ER	estrogen receptor
ErbB2/ERBB2/HER2	*human epidermal growth factor receptor 2*
ErbB3/ERBB3/HER3	*human epidermal growth factor receptor 3*
FACS	fluorescence-activated cell sorting (flow cytometry)
FFPE	formalin-fixed paraffin-embedded
FLRF	fetal liver ring finger
HE	hematoxylin-eosin
HER1/2/3/4	human epidermal growth factor receptor 1/2/3/4
HER2+	HER2-positive
HER2-	HER2-negative

HER3-C	cytoplasmic HER3
HER3-M	membranous HER3
HER3-T	total cellular HER3
HIER	heat-induced epitope retrieval
HR	hormone receptor
HRGβ1	heregulin-β1 (neuregulin-1)
HRP	horseradish peroxidase
IDC	invasive ductal carcinoma
IF	immunofluorescence
IGF1R	insulin-like growth factor 1 receptor
IHC	immunohistochemistry
ILC	invasive lobular carcinoma
ISH	*in situ* hybridization
LI	labelling index (percentage of labelled cells)
LMW-E	low molecular weight cyclin E1
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
MCM	minichromosome maintenance protein family
MCM2	*minichromosome maintenance protein 2*
MCM2	minichromosome maintenance protein 2
MCM2-7	minichromosome maintenance proteins 2, 3, 4, 5, 6, 7
MFI	mean fluorescence intensity
NBF	neutral-buffered formalin
NEDD4-1	neural precursor cell expressed developmentally downregulated 4-1 protein
NRDP1	neuregulin receptor degradation protein 1
NRDP1-C	cytoplasmic NRDP1
NRDP1-N	nuclear NRDP1
NRG1	neuregulin-1 (heregulin-β1)
ORC	origin recognition complex
ORI	origin of replication
OS	overall survival
pAb	polyclonal antibody
PBS	phosphate buffered saline
pCR	pathologically complete response/remission
PCR	polymerase chain reaction
PFS	progression-free survival

PI3K	phosphoinositide-3 kinase
PLA	Proximity Ligation Assay
PR	progesterone receptor
pRb	Retinoblastoma protein
pre-RC	pre-replication complex
RFS	recurrence-free survival
RNF41	ring finger protein 41
ROC	Receiver Operating Characteristic analysis
ROI	region of interest
RT	room temperature
RTK	receptor tyrosine kinase
SNP	single nucleotide polymorphism
SSC	standard saline citrate
TAIC	tumour-associated immune cell
TK	tyrosine kinase
TKI	tyrosine kinase inhibitor
TMA	tissue microarray
TNBC	triple-negative breast cancer (ER-, PR-, HER2-)
TNM	tumour size (=T), lymph node status (=N), metastasis (=M)
TPD	trastuzumab, pertuzumab and docetaxel -based regimen
VA	visual assessment (microscopy)
WSI	whole slide image
WTS	whole tissue section

1 INTRODUCTION

Breast cancer is the most common cancer among females and the most frequent reason of cancer-related deaths in women worldwide (Global Burden of Disease Cancer Collaboration et al., 2018). In Finland, 4984 new breast cancers were diagnosed, and 895 patients were confirmed to die for this disease in 2016. Overall, breast cancer is by far the most frequently diagnosed cancer in Finnish women; its incidence represented one third (30%) of all confirmed carcinomas among females in 2016. The number of alive females with past breast cancer diagnosis was 69532, which represents nearly half (45%) of the total cancer prevalence in women in 2016. Concerning mortality, 15% of cancer-associated deaths in females were due to breast cancer. The relative 5-year breast cancer specific survival (BCSS) rate was particularly high, 91.3%. (Finnish Cancer Registry, November 2018).

Although breast cancer mortality has decreased during the last decades (Autier et al., 2010), a remarkable proportion of patients experience eventually mortal disease recurrence (Tevaarwerk et al., 2013). Especially, breast carcinomas characterized with *HER2* (*human epidermal growth factor receptor 2*) gene amplification and HER2 protein over-expression are associated with poor clinical outcome (Molnar et al., 2017; S. Park et al., 2012). Approximately 15 to 20% of breast carcinomas represent this biologically aggressive HER2-positive (HER2+) subtype (S. Giuliani et al., 2016; Köninki, Tanner, Auvinen, & Isola, 2009). Addition of trastuzumab (HER2-targeting agent) to standard therapy has dramatically improved the prognosis (Cameron et al., 2017; Perez et al., 2014), but HER2-driven tumours are often refractory to trastuzumab because of *de novo* (primary) or acquired resistance (Pernas, Barroso-Sousa, & Tolaney, 2018). Approximately 25% of early HER2+ breast cancer patients treated with adjuvant trastuzumab therapy experience disease recurrence within four to six years after the diagnosis (Gallagher et al., 2016; Zurawska et al., 2013). Recurrence risk is particularly high in HER2+ breast cancers characterized with low hormone receptor expression (Strasser-Weippl et al., 2015). New biomarkers, besides HER2 itself, are therefore needed to define clinically more aggressively behaving HER2+ breast cancers (Duffy et al., 2017; Lambertini, Ponde, Solinas, & de Azambuja, 2017). In the era of precision medicine, "*companion diagnostics*" with applicable predictive biomarkers is needed to find out patients who

will best respond to targeted therapies, *e.g.* anti-HER3 agents. Considering a biologically heterogeneous group of HER2+ breast carcinomas, it is challenging to decipher the most efficient therapy for carcinomas exhibiting distinct molecular characteristics that influence on therapy response and prognosis (Loi & Savas, 2016).

Research on breast cancer biology has denoted that diverse molecular aberrations affecting cell signaling pathways and control mechanisms confer preference for cell growth and thus enable malignant transformation. Several mechanisms in the cell cycle regulation are characteristically disrupted in cancer leading to uncontrollable and sustained cell proliferation, denominated as one of the cancer hallmarks (Hanahan & Weinberg, 2011). Intrinsic factors contributing to cell proliferation are therefore mostly attracted as biomarkers to define breast cancer aggressiveness. The concept of *biomarker* is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Biomarkers Definitions Working Group., 2001). From this point of view, the aim of this doctoral thesis was to clarify the clinical relevance of cyclin E1, HER3, NEDD4-1, NRDP1, and MCM2 as prospective biomarkers in *HER2*-amplified breast cancer subtype.

Cyclin E1 and MCM2 are crucially involved in the regulation of cell cycle progression by controlling G1/S transition and initiation of DNA synthesis (Neves & Kwok, 2017; Siu, Rosner, & Minella, 2012). HER3 is particularly important in mediating cell growth promoting signaling together with other HER family members (Mujoo, Choi, Huang, Zhang, & An, 2014). NEDD4-1 and NRDP1 contribute to HER3 function by regulating its degradation through ubiquitination, but their exact role and significance in breast carcinogenesis is unconfirmed (Carraway, 2010). Although dysregulation of these markers has been confirmed or hypothesized to attribute to breast malignant transformation, their clinical relevance is not properly defined in cancer progression and therapy response. This retrospective study was established to clarify the clinical significance of selected markers in predicting early recurrence during the first 5 years post-surgery with respect to short-term (9-wk) adjuvant trastuzumab therapy. In addition, by comparing biomarker expression profiles with conventional histopathologic parameters, the aim was to clarify if these markers could define more aggressive phenotypes within heterogeneous group of *HER2*-amplified breast carcinomas.

2 REVIEW OF THE LITERATURE

2.1 HER2-positive breast carcinoma

HER2+ breast carcinoma is one of the four molecular subtypes of breast cancer that differ in respect of biology, prognosis and therapy implications (Feng et al., 2018). HER2+ breast cancer cells show over-expression of HER2 receptors on their surface, and consequently exhibit intensified growth-promoting signaling through HER2 receptor-mediated pathways (Gutierrez & Schiff, 2011). This doctoral thesis is focused particularly on HER2+ breast carcinoma.

2.1.1 Molecular pathogenesis and incidence

HER2 (*human epidermal growth factor receptor 2*, ErbB2) protein is a receptor tyrosine kinase (RTK) that shares homologous structure with other ErbB/HER family members, EGFR/HER1 (ErbB1), HER3 (ErbB3) and HER4 (ErbB4). HER proteins are structurally composed of an extracellular ligand-binding domain (ECD), a transmembrane lipophilic segment and an intracellular domain having tyrosine kinase (TK) catalytic activity. Unlike other HERs, HER2 do not have a specific ligand but its activation is triggered either by autophosphorylation (homodimerization) or heterodimerization with other HER family members or insulin-like growth factor 1 receptor (IGF1R). These interactions result in phosphorylation of tyrosine residues within the receptor intracellular domains and induce various signaling pathways that eventually activate various effectors and transcription factors. Followingly, transcription factors are transferred into nucleus to induce expression of genes that crucially regulate cell growth, proliferation and differentiation. Two key signaling cascades are phosphoinositide-3 kinase (PI3K)/Akt and Ras/Raf/MEK/MAPK (mitogen activated protein kinase) pathways that are activated both under normal and pathological conditions, Figure 2 (p.44). In addition, HER receptors can activate downstream signaling through JAK (Janus kinase)/STAT (signal transducer and activator of transcription) and PLC-γ1 (phospholipase C-γ1)/PKC (protein kinase C) pathways. (Dey, Williams, Leyland-

Jones, & De, 2015; Jacobi, Seeboeck, Hofmann, & Eger, 2017). Excessive HER2 function through mutational activation and/or over-expression is a firmly established oncogenic mechanism that enables uncontrollable cell growth. Several *in vitro* and *in vivo* studies have confirmed that HER2 contributes crucially to early phases of breast malignant transformation. (Iqbal & Iqbal, 2014).

HER2 coding gene, *ERBB2* (*HER2*), is located at chromosome band 17q12. In breast cancer, *ERBB2* is one of the most frequently amplified genes, and it is recognized as an important proto-oncogene. (Moelans, de Weger, Monsuur, Vijzelaar, & van Diest, 2010; Slamon et al., 1989). A breast tumour characterized with increased *ERBB2* copy number (amplification) and/or high HER2 protein expression is denoted as HER2+ breast carcinoma. More precisely, *HER2*-amplified breast carcinoma is confirmed to carry *ERBB2* amplification that is often associated with high level expression of HER2 protein. The molecular pathogenesis of HER2+ breast cancer is predominantly associated with intensified HER2 function that confers proliferative and anti-apoptotic signaling and drives tumour development and progression. (Iqbal & Iqbal, 2014). Approximately one fifth (15 to 20%) of all primary breast carcinomas represents this biologically aggressive subtype, generally characterized with large size, high histological grade, lymphovascular invasion and infiltration to axillary lymph nodes (S. Giuliani et al., 2016; Killelea, Chagpar, Horowitz, & Lannin, 2017; Köninki et al., 2009). Fundamentally, patients diagnosed with HER2+ breast cancer have poor clinical outcome because of higher risk of recurrence and mortality, but they respond to HER2-targeted therapies, which has remarkably improved patients' survival (Pernas et al., 2018). Accordingly, HER2 status has both prognostic and predictive implications (Kos & Dabbs, 2016).

2.1.2 Diagnostic histopathology with clinical implications

Histopathologic breast cancer diagnostics is commonly based on morphological evaluation and ancillary biomarker (ER, PR, HER2, Ki-67) characterization of breast tumour biopsy or surgically removed breast tissue prepared as formalin-fixed paraffin-embedded (FFPE) specimen. Diagnostic markers provide also predictive and prognostic information that contribute to therapy decisions and determines breast cancer prognosis. (Finnish Breast Cancer Group, 2018; Kos & Dabbs, 2016).

2.1.2.1 Morphology and histological grade

Breast tumour morphology is evaluated on hematoxylin-eosin (HE) stained tissue slides using microscopy. Microscopical examination considers tumour cell type and morphology, localization, size and extent (excision margins), growth pattern (infiltrative/invasive, non-invasive/*in situ*), multifocality, mitotic activity (number of mitotic figures), and lymphovascular invasion. The most common (70 to 80%) breast cancer type is invasive breast carcinoma of no special type (NST) that originates from epithelial cell layer lining the breast ducts ('ductal carcinoma'). Approximately 10 to 15% of all breast carcinomas is classified as invasive lobular breast carcinoma (ILC) that arises from the epithelial cell layer of breast lobular structures (absence of E-cadherin). Classification is based on WHO Classification of Tumours of the Breast. (Feng et al., 2018; Lakhani, Ellis, Schnitt, Tan, & van de Vijver, 2012).

Histological grade of malignancy is determined on basis of combined scores given for tubule/gland formation, nuclear pleomorphism and mitotic count, each scored from 1 to 3. Grade I (scores 3 to 5) denotes good, grade II (scores 6 to 7) moderate, and grade III (scores 8 to 9) poor cell differentiation. (International Academy of Pathology Finnish Division, 2018). Well-differentiated grade I tumours closely resemble histologically normal breast tissue and are associated with favorable prognosis, while grade III denotes more aggressively behaving cancer type (Geurts et al., 2017; A. E. Giuliano et al., 2017). HER2+ breast carcinoma is commonly characterized with high histologic grade (S. Giuliani et al., 2016; Killelea et al., 2017).

2.1.2.2 pTNM Classification

Breast carcinomas are classified according to the tumour size and its metastases in the regional lymph nodes and distantly localized organs. Pathological classification is based on pTNM system of American Joint Committee on Cancer (AJCC), presented in Table 1 (p.24). In the pTNM Classification system, 'p' refers to pathologic-anatomic examination by microscopy, 'T' to tumour size, 'N' to lymph nodal status and 'M' to metastasis. pTNM offers prognostic information on how aggressively the tumour may behave clinically. (A. E. Giuliano et al., 2017). Large breast tumour size (>2 cm, ≥pT2) and positive lymph nodal status (≥pN1a) are firmly established prognostic biomarkers for early recurrence (Geurts et al., 2017; Wangchinda & Ithimakin, 2016). These are also typical features and negative prognostic factors in HER2+ breast cancers (S. Giuliani et al., 2016; Killelea et al., 2017; H. J. Lee et al., 2014).

Table 1. pTNM Classification for Breast Cancers by AJCC (A. E. Giuliano et al., 2017).

pTNM class	*Classification criteria*
pTX	The size of primary tumour can not be determined
pT0	No evidence of primary tumour
pTis	pTis(DCIS) ductal carcinoma in situ / pTis(Paget) Paget's disease
pT1	Tumour size ≤20 mm (in greatest dimension)
pT1mi	Microinvasion ≤1 mm
pT1a	Tumour size >1 mm to ≤5 mm
pT1b	Tumour size >5 mm to ≤10 mm
pT1c	Tumour size >10 mm to ≤20 mm
pT2	Tumour size >20 mm to ≤50 mm (in greatest dimension)
pT3	Tumour size >50 mm (in greatest dimension)
pT4	Any size tumour with direct extension to chest wall (pT4a) OR skin (pT4b)
pT4a	Tumour extended to chest wall
pT4b	Tumour extended to skin: edema, ulceration, satellite skin nodule OR peau d'orange
pT4c	Both pT4a and pT4b
pT4d	Inflammatory breast carcinoma
pNX	Regional (=ipsilateral axilla) lymph nodes can not be assessed
pN0	No regional lymph node metastasis OR only ITC ≤0.2 mm cluster / <200 cells
pN1mi	Micrometastasis >0.2 mm to ≤2 mm AND/OR >200 cells in ≤2 mm area
pN1a	Metastasis in 1-3 regional lymph nodes >0.2 mm, at least one node >2 mm
pN1b	Metastasis in parasternal SLN detected by microscopy, ITC excluded
pN1c	pN1a and pN1b combined
pN2a	Metastasis in 4-9 regional lymph nodes, at least one node >2 mm
pN2b	Clinically apparent metastasis in parasternal lymph nodes (with or without microscopic confirmation) with pathologically negative axillary lymph nodes
pN3a	Metastasis in ≥10 regional lymph nodes, at least one node >2 mm OR metastasis in ipsilateral infraclavicular lymph nodes
pN3b	Clinically apparent metastasis in parasternal lymph nodes with ≥1 axillary lymph node metastasis OR microscopically detected metastasis in parasternal lymph nodes with >3 axillary lymph node metastases
pN3c	Metastasis in ipsilateral supraclavicular lymph node
pMX	Distant metastasis (beyond the regional axillary lymph nodes) can not be assessed
M0	No distant metastasis
pM1	Distant metastasis (microscopically confirmed)

Abbreviations: ITC: isolated tumour cells; SLN: sentinental lymph node

2.1.2.3 Estrogen and progesterone receptors

Over-expression of estrogen (ER) and progesterone (PR) hormone binding receptors is a fundamental oncogenic mechanism that contributes to breast cell proliferation, inhibition of apoptosis, invasion and angiogenesis (Feng et al., 2018). Expression of ER (ERα isoform) and PR receptors is routinely determined for all

breast carcinomas using immunohistochemistry (IHC). Scoring is based on counting the percentage of stained tumour cell nuclei. (Duffy et al., 2017; Kos & Dabbs, 2016). There is no consensus cut-off value to define negative (HR-) and positive (HR+) hormone receptor status, but 1% and 10% are the mostly used cut-offs in clinical practice to select patients that are likely responsive to endocrine therapy (Fujii et al., 2017; Goldhirsch et al., 2007; Hammond et al., 2010). Approximately 50% of HER2+ breast carcinomas are defined with HR+ status (mostly ER+) (Howlader et al., 2014; Y. H. Park et al., 2010; Vici et al., 2015), albeit ER is present at lower level than in ER+HER2- breast carcinomas (G. Konecny et al., 2003). Negative HR status, especially ER-, is predictive of early recurrence (Strasser-Weippl et al., 2015; Zurawska et al., 2013), reduced post-relapse survival (Y. H. Park et al., 2010) and death (Vaz-Luis et al., 2012) in HER2+ breast cancer type. Accordingly, HR-HER2+ breast cancers are often characterized with large size (≥pT3), high grade (III) and lymph nodal involvement (H. J. Lee et al., 2014; Vaz-Luis et al., 2012). Endocrine therapy has been shown to confer a survival benefit in ER+HER2+ breast carcinomas when combined with adjuvant trastuzumab and chemotherapy (Hayashi, Niikura, Yamauchi, Nakamura, & Ueno, 2013). This has been suggested to premise on bidirectional crosstalk between HER2 and ER signaling pathways (M. Giuliano, Trivedi, & Schiff, 2013).

2.1.2.4 HER2 receptor

Determination of HER2 status is a mandatory for all breast tumours because HER2+ carcinomas have different therapy options and prognosis than carcinomas characterized with normal HER2 status, denoted as HER2 negative (HER2-) (Duffy et al., 2017; Gutierrez & Schiff, 2011). The diagnostic criteria for HER2 positivity are defined in guidelines by the American Society of Clinical Oncology (ASCO) and College of American Pathologists (CAP), presented in Table 2 (p.26). HER2 protein expression is routinely determined by IHC, and cell membrane staining pattern is evaluated from the invasive cancer region. *HER2* amplification status is determined by *in situ* hybridization (ISH) technique using chromogen (CISH) or fluorescence (FISH) -based detection. ISH can be performed using either single-probe (for *HER2*) or dual-probe (both for *HER2* and centromere 17) ISH assay. Analysis is based on counting of cellular *HER2* gene copies only or in relation to chromosome 17 centromere (CEP17) copies (HER2:CEP17 ratio). (Duffy et al., 2017; Wolff et al., 2018). Breast carcinomas characterized with HER2 protein over-expression (scored as 3+ by IHC) and/or amplified *HER2* (≥6 copies/cell or HER2:CEP17

ratio ≥2.0 by ISH) are considered HER2-positive (Wolff et al., 2018). HER2 protein over-expression is attributable to *HER2* amplification; high HER2 expression with coincidentally normal *HER2* status is infrequent in breast cancers (Pauletti et al., 2000; Sircoulomb et al., 2010).

Table 2. Scoring criteria for defining HER2 status by IHC and ISH (single-probe) in breast carcinomas according to the recent ASCO/CAP recommendation for clinical practice (Wolff et al., 2018).

Score	*Description*	*HER2 status*
Immunohistochemistry (HER2 protein expression)		
0	Absent or faint incomplete staining reaction in ≤10% of tumour cells	Negative
1+	Weak incomplete membrane staining in >10% of tumour cells	Negative
2+	Weak to moderate complete membrane staining in >10% of tumour cells	Equivocal*
3+	Strong complete circumferential membrane staining in >10% of tumour cells	Positive
In situ hybridization with single-probe (HER2 gene copy number/cell)		
<4	HER2 signals <4 per cell	Negative
≥4 to <6	HER2 signals 4-5 per cell	Equivocal**
≥6	HER2 signals ≥6 per cell	Positive

*Samples with equivocal IHC result should be confirmed for HER2 status by ISH (count ≥20 cells from the tissue area determined with equivocal IHC reaction)
** Samples with equivocal ISH result should be confirmed by retesting/another observer if concurrent IHC score is 2+ (equivocal ISH with 3+ IHC → HER2+, equivocal ISH with 1+ IHC → HER2-)

The risk of disease recurrence in HER2+ breast cancer is manifold higher than in luminal A breast cancers (HR+, HER2-, low Ki67) at 10 years after the diagnosis of primary disease (K. D. Voduc et al., 2010). Approximately 25% of early HER2+ breast cancer patients that are treated with adjuvant trastuzumab therapy experience disease recurrence within four to six years after the diagnosis (Gallagher et al., 2016; Zurawska et al., 2013). In general, during the 15-year follow-up, 16% and 38% of adjuvant chemotherapy treated breast cancer patients experience either local or regional metastasis, respectively (Early Breast Cancer Trialists' Collaborative Group (EBCTCG), 2018). HER2 status substantively determines breast cancer prognosis, and is the most prominent biomarker to predict response to anti-HER2 therapies (Duffy et al., 2017; Gutierrez & Schiff, 2011; Wolff et al., 2018).

2.1.2.5 Ki-67 protein

Ki-67 protein, encoded by *MKI67* gene, is universally expressed in proliferating cell nuclei throughout the cell cycle, with exception of early G1 and G0 phases, which are typical for slowly-growing and quiescent cells. Proliferating cells exhibit the lowest Ki-67 levels in G1 and early S, and the most intensive Ki-67 expression in M phase. (Gerdes et al., 1984; Lopez et al., 1991). The exact mechanism by which Ki-67 functions in cell division is not completely elucidated (X. Sun & Kaufman, 2018). It has been shown that by coating condenced chromosomes, Ki-67 prevents them from sticking together, which enables chromosome motility during the karyokinesis (Cuylen et al., 2016). Ki-67 has also been suggested to organize heterochromatin and thereby control gene expression (Sobecki et al., 2016). High Ki-67 expression is typical in malignancies, and associates with aggressive features (large tumour size, high grade, HR negativity, HER2+ status) and poor survival (Knutsvik et al., 2014; Nishimura et al., 2010). Ki-67 is recommended and widely used as a biomarker to define aggressive growth pattern in breast cancers (Duffy et al., 2017).

Ki-67 expression is generally analyzed using IHC. Percentage of stained cancer cells (Ki-67 labelling index, Ki67-LI) is determined and compared to cut-off value. (Kos & Dabbs, 2016). A cut-off point of 20% has been regarded clinically valid to determine low (<20%) and high (≥20%) expression level (Goldhirsch et al., 2013), both in HER2- (Bustreo et al., 2016; Tashima et al., 2015) and HER2+ (Muftah et al., 2017) breast cancers. A cut-off value of 14% has been shown applicable in differentiating between luminal A (low Ki-67) and luminal B (high Ki-67) breast carcinomas (Healey et al., 2017), but should be higher in aggressive HER2+ breast cancer subtype (Nishimura et al., 2010). Ki-67 is used in stratifying patients that are most likely to benefit from neoadjuvant (R. Chen et al., 2018) and adjuvant chemotherapy (Nitz et al., 2014), and to evaluate response to neoadjuvant therapy based on Ki-67 expression in the residual tumour (Cabrera-Galeana et al., 2018).

Although widely used, the prognostic significance of Ki-67 is controversial, and has been criticized, especially in aggressive HER2+ breast cancers (Aleskandarany et al., 2012; Kontzoglou et al., 2013; Niikura et al., 2014). Discrepancies in detection methods and interpretation have undermined its clinical applicability, and therefore standardization is needed to increase inter-laboratory assay consistency (Dowsett et al., 2011; Duffy et al., 2017). Use of digital image analysis (DIA) has been demonstrated to increase Ki-67 assay accuracy and reproducibility in comparison with conventional microscopy (Joshi et al., 2015; Stålhammar et al., 2018; Tuominen, Ruotoistenmäki, Viitanen, Jumppanen, & Isola, 2010; Zhong et al., 2016).

2.1.3 HER2-targeted therapy for early breast carcinoma

Targeting of over-expressed HER2 receptors with anti-HER2 therapy approaches has significantly increased patients' clinical outcome. Anti-HER2 therapeuticals that are currently in the clinics include humanized monoclonal antibodies against HER2 (trastuzumab, pertuzumab), antibody-drug conjugate (T-DM1), and tyrosine kinase inhibitors (TKI, lapatinib, neratinib). (Pernas et al., 2018). Additionally, a plethora of novel anti-HER2 agents are under investigation (Escriva-de-Romani, Arumi, Bellet, & Saura, 2018). Standard treatment of early (M0) HER2+ breast cancer is based on combination of adjuvant systemic chemotherapy and one-year trastuzumab therapy. Addition of endocrine therapy is recommended if the tumour is confirmed positive for HR status (ER ≥1%). (Finnish Breast Cancer Group, 2018). De-escalation approach recommends anti-HER2 therapy only for patients diagnosed with very early-stage (pT1apN0) low-risk HER2+ breast cancer. Neoadjuvant (preoperative) therapy is recommended for patients with locally advanced or non-operable HER2+ breast carcinoma. According to current treatment guidelines, neoadjuvant therapy consists of dual anti-HER2 therapy (trastuzumab plus pertuzumab) combined with chemotherapy. (Curigliano et al., 2017; Finnish Breast Cancer Group, 2018).

2.1.3.1 Trastuzumab

Trastuzumab (Herceptin®) is a humanized monoclonal antibody that binds to ECD subdomain IV in HER2 receptor (Cho et al., 2003). Proposed mechanisms of action include blocking of HER2 heterodimerization leading to subsequently hindered signaling via PI3K/Akt pathway, antibody-dependent cellular cytotoxicity (ADCC), and increased endocytosis of HER2 receptors. Adjuvant trastuzumab in combination with chemotherapy is a standard treatment, and thus far the only approved anti-HER2 therapy for early HER2+ breast cancer. (Lv et al., 2016; Pernas et al., 2018). Adjuvant trastuzumab has been shown to remarkably improve patients' survival during the long-term follow-up (Cameron et al., 2017; Perez et al., 2014). One year is recommended duration for adjuvant trastuzumab therapy (Denduluri et al., 2016; Pernas et al., 2018), although several trials have studied if comparable effect can be achieved with shorter administration and fewer side effects. FinHer Trial demonstrated that 9-wk course of adjuvant trastuzumab with vinorelbine or docetaxel significantly improved patients' recurrence-free survival (RFS) during the 5-year follow-up (Joensuu et al., 2009). Recently published results of SOLD trial (Joensuu et al., 2018) and Short-HER study (Conte et al., 2018) confirmed that one-

year trastuzumab schema is still preferable to 9-wk therapy. On the other hand, extending adjuvant trastuzumab therapy to last for two years was not either shown to remarkably improve patients' survival (Cameron et al., 2017). Neoadjuvant trastuzumab therapy is recommended for locally advanced (pT>2cm, pN+) early HER2+ breast cancer on basis of results achieved in NOAH (Gianni et al., 2014) and GeparQuattro (Untch et al., 2010) trials.

Although trastuzumab has remarkably increased patients' survival, its efficacy is limited by acquired or *de novo* resistance. A remarkable proportion of HER2+ breast cancer patients are intrinsically resistant or develop resistance after initial response to trastuzumab. (de Melo Gagliato, Jardim, Marchesi, & Hortobagyi, 2016). HERA (Cameron et al., 2017), NSABP B-31 and NCCTG N9831 trials (Perez et al., 2014) showed that 25 to 30% of adjuvant trastuzumab-treated early HER2+ breast cancer patients experience disease progression during the 10-year follow-up. Other studies have shown that 25% of adjuvant trastuzumab treated early HER2+ breast cancers recur within four to six years from the diagnosis (Gallagher et al., 2016; Zurawska et al., 2013). Proposed resistance mechamisms include deteriorated antibody-receptor binding (due to truncated HER2 receptor p95HER2 or HER2 epitope masking by MUC4), increased signaling through alternative RTKs (HERs, c-MET, IGF1R), upregulation of PI3K/Akt pathway due to *PIK3CA* mutation or PTEN loss and low immune response (de Melo Gagliato et al., 2016). To circumvent resistance, trastuzumab is being studied in combination with other HER-targeting agents, PI3K, c-MET and IGF1R inhibitors (Elster et al., 2015; Pernas et al., 2018).

2.1.3.2 Other HER2-targeting therapies

Excluding trastuzumab, other HER2-targeting therapeuticals are mainly used for the treatment of advanced metastatic and refractory HER2+ breast cancers. Lapatinib ditosylate (Tykerb®) is a dual TKI that binds to intracellular TK domains and thus renders autophosphorylation and subsequent signaling via EGFR and HER2 receptors (G. E. Konecny et al., 2006). Lapatinib has not been approved for the treatment of early HER2+ breast cancer (Pernas et al., 2018). Pertuzumab (Perjeta®) is a humanized monoclonal antibody that binds to HER2 ECD subdomain II and thus hinders HER2 heterodimerization, mostly with HER3 (Franklin et al., 2004). This inhibits activation of PI3K/Akt and Ras/Raf/MEK/MAPK pathways but also activates ADCC reaction (Harbeck et al., 2013). The CLEOPATRA trial has proven the therapeutical efficacy and clinical benefit of trastuzumab plus pertuzumab plus docetaxel (chemotherapeutic agent), TPD regimen, as a first-line therapy for

metastatic HER2+ breast cancers (Baselga et al., 2012). In early HER2+ breast cancer, pertuzumab is approved only as a neoadjuvant therapy with trastuzumab and chemotherapy (Gianni et al., 2016; Schneeweiss, Chia et al., 2018). Ado-trastuzumab emtansine T-DM1 (Kadcyla®) is an antibody-drug conjugate that targets subdomain IV in HER2 receptor by trastuzumab and delivers cytotoxic DM1 (maytansinoid derivative) drug into HER2+ cancer cells. Antiproliferative effects of T-DM1 are attributed to inhibition of microtubules leading to mitotic arrest, inhibition of HER2 shedding, prevented signaling via PI3K/Akt pathway and activation of ADCC. (Junttila, Li, Parsons, Phillips, & Sliwkowski, 2011). T-DM1 is not currently approved for the treatment of early HER2+ breast cancers (Pernas et al., 2018). Neratinib maleate (Nerlynx®) is a pan-TKI that inhibits EGFR, HER2 and HER4 receptors and thus prevents associated downstream signaling pathways (Tiwari, Mishra, & Abraham, 2016). Neratinib is approved for the extended adjuvant treatment of early HER2+ breast cancer patients who have already completed one-year trastuzumab therapy. ExteNET Phase III study showed that especially patients with HR+ and poorly differentiated breast tumour and multiple positive lymph nodes benefit most from neratinib therapy. (Martin et al., 2017).

2.2 Cyclin E1 in cell cycle control and breast carcinogenesis

Cell cycle is a strictly controlled and highly organized process that ensures cell genomic duplication and division. Cyclins A, B, D and E are well-characterized proteins that critically drive cell cycle progression. To accomplish their regulatory functions, cyclins interact with their catalytic partners, cyclin-dependent kinases (Cdk), to form active cyclin-Cdk complexes that phosphorylate a plethora of cell cycle-related proteins. Cyclins act sequentially to enhance cell proliferation by accelerating transition through consecutive cell cycle phases, G1 (growth), S (DNA replication), G2 (growth), and M (mitosis), Figure 1 (p.31). Quiescent non-dividing cells rest in G0 phase and can re-enter the cycle if proliferation stimuli exist (Satyanarayana & Kaldis, 2009). Expression and concerted activation of cyclin proteins is strictly controlled. Dysregulation in cell-cycle regulatory machinery, *e.g.* cyclin expression, contributes to cancer initiation and progression. Accordingly, cyclins are commonly over-expressed in carcinomas. (Malumbres & Barbacid, 2009). Cyclins and Cdks have been widely studied to elucidate their role in breast pathogenesis and as cancer therapy targets. Herein, cyclin E1 is described for its function in cell cycle control, particularly in breast cancer context.

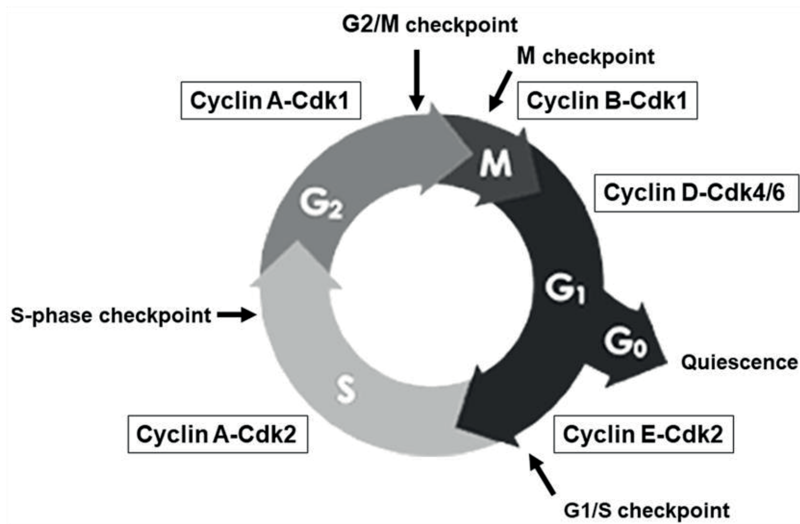

Figure 1. Cyclins and Cdks regulate cell cycle progression. Sequential accumulation of certain cyclins and cyclin-Cdk complexes enables transition between the cell cycle checkpoints.

2.2.1 Mechanisms of cell cycle (de)regulation by cyclin E1

Cyclin E1 is encoded by the *CCNE1* gene located at 19q12 chromosomal locus. *CCNE1* operates as one of the driver genes at this locus, and has been shown crucial for breast cancer cell survival (Natrajan et al., 2012). Cyclin E1 regulates cell transition from G1 to S phase to commence DNA replication. *CCNE1* transcription is triggered by mitogenic growth factor stimuli and cyclin D-Cdk4/6 induced release of E2F transcription factors. Cyclin E1 expression is at its peak in G1/S boundary, which promotes Cdk2 activity and formation of cyclin E1-Cdk2 complexes. Mechanistically, cyclin E1-Cdk2 complexes inactivate tumour suppressor Retinoblastoma protein (pRb) by phosphorylation which again induces E2F release. (Hwang & Clurman, 2005; Koff et al., 1992; Ohtsubo, Theodoras, Schumacher, Roberts, & Pagano, 1995). Along with cyclin E1, this promotes expression and phosphorylation of a diversity of additional factors that drive S-phase initiation and progression, *e.g.* Smad3, CBP/p300, E2F-5 and $p220^{NPAT}$. Once cyclin E1 accumulates up to a critical level and cyclin E1-Cdk2 complexes phosphorylate Cdk2 inhibitors, specifically p21(WAF-1/Cip-1) and p27(Kip-1), G1/S transition becomes irreversible, and cell enters to S-phase. (Sheaff, Groudine, Gordon, Roberts, & Clurman, 1997; Siu et al., 2012; Zhu, Nie, & Maki, 2005). Inhibition of cyclin E1 and Cdk2 has been shown to result in G1 arrest *in vitro* (Ohtsubo et al., 1995), confirming

that cyclin E1 is critical for cell cycle progression. Cyclin E1 involves also DNA replication initiation by regulating Minichromosome Maintenance Proteins (MCM) (Ekholm-Reed et al., 2004; Jones et al., 2013). In addition, cyclin E1-Cdk2 complexes promote centrosome duplication by phosphorylating nucleophosmin NPM/B23 and CP110, which are consequently dissociated from centrosome structures to allow chromosomal duplication (Z. Chen, Indjeian, McManus, Wang, & Dynlacht, 2002; Okuda et al., 2000).

Cyclin E1 expression and cyclin E1-Cdk2 activity is controlled by several mechanisms. Dysfunction in any of these control mechanisms influences cyclin E1 and may promote oncogenesis. (Shaye et al., 2009; Siu et al., 2012). Endogenous Cdk2 inhibitors, p21 and p27, can disturb cyclin E1 function by inhibiting cyclin E1-Cdk2 complexes (Caldon, Daly, Sutherland, & Musgrove, 2006). Trancription factor DEC1 has been shown to essentially regulate cyclin E1. In a study by Bi et al. (2015), DEC1 over-expression was shown to enhance cyclin E1-Cdk2 complex formation and prevent cyclin E1 degradation by hindering its interaction with ubiquitin ligase (Fbw7). All these effects were shown to prolong S-phase and suppress breast tumour growth in a mouse xenograft model *in vivo*. The amount of cyclin E1 is tightly controlled by two ubiquitin-proteasome pathways to ensure periodicity and cell cycle progression. In the early S-phase, cyclin E1 is quickly degraded in a process triggered by SCF (Skp1-Cullin1-F-box-protein) and BCR (BTB-Cul-3-Rbx1)-type E3 ubiquitin ligases. BCR-dependent pathway destroys monomeric cyclin E1, while SCF-type ubiquitin ligases (Fbw7 and Skp2) target Cdk2-bound phosphorylated cyclin E1. (Koepp et al., 2001; Nakayama et al., 2000; Siu et al., 2012). After completion of DNA replication, cyclin E1 level is normally declined to undetectable level, which allows cell cycle progression (Ekholm, Zickert, Reed, & Zetterberg, 2001). Cyclin E1 degradation is often disturbed in cancer. One suggested mechanism is defunctional Fbw7 (hCdc4), as was demonstrated in breast cancer cell line that expressed strongly cyclin E1 due to mutated *hCDC4* gene (Strohmaier et al., 2001).

Cyclin E1 is infrequently expressed in histologically normal breast tissue but is often over-expressed in breast malignancies. The fundamental role of deregulated cyclin E1 in early breast carcinogenesis is well established. (Scott & Walker, 1997; Shaye et al., 2009). Constitutive cyclin E1 expression increases Cdk2 activity, and therefore cyclin E1-Cdk2 complexes are available throughout the cell cycle to accelerate G1-S transition. This promotes cell proliferation and enables malignant cell growth. (Harwell, Mull, Porter, & Keyomarsi, 2004; Hwang & Clurman, 2005). Experiments with transgenic mice carrying human *CCNE1* have confirmed that cyclin E1 over-expression induces mammary tumour development (Bortner &

Rosenberg, 1997). Vice versa, targeting of over-expressed cyclin E1 with specific small interfering RNAs (siRNA) has been shown to reduce breast cancer cell growth *in vitro* and suppress tumour formation in mouse xenografts *in vivo* (Liang et al., 2010).

During the S-phase, cyclin E1 deregulation leads to inefficient assembly of pre-replication complex (pre-RC) and impaired replication fork movement, which then induce cell replication stress and contribute to genomic instability in breast cancer cells. Mechanistically, cyclin E1 over-expressing cells enter prematurely into the M-phase with incompletely-replicated genomes leading to chromosome segregation anomalies and loss of genomic regions. (Ekholm-Reed et al., 2004; Spruck, Won, & Reed, 1999; Teixeira et al., 2015). Teixeira et al. (2015) have shown that *CCNE1* copy number gain is associated with numerous genomic aberrations in breast cancer cells. Deregulated cyclin E1 expression in transgenic mice has been associated with loss of regulatory control through *p53* tumour suppressor gene (A. P. Smith et al., 2006). Under normal conditions, DNA damage induces *p53* expression, which then inhibits cyclin E1-Cdk2 activity and leads to G1 arrest to allow DNA repair before entering into S-phase (He et al., 2005). By all these mechanisms cyclin E1 over-expression causes genetic instability.

2.2.2 Cyclin E1 expression with clinicopathological associations

Cyclin E1 expression has been widely studied in breast carcinomas (Table 3, p.35), mostly by IHC. The cut-offs used to define low versus high cyclin E1 expression vary considerably among the reviewed studies, ranging from 2 to 50%. The proportion of cyclin E1 over-expressing carcinomas vary from 10 to 60%, depending on the study setting and breast cancer type. Approximately 50% of HER2+ breast cancers have been shown to display cyclin E1 over-expression (Mittendorf et al., 2010). High cyclin E1 has been significantly associated with aggressive breast cancer phenotype, characterized by high grade, ER negativity, high Ki-67, metastasis and large tumour size (Bostrom et al., 2009; Donnellan, Kleinschmidt, & Chetty, 2001; Lindahl et al., 2004; Potemski et al., 2006). Additionally, high cyclin E1 has been related to basal-like phenotype, TNBC subtype, HER2+ status and young age at disease onset (Aaltonen et al., 2009; Bostrom et al., 2009; Fredholm et al., 2017; Zagouri et al., 2017). Breast carcinomas carrying *p53* (Lindahl et al., 2004) and *BRCA1* (Aaltonen et al., 2008; Chappuis et al., 2005) mutations have been confirmed to typically exhibit cyclin E1 over-expression. High cyclin E1 has also been

associated with high co-expression of other cyclins and low-level expression of p21 and p27 (Bostrom et al., 2009; S. Han et al., 2003; Zagouri et al., 2017).

Cyclin E1 has been profoundly studied for its association with breast cancer clinical outcome but remains mostly uncertain for its prognostic utility (Table 3, p.35). Meta-analysis (Gao, Ma, & Lu, 2013) comprising of 7759 breast cancer patients from 23 eligible studies demonstrated that cyclin E1 over-expression is an independent prognostic factor of reduced breast cancer-specific survival (BCSS). Nevertheless, cyclin E1 has not been established generally applicable prognostic biomarker due to the conflicting results achieved in the previous studies (Table 3, p.35). Only two earlier studies have concentrated particularly on HER2+ breast cancer type. Mittendorf et al. (2010) showed that high cyclin E1 expression significantly associates with decreased 5-year BCSS in *HER2*-amplified breast carcinomas. Duchnowska et al. (2016) found that high cyclin E1 predicted disease recurrence and and reduced overall survival (OS) in lapatinib-treated ER-HER2+ breast cancer patients. In general, high cyclin E1 has been associated with increased risk of disease progression in most of the previous studies. On the contrary, some studies suggest that low cyclin E1 is predictive of poor clinical outcome, and certain studies have even contradicted the clinical relevance of cyclin E1.

Table 3. Overview of earlier published studies clarifying the prevalence and prognostic role of cyclin E1 expression in breast carcinomas.

Published by	Method	Cohort description		Prognostic role of cyclin E1 expression (% of cell nuclei / cut-off)
(Fredholm et al., 2017)	IHC[1]	BCA (*n*=857)	↑ ↓	High cyclin E1 (>10%) was demonstrated in 50.8% of all patients and associated with worse DDFS in ≥40 years patients (F 10.3 y). Low cyclin E1 (≤10%) associated with reduced DDFS in luminal B PR- tumours in <40 years patients (F 9.6y).
(Zagouri et al., 2017)	IHC[2]	BCA (*n*=936) HE10/97, HE10/00 trials	↓	Low cyclin E1 (≤50%) was shown in 53.5% of carcinomas and predicted poorer DFS (F 10.1y).
(Duchnowska et al., 2016)	IHC[1]	HER2+BCA (*n*=180) lapatinib	↑	High cyclin E1 (H-score ≥200) associated with reduced PFS and OS in patients with ER- carcinoma treated with lapatinib (F 0.6-20.2y).
(Lundgren et al., 2015)	IHC[1]	BCA N0 (*n*=372)	↑	High cyclin E1 (>20.45% nuclei/cytoplasm) associated with increased risk for breast cancer death (reduced OS).
(Jansen et al., 2012)	qRT-PCR	ER+BCA (*n*=65) tamoxifen monotherapy	↑	High cyclin E1 mRNA expression associated with shorter PFS in patients with advanced breast cancer treated with tamoxifen monotherapy as a first-line therapy.
(Pillay, McCleod, Chetty, & Hall, 2011)	IHC[3]	BCA (*n*=66)	=	High cyclin E1 expression (≥5%) was demonstrated in 13.6 % of carcinomas, no prognostic significance on DDFS (F 4.4y).
(Mittendorf et al., 2010)	WB	HER2+BCA (*n*=117)	↑	High expression of total cyclin E1 and LMW cyclin E1 was demonstrated in 50.4% and 42.7% of HER2-positive carcinomas and was associated with shorter 5-year BCSS.
(Aaltonen et al., 2009)	IHC[1]	BCA (*n*=797)	↑	High cyclin E1 expression (>6.8%) correlated with poor MFS (both in uni- and multivariate analysis) and OS (only in univariate analysis) (F 7.8y).
(Agarwal et al., 2009)	RPPA	BCA (*n*=779)	↑ =	High cyclin E1 associated with poor RFS and OS, but neither among subtypes of ER+ primary breast carcinomas treated with adjuvant tamoxifen nor in TNBC. Co-overexpression of cyclins E1 and B1 associated with the worst clinical outcome.
(Potemski et al., 2009)	IHC	BCA (*n*=174)	↑	High cyclin E1 expression (≥2%), demonstrated in 56.9% of carcinomas, associated with poor BCSS in the whole cohort, and with higher risk of death in N+ group (multivariate analysis, *n*=174) (F 4.8y).

Overview of earlier published studies clarifying the prevalence and prognostic role of cyclin E1 expression in breast carcinomas (Continued).

Published by	Method	Cohort description	Prognostic role of cyclin E1 expression (% of cell nuclei / cut-off)	
(Berglund et al., 2008)	IHC[1]	BCA premenopausal stage II (*n*=385)	↑	High cyclin E1 (≥11% of cell nuclei) was demonstrated in 40% of carcinomas and was prognostic of worse RFS only among the carcinomas showing infiltrative growth pattern.
(D. Voduc, Nielsen, Cheang, & Foulkes, 2008)	IHC[3]	BCA (*n*=319)	↑	High cyclin E1 (>10%) was demonstrated in 46% of carcinomas. High cyclin E1 expression associated with inferior BCSS (only in univariate analysis) (F 15.4y).
(Desmedt et al., 2006)	RT-PCR	BCA (*n*=205)	↑	High cyclin E1 mRNA was associated with reduced RFS and predicted tamoxifen resistance (shorter RFS among tamoxifen-only treated ER+ cohort, *n*=110) (F 8.1y).
(P. L. Porter et al., 2006)	IHC	BCA (*n*=2032) Trial S9313	=	High cyclin E1 was demonstrated in 46% of carcinomas, no prognostic significance on DFS and OS (F 7.0y).
(Potemski et al., 2006)	IHC	BCA (*n*=189)	↑	High cyclin E1 (≥2%) was demonstrated in 58.2% of carcinomas. High cyclin E1 expression predicted reduced 5-year BCSS in HR- carcinomas.
(Sieuwerts et al., 2006)	RT-PCR	BCA N0 (*n*=635)	↑	High cyclin E1 mRNA associated with shorter 5-year DDFS and OS. The prognosis was worst in ER- and small (pT1) tumours characterized with high cyclin E mRNA.
(Callagy et al., 2005)	IHC[3]	BCA (*n*=182)	=	High cyclin E1 (>25%), demonstrated in 25.3% of carcinomas, was not prognostic of disease-specific death (F 4.8y).
(Chappuis et al., 2005)	IHC[1]	BCA (*n*=253)	↑	High cyclin E1, demonstrated in 26% of carcinomas, predicted poorer BCSS (univariate analysis) but was shown independent prognosticator only in patients who did not receive adjuvant chemotherapy and who were treated with adjuvant endocrine therapy (multivariate analysis) (F 8.0y).
(Lindahl et al., 2004)	IHC[1]	BCA (*n*=270)	↑	High cyclin E1 (≥50%) was observed in 10% of carcinomas and associated with poor OS (F 10.2y).
(Peters et al., 2004)	IHC	BCA (*n*=56)	↓	High cyclin E1 was associated with longer RFS (only in univariate analysis).

Overview of earlier published studies clarifying the prevalence and prognostic role of cyclin E1 expression in breast carcinomas (Continued).

Published by	Method	Cohort description		Prognostic role of cyclin E1 expression (% of cell nuclei / cut-off)
(S. Han et al., 2003)	IHC	BCA N0 (*n*=175) CMF, tamoxifen (ER+)	↑	High cyclin E1 (≥10%) was demonstrated in 27.4% of carcinomas and associated with reduced OS (F 4.9y).
(Kuhling et al., 2003)	IHC	BCA N0 pT1-pT2 (*n*=332)	↑	High cyclin E1 (>10%) was observed in 44.3% of carcinomas and predicted shorter MFS and BCSS (Ki67-LI omitted in multivariate analysis) or only shorter MFS (Ki67-LI included in multivariate analysis) (F 8.3y).
(Span et al., 2003)	RT-PCR	BCA (*n*=277)	= ↑	Cyclin E mRNA expression was not associated with RFS nor OS but associated with poor RFS among patients treated with adjuvant endocrine therapy (*n*=108) (F 6.3y).
(Keyomarsi et al., 2002)	WB IHC	BCA (*n*=395) BCA (*n*=256)	↑	High expression of full-length cyclin E1 and LMWs (by WB, shown in 27% of cases) and total cyclin E1 (by IHC, shown in 53.1% of cases) were associated with reduced 5-year BCSS and OS both in uni- and multivariate analyses (F 6.4y).
(Bukholm, Bukholm, & Nesland, 2001)	IHC	BCA (*n*=170)	=	Cyclin E1 expression (≥15%) was demonstrated in 21.8% of samples, no prognostic significance on RFS (F 4.9y).
(Donnellan et al., 2001)	IHC[3]	Ductal BCA (*n*=156)	↑	High cyclin E1 expression (≥5%) was demonstrated in 46% of samples and associated with poor clinical outcome (only in univariate analysis) (F 3y).
(H. K. Kim et al., 2001)	IHC[4]	BCA (*n*=128)	↑	High cyclin E1 (>50%), demonstrated in 40.6% of carcinomas, predicted independently disease recurrence, especially distant (visceral) metastasis (F 5.8y).
(Nielsen, Arnerlov, Emdin, & Landberg, 1996)	WB	BCA (*n*=114)	↑	High cyclin E1 expression was demonstrated in 29.8% of samples and associated with reduced RFS and OS (only in univariate analysis, *n*=100) (F 4.4y).

Abbreviations: BCA: primary breast cancer (subtype not specified); BCSS: breast cancer-specific survival; DDFS: distant disease-free survival; DFS: disease-free survival; F: follow-up (median, in years); HER2+: HER2-positive; HR-/+: hormone receptor negative/positive; IHC: immunohistochemistry; LMW: low-molecular weight isoform; MFS: metastasis-free survival; N0: node-negative; N+: node-positive; PFS: progression-free survival; qRT-PCR: Real-Time Quantitative Polymerase Chain Reaction; RFS: recurrence-free survival; RPPA: reverse phase protein lysate microarray; WB: Western blot, quantitation with densitometry; ↓: low cyclin E1 (mRNA/protein) is associated with worse clinical outcome; ↑: high cyclin E1 (mRNA/protein) is associated with worse clinical outcome; =: cyclin E1 is not associated with breast cancer prognosis *Cyclin E1 antibody for IHC* (if specified): [1)] HE12 clone mAb (EL+LMW-E), [2)] EP435E clone mAb, [3)] 13A3 clone mAb (EL) [4)] M20 pAb (EL)

2.2.3 Amplification of the cyclin E1 gene (*CCNE1*)

Gene amplification is a prominent mechanism for oncogene activation in cancer cells (Santarius, Shipley, Brewer, Stratton, & Cooper, 2010). *CCNE1* amplification has been shown to cause cyclin E1 over-expression in breast cancer due to enhanced transcription of multiple gene copies (Callagy et al., 2005; Keyomarsi & Pardee, 1993; Natrajan et al., 2012). Natrajan et al. (2012) demonstrated that survival of breast cancer cells carrying *CCNE1* amplification is dependent also on Cdk2 expression and kinase activity *in vitro*.

Prevalence of *CCNE1* amplification has not been profoundly studied in breast cancer, especially in distinct subtypes, although some breast cancer cell lines have been shown to carry amplified *CCNE1* (Keyomarsi & Pardee, 1993; Natrajan et al., 2012). Based on the literature, *CCNE1* amplification seems relatively uncommon in breast cancers (Table 4, p.39). In the earlier studies, the number of *CCNE1*-amplified breast carcinomas varied from 1.6% to 29.1%. Co-amplification of *CCNE1* and *HER2* was shown in 7% (*n*=2/27) of breast carcinomas (Moelans et al., 2010). Scaltriti et al. (2011) found *CCNE1* amplification/cyclin E1 over-expression (not specified which one) in 35% of *HER2*-amplified breast cancers. They analyzed also an additional aCGH data set comprising of 595 breast carcinomas (Russnes et al., 2010), in which *CCNE1* amplifications were found in 20% (*n*=13/64) of *HER2*-amplified carcinomas. On the contrary, Natrajan et al. (2012) confirmed *CCNE1* amplification exclusively in one of 64 examined HER2+ breast carcinomas. *CCNE1* amplification has been shown to associate with aggressive breast cancer features; high histological grade, ER negativity, high cyclin E1 expression (Callagy et al., 2005; Natrajan et al., 2012; Scaltriti et al., 2011), large tumour size (Moelans et al., 2010), basal-like phenotype and TNBC (Agarwal et al., 2009). Additionally, *CCNE1* copy number gains have been shown to be more common in metastatic lesions than in primary breast tumours (Moelans et al., 2014). Previous studies, albeit few at number, have not found independent prognostic value for *CCNE1* amplification (Table 4, p.39), and therefore the clinical significance is still unknown.

Table 4. Overview of published studies clarifying the prevalence and/or prognostic role of *CCNE1* amplification in breast carcinomas.

Published by	Detection method	Sample cohort	***CCNE1*** **amplification (%)**	**Prognostic role of** ***CCNE1*** **amplification**
(Moelans et al., 2014)	MLPA	BCA+M (*n*=55)	5.5% (BCA) 12.7% (M)	*CCNE1* amplifications were more frequent in metastases compared to the corresponding primary breast tumours.
(Natrajan et al., 2012)	aCGH	BCA (*n*=313 of which HER2+BCA *n*=64)	1.6% (whole BCA cohort) 1.6% (HER2+BCA subgroup)	NA
(Scaltriti et al., 2011)	FISH	HER2+BCA (*n*=55 of which *n*=34 HER2+T)	35.0% exp/amp	*CCNE1* amplification/overexpression was related to worse clinical benefit from trastuzumab therapy and lower PFS.
	aCGH data analysis	BCA (*n*=595 of which HER2+BCA *n*=64)	20% (HER2+BCA subgroup)	
(Moelans et al., 2010)	MLPA	BCA (*n*=104, of which HER2+BCA *n*=27)	12% (whole BCA cohort) 7% (HER2+BCA subgroup)	NA
(Husdal, Bukholm, & Bukholm, 2006)	RT-PCR	BCA (*n*=81)	29.1%	*CCNE1* amplification did not predict survival during long-term follow up (>14 years).
(Callagy et al., 2005)	FISH	BCA (*n*=187)	6.0%	*CCNE1* amplification was not associated with prognosis (median follow-up 4.8 years).

Abbreviations: aCGH: microarray comparative genomic hybridization; BCA: primary breast cancer (not specified subtype); DCIS: ductal carcinoma *in situ*; DMFS: distant metastasis-free survival; exp/amp: cyclin E1 over-expression or *CCNE1* amplification; FISH: fluorescent *in situ* hybridization; HER2+BCA: HER2-positive breast cancer; HER2+T: trastuzumab-treated HER2-positive breast cancer; IC: invasive breast carcinoma; M: first distant metastasis corresponding to primary breast cancer; MLPA: multiplex ligation-dependent probe amplification; MPBC: metastatic metaplastic breast carcinoma; NA: not analyzed; PFS: progression-free survival; RT-PCR: Real-Time Quantitative Polymerase Chain Reaction

2.2.4 Cyclin E1 low-molecular weight isoforms

Alternative post-translational cleavage of full-length cyclin E1 (EL) produces low molecular weight isoforms (LMW-E) in cancer cells. LMW-E lack a specific NH_2-terminal sequence that guides protein transfer into nucleus, and therefore accumulate mainly in cytoplasm, while EL localizes predominantly in nuclei. (Delk, Hunt, & Keyomarsi, 2009; D. C. Porter et al., 2001). Compared to EL, LMW-E bind more efficiently to Cdk2, which leads to increased cyclin E-Cdk2 activity and resistance to inhibitory effects of p27 and p21. LMW-E also phosphorylate substrates, like pRb, more efficiently (D. C. Porter et al., 2001; Rath & Senapati, 2014; Wingate et al., 2009). Additionally, LMW-E are not susceptible to degradation mediated by nuclear ubiquitin ligases, and thus appear stable long time (Delk et al., 2009).

Role of LMW-E in breast carcinogenesis

LMW-E appear more frequently in breast malignancies than in histologically normal breast (Harwell, Porter, Danes, & Keyomarsi, 2000; Wingate et al., 2009). LMW-E can induce breast tumorigenesis by several mechanisms (Caruso, Duong, Carey, Hunt, & Keyomarsi, 2018; Loeb & Chen, 2012). Biologically hyperactive LMW-E, together with Cdk2, effectively increase G1/S transition, which promotes cell proliferation. Transfection of LMW-E into normal breast epithelial cells was shown to induce cell growth *in vitro*. (Akli, Van Pelt, Bui, Meijer, & Keyomarsi, 2011; Wingate et al., 2009). Transgenic xenograft mice over-expressing LMW-E were shown to have more mammary tumours and distant metastases compared with mice expressing predominantly EL (Akli et al., 2007; Duong et al., 2012). On the other hand, tumour progression in these xenograft mice was delayed by administration of Cdk2 inhibitors (Akli et al., 2007; Akli et al., 2011). LMW-E have been shown to critically deregulate B-Raf-ERK1/2-mTOR signaling pathway and promote cancer stem cell (CSC) properties in breast cancer (Duong et al., 2012; Duong et al., 2013). Earlier studies have demonstrated that LMW-E induce resistance to growth-inhibitory effects of antiestrogens in breast cancer cells *in vitro* (Akli et al., 2004; Akli et al., 2010). A recent study demonstrated that upon LMW-E induction, mouse tumour xenografts were unresponsive to aromatase inhibitors *in vivo*. This result was confirmed also in HR+ breast cancers showing high LMW-E expression. (Doostan et al., 2017). In general, presence of LMW-E has been associated with aggressively

behaving breast cancer type and decreased survival (Duong et al., 2012; Keyomarsi et al., 2002; Mittendorf et al., 2010). Mittendorf et al. (2010) reported that nearly half (42.7%) of HER2+ breast carcinomas exhibit high LMW-E expression. Two recent studies have shown that high-level expression of cytoplasmic cyclin E1 (LMW-E) significantly predicts increased risk of disease-specific recurrence in large breast cancer cohorts (Hunt et al., 2017; Karakas et al., 2016).

2.2.5 Interaction between cyclin E1 and HER2

In HER2-driven breast cancers, cyclin E1 and HER2 are proposed to vicariously interact with each other, but the exact mechanism is unknown. Both cyclin E1 and HER2 are cell growth regulators that enhance cell cycle progession by accelerating G1/S transition. HER2 over-expression has also been shown to increase Cdk2 activity (Timms, White, O'Hare, & Waterfield, 2002). Due to this bidirectional interaction, cyclin E1 over-expression has been suggested to counteract the antiproliferative effect of anti-HER2 therapy, and consequently predict resistance to these therapies.

Mittendorf et al. (2010) have demonstrated that cyclin E1 is highly expressed in HER2+ breast carcinomas and has an unfavourable impact on patient survival. Additionally, cyclin E1 expression, particularly of its LMW isoforms, was shown to decrease upon HER2 downregulation by specific siRNA and trastuzumab both in breast cancer cells *in vitro* and in mouse breast cancer xenograft model *in vivo*. Synergistic interaction was achieved by exposing cells to a combination of trastuzumab and roscovitine (Cdk2 inhibitor) *in vitro* (Mittendorf et al., 2010). Mechanistically, HER2 blocking causes accumulation of p27, inactivation of cyclin E1-Cdk2 complexes, and cell cycle arrest in G1, as has been shown in breast cancer cells *in vitro*. Accordingly, constantly active HER2 signaling is suggested to directly increase cyclin E1 expression, especially of LMW-E, and by this mechanism contribute to G1/S transition and cell proliferation. (Lane et al., 2000; Le et al., 2006; Mittendorf et al., 2010). Scaltriti et al. (2010) showed that high cyclin E1 exerts a control over HER2-mediated signaling. Additionally, cyclin E1 over-expression or *CCNE1* amplification was demonstrated to predict reduced progression-free survival (PFS) in *HER2*-amplified breast cancer patients receiving adjuvant trastuzumab. Presence of acquired *CCNE1* amplication and total cyclin E1 over-expression was confirmed also in trastuzumab-exposed BT474 breast cancer cells that were induced trastuzumab-resistant. Inhibition of cyclin E1-mediated signaling

by knockdown or by Cdk2 inhibitors remarkably decreased cell proliferation and enhanced apoptosis *in vitro*. Cdk2 inhibition reduced also tumour growth *in vivo* in trastuzumab-resistant mouse xenograft model. (Scaltriti et al., 2011). Another study (Nahta, Takahashi, Ueno, Hung, & Esteva, 2004) has shown increased Cdk2 activity accompanied with p27 downregulation in trastuzumab-resistant breast cancer cells. A recent study (Duchnowska et al., 2016) found correlation between cyclin E1 expression and shorter PFS in a cohort of HER2+ advanced breast cancers treated with lapatinib plus capecitabine.

2.3 HER3 receptor in breast carcinoma

HER3 (*human epidermal growth factor receptor 3*, ErbB3) is a RTK encoded by the *ERBB3* gene that is localized at chromosome band 12q13. HER3 is a structurally homologous relative to other HER family members (EGFR/HER1, HER2 and HER4), which are composed of ligand-binding ECD domain, a transmembrane region and an intracellular domain with tyrosine kinase property. All HER receptors contribute to various downstream signaling networks that regulate normal (breast) epithelial cell growth and differentiation. Excessive HER function through mutational activation and/or over-expression is a firmly established oncogenic mechanism. (Hynes & MacDonald, 2009; Roskoski, 2014; Stern, 2008). Accordingly, also HER3 has been widely studied to confirm its role in breast pathogenesis, and is currently drawing attention also as a fascinating therapy target (Mota et al., 2017).

2.3.1 HER3 receptor and associated signaling pathways

HER receptors typically interact with each other and are activated through reciprocal dimerization. Unlike other HERs, HER3 lacks intrinsic TK catalytic activity which renders it unable for autophosporylation to orchestrate its own activity (Berger, Mendrola, & Lemmon, 2004; Jura, Shan, Cao, Shaw, & Kuriyan, 2009). For this reason, HER3 pseudokinase is activated only through dimerization with other HER members, most preferably with HER2 (Holbro et al., 2003; Menendez & Lupu, 2007) and EGFR (Littlefield et al., 2014). However, recent *in silico* modelling studies have disproved this concept by presenting preliminary data on HER3 homointeractions in certain conditions upon ligand stimulation (Kerketta, Halasz, Steinkamp, Wilson, & Edwards, 2016; Steinkamp et al., 2014). Homodimerization

may be associated with gain-of-function mutation in HER3 kinase domain (McCabe Pryor et al., 2015). HER3-mediated signaling is generally induced when HER3 binds its ligand, heregulin β-1 (HRGβ1), called also as neuregulin-1 (NRG-1). HRGβ1 is responsible for the recruitment of dimerization partner for HER3 (Mujoo et al., 2014). In *HER2*-amplified breast cancers, HER2:HER3 heterodimerization occurs also independent of HRGβ1 presence (Junttila et al., 2009; Mukherjee et al., 2011).

As a result of HER3-involved dimerization, tyrosine residues in its intracellular domain become phosphorylated. This triggers initiation of signaling cascades, most prominently through PI3K/Akt and Ras/Raf/MEK/MAPK pathways (Figure 2, p.44). Downstream signaling via these pathways crucially promotes cell proliferation and survival, inhibition of apoptosis, angiogenesis and invasion. (Dey et al., 2015; Mujoo et al., 2014). Akt activates also HSF1 (heat shock factor 1)-SLUG signaling pathway leading to epithelial-mesenchymal transition (EMT) (Carpenter, Paw, Dewhirst, & Lo, 2015). Of all HER family members, HER3 is the most competent to activate PI3K/Akt downstream signaling because it carries six motifs that bind to p85 subunit of PI3K (Schoeberl et al., 2009). Furthermore, HER2:HER3 dimers are the most potent complexes to activate these signaling events, and have been proposed to form 'oncogenic units' that significantly contribute to breast cancer cell proliferation (Lee-Hoeflich et al., 2008; Tzahar et al., 1996). HER3:HER2 complex is intertwined also with JAK/STAT and PLC-γ1/PKC signaling pathways (Lyu, Han, Polsdofer, Liu, & Liu, 2018; Mota et al., 2017).

Like HER2, HER3 is a transmembrane protein, but it is not as tightly attached to the cell membrane as HER2 does (Bertelsen & Stang, 2014; Jeong, Kim, Kim, VanHouten, & Wysolmerski, 2017). HER3 receptors are constitutively internalized from the cell membrane into cytoplasm and nucleus, especially in proliferating cells (Reif et al., 2016; Sak et al., 2012). Regulatory mechanisms influencing subcellular localization of HER3 receptors are mostly unknown. Abundancy of HRGβ1 has been shown to directly increase the quantity of membranous HER3 receptors (Offterdinger, Schofer, Weipoltshammer, & Grunt, 2002). Also, lipid raft-associated flotillin-1 and flotillin-2 proteins are suggested to regulate HER3 membrane localization in breast cancer cells. Flotillins form complexes with HER3 to delay its recidence at plasma membrane and thus indirectly enhance *e.g.* HER3 dimerization with other RTKs. Vice versa, depletion of flotillins has been shown to result in HER3 downregulation, reduced HER2:HER3 dimerization and inhibition of PI3K/Akt and Ras/Raf/MEK/MAPK signaling cascades (Asp, Pust, & Sandvig, 2014).

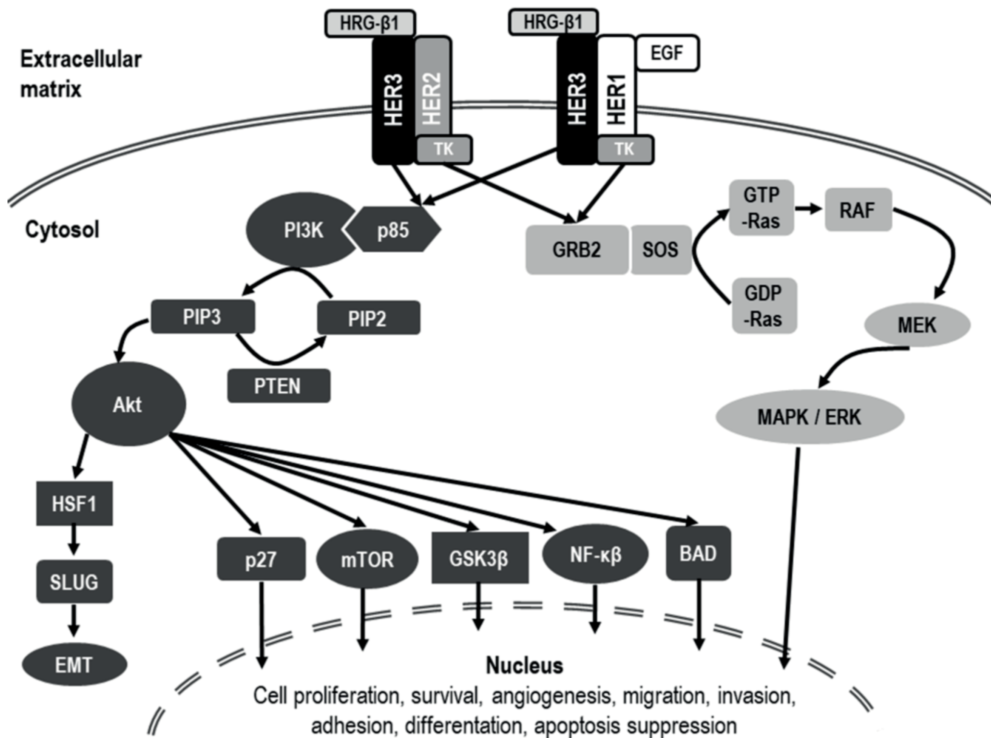

Figure 2. Overview of HER3-involved signaling pathways. Ligand (HRGβ1, EGF) binding triggers heterodimerization (HER3:HER2, HER3:HER1) leading to receptor phosphorylation and downstream signaling through PI3K/Akt and Ras/Raf/MEK/MAPK pathways. Activated Akt phosphorylates various cellular substrates (mTOR, p27, BAD, NF-κβ, GSK3β). Nuclear translocation of these downstream signaling effectors and MAPK permits transcription of several genes that are involved *e.g.* in cell proliferation, differentiation, migration, adhesion, apoptosis suppression, angiogenesis and metabolic pathways. (*Modified from reviews by Jacob et al. 2018, Lyu et al. 2018, and Dey et al. 2015*).

2.3.2 HER3-related alterations in breast carcinoma

In histologically normal breast, HER3 protein is highly expressed in luminal epithelium, while basal epithelial cells display low level HER3 expression. HER3 has been suggested to crucially regulate the balance between luminal and basal cell types within the breast epithelium by its own expression pattern. (Balko et al., 2012). High HER3 expression was firstly demonstrated in human breast tumours at the time when the *ERBB3* gene was characterized (Kraus, Issing, Miki, Popescu, & Aaronson, 1989). Currently, HER3 - especially in association with HER2 - is established as a key oncogene in breast malignancies (Dey et al., 2015). Still, the exact mechanisms

that regulate cellular HER3 expression, function and underlying cancer biology have not been fully elucidated. Anyway, HER3 dysregulation has been affiliated with miscellaneous alterations in HER2-driven breast carcinomas contributing to HER3 expression, localization, phosphorylation and downstream signaling (Amin, Sergina, Lim, Goga, & Moasser, 2012).

2.3.2.1 Interaction between HER3 and HER2

HER3, an allosteric activator of HER2, is required for the maintenance of active HER2-mediated signaling (Lee-Hoeflich et al., 2008; Liu et al., 2009). HER3 over-expression has been shown to commonly co-occur with *HER2* amplification and high HER2 expression. Accordingly, HER3 is thought to centrally contribute to the pathogenesis of HER2+ breast cancer subtype. (Amin et al., 2012; Dey et al., 2015). HER2 activates HER3 in a ligand-indepent manner, and this interaction drives effectively breast cancer cell proliferation (Holbro et al., 2003; Vaught et al., 2012). In practice, Holbro et al. (2003) and Lee-Hoeflich et al. (2008) demonstrated that HER3 is as critical as HER2 to maintain growth of HER2+ breast cancer cells by applying receptor knockdown. Co-expression of HER2 and HER3 has also been demonstrated to induce breast cancer cell migration and invasiveness in 3D cultures (Aceto et al., 2012). Again, studies with mice tumour models have shown that elevated co-expression of HER2 and HER3 crucially attributes to the development of mammary tumours *in vivo*. Vice versa, tumour formation was substantially reduced upon HER3 ablation, which provides evidence for the interdependency between HER2 and HER3 during the breast malignant transformation. (Lee-Hoeflich et al., 2008; Lyu et al., 2015; Siegel, Ryan, Cardiff, & Muller, 1999; Vaught et al., 2012).

On the other hand, inhibition of HER2 in HER2-dependent breast cancer cells *in vitro* has been shown to result in compensatory upregulation of HER3 in order to maintain active PI3K/Akt signaling for cell growth and survival (Garrett et al., 2011; Liu et al., 2007; Sergina et al., 2007). Additionally, downregulation of HER3 in HER2/HER3 co-expressing breast cancer cells resulted in reduced phosphorylation of both receptors (Liu et al., 2007). These findings imply that HER2-dependent breast cancer cells rely mostly on HER3-mediated signaling to steer their growth and survival. Experiments with xenograft mice bearing HER2+ tumours confirmed that blocking of both HER2 and HER3 receptors is more effective than inhibition of HER2 only (Garrett, Sutton, Kuba, Cook, & Arteaga, 2013). In this study, HER3 downregulation by specific U3-1287 antibody was synergized with dual anti-HER2 therapy (trastuzumab plus lapatinib). Mice treated with this combination exhibited

fewer recurrences and better survival compared with those treated with trastuzumab plus lapatinib only. Additionally, pertuzumab has been shown to effectively induce tumour growth in combination with trastuzumab (Lee-Hoeflich et al., 2008). These results provide *in vivo* evidence for the oncogenic HER2:HER3 interaction and necessity to therapeutically block the compensatory HER3 route to completely inhibit HER2 signaling.

Increased HER2:HER3 dimerization, resulting from high expression of HER2, HER3 and/or HRGβ1, leads to HER3-mediated oncogenic activation of PI3K/Akt pathway, which is critical for HER3-driven early breast tumorigenesis (Kirouac et al., 2016). PI3K/Akt downstream signaling triggered by HER3 was shown to increase breast cancer cell motility and ability to invade and metastasize (Smirnova et al., 2012). HER3 dimerization pattern has been recently studied in breast cancer tissues by several researchers using a novel technique called Proximity Ligation Assay (PLA), which allows accurate dimer quantification *in situ*. Frequent expression of HER2:HER3, but also of EGFR:HER3 heterodimers, was shown useful to identify more aggressively behaving HER2+ breast cancer types (Barros et al., 2014; Karamouzis et al., 2016). More precisely, 66% of HER2+ breast carcinomas were shown to highly express HER2:HER3 heterodimers (Barros et al., 2014). Presence of HER2:HER3 dimers was also found to correlate with aggressive features, such as *HER2* amplification, HR negativity and high histological grade (Barros et al., 2014; Spears et al., 2012). Additionally, abundant HER2:HER3 heterodimerization was associated with poor BCSS (Spears et al., 2012), and was suggested to predict poor response to adjuvant trastuzumab therapy in *HER2*-amplified breast cancers (Green et al., 2014). However, Barros et al. (2014) were not able to confirm this result.

Interestingly, a recent *in vitro* study (Ruiz-Saenz et al., 2018) performed with *HER2*-amplified *ERBB3*-silenced breast cancer cells, strongly suggest that HER2-driven tumorigenesis is not fully dependent on HER3. HER3 knockout in HER2+ HCC1569 cells was shown to transiently reduce cell proliferation but was abrogated by intensified HER2 expression and homodimerization. This study claimed also that abundant HER2 can directly activate the oncogenic PI3K/Akt pathway in the absence of HER3, but the exact mechanism remains unknown. Similarly, Spears et al. (2012) and Barros et al. (2014) revealed a fraction of *HER2*-amplified breast carcinomas that were not expressing heterodimers but were rather driven by HER2 autophosphorylation. On the other hand, it has been speculated that low HER2 expression may favor HER2:HER3 heterodimer formation, and therefore HER3 may have a role in the pathogenesis of HER2-low breast cancers (Collins et al., 2017).

Since HRGβ1 binds to HER2:HER3 heterodimers, genetical alterations in *NRG1* (heregulin-1 coding gene) may be associated with aberrant HER2-HER3 signaling in breast cancers (Drilon et al., 2018). Also, HRGβ1-induced HER3 degradation is remarkably slower in HER2+ cancer cells (Sak et al., 2013). In addition, certain intrinsic factors interacting with HER3 may attribute to the pathogenesis of HER3-expressing carcinomas. For example, upregulated APIP (*Apaf-1-interacting protein*) has been demonstrated to efficiently bind HER3 receptors upon HRGβ1 stimulation, resulting in increased HER2:HER3 dimerization in gastric cancer cells *in vitro* (Hong et al., 2016). Similarly, over-expression of DJ-1/PARK7 (*Parkinson Protein 7* or *Protein 7*) has been shown to increase HER3 levels and was associated with increased breast cancer cell proliferation *in vitro* and tumour growth *in vivo*, albeit the effect was diminished in the abundancy of HRGβ1. Mechanistically, DJ-1 binds to HER3, which protects it from ubiquitination (S. Zhang et al., 2016). This study confirmed also that DJ-1 is often co-expressed with HER3 in breast cancer tissues. Another study (Y. Wang et al., 2017) reported that during breast cancer progression, DJ-1 expression decreases while HER3 expression inversely increases.

2.3.2.2 Expression and clinicopathological associations

HER3 (mRNA/protein) over-expression is common in breast cancers, as has been shown in a plethora of previous studies (Table 5, p.51). High HER3 expression has been confirmed in approximately 50% of breast carcinomas (Abd El-Rehim et al., 2004; Baselga et al., 2014; Bieche et al., 2003; Larsen et al., 2012), but notable variation exists depending on the study setting. Bae et al. (2013) have shown that HER3 expression significantly differs across breast cancer subtypes depending on HR and HER2 statuses. In their large study cohort, proportions of HER3-expressing carcinomas were as follows: 55% (HR+HER2-), 77% (HR+HER2+), 41% (HR-HER2-/TNBC), 67% (HR-HER2+), and 72% (HER2+). Most of earlier studies, especially those on HER2+ breast cancer subtype, have analyzed HER3 expression in metastatic carcinomas, while studies on early (M0) HER2+ breast cancer cohorts are few. This may explain the differences in HER3 expression pattern since tumour model studies have shown the importance of HER3 particularly in the early phases of breast carcinogenesis (Stern, 2008; Vaught et al., 2012). HER3 over-expression has been suggested to define aggressive breast cancer phenotype. The majority of HER2+ breast carcinomas exhibit high HER3 expression, measured either as increased mRNA (Bieche et al., 2003; Koutras et al., 2015) or protein (Bae et al., 2013; Berghoff et al., 2014; Chiu et al., 2010) level. Irrespective of HER2 status, high

HER3 expression has been associated with HR+ status, low histological grade, and lymphovascular invasion (Bieche et al., 2003; Chiu et al., 2010; Koutras et al., 2015; Larsen et al., 2012; Y. Lee et al., 2007).

Comparison between the previous studies is complicated, not only for the cohort differences, but also due to methodological discrepancies and lack of standardized analyzing criteria to determine biologically relevant HER3 expression (Nuciforo, Radosevic-Robin, Ng, & Scaltriti, 2015). Most of the previous studies have applied IHC or VeraTag® technology to detect HER3 protein in breast cancer tissues. HER3 transcript (mRNA) levels were mostly quantified by RT-PCR. In IHC-studies, various antibodies and scoring methods have been applied. Additionally, varying cut-off values, ranging from 1 to 50%, have been applied to distinguish between low and high HER3 expression. As HER signaling is known to depend on the specific breast cancer type, HER3 may have biologically distinct expression profile and role in distinct subtypes (Hynes & MacDonald, 2009; Ribelles et al., 2013).

The prognostic role of HER3

The prognostic significance of HER3 has been widely studied in breast cancers (Table 5, p.51). Still, the clinical relevance of HER3 - especially in *HER2*-amplified subtype - remains controversial. A recently published comprehensive meta-analysis of 32 related studies did not find any association between HER3 status and BCSS (H. J. Kim et al., 2015). However, many individual studies have found significant association between HER3 and clinical outcome. Some studies have proposed that characterization of HER family co-expression may be more predictive of clinical outcome than profiling of single HER receptor (Bianchi et al., 2006; Giltnane, Moeder, Camp, & Rimm, 2009; Suo et al., 2002; Wiseman et al., 2005). High co-expression of HER2 and HER3 has been demonstrated to predict reduced survival and also anti-HER2 therapy resistance (Abd El-Rehim et al., 2004; Bae et al., 2013; Berghoff et al., 2014; Giltnane et al., 2009; S. W. Han et al., 2012; Y. H. Park et al., 2014). Giltnane et al. (2009) reported that 5-year and 10-year BCSS were 45% and 39% in breast cancer patients carrying carcinomas that display high HER2/HER3 co-expression. Comparative survival data for HER2+HR- subtype was reported by Bae et al. (2012). In their patient cohort, 5-year disease-free survival (DFS) was 59% (*vs* 83%) and 10-year DFS was 55% (*vs* 79%) stratified for high and low HER3 expression level, respectively. HER3 over-expression has been shown predictive of reduced survival also in HER2- breast carcinomas (Chiu et al., 2010), TNBC (Bae et al., 2013), and TNBC subgroup displaying concurrently high EGFR expression

(Giltnane et al., 2009). Some previous studies have adversely associated low HER3 mRNA (Baselga et al., 2014; Pawlowski, Revillion, Hebbar, Hornez, & Peyrat, 2000) or protein (S. W. Han et al., 2012; Y. Lee et al., 2007; Takada et al., 2018) expression with unfavourable breast cancer outcome, irrespective of HER2 status. Furthermore, many studies (Bianchi et al., 2006; Gori et al., 2012; Haas et al., 2009; Larsen et al., 2012) have not found any demonstrable relationship between HER3 and breast cancer survival.

The predictive role of HER3

High HER3 expression has been suggested as a notable resistance mechanism against therapies that target HER2 and EGFR (Garrett et al., 2013). HER3 over-expression has been shown to induce inherent or acquired resistance to trastuzumab (Yang et al., 2017), lapatinib (Vaught et al., 2012; Xia et al., 2013) and gefinitib (Sergina et al., 2007) in HER2+ breast cancer cells *in vitro* and in tumour models *in vivo*. More closely, anti-HER2 therapies have been shown to induce expression and heterodimerization of HER3 and EGFR, and sensitize breast cancer cells to EGFR-inhibitors, gefinitib and cetuximab (B. K. Choi, Fan, Deng, Zhang, & An, 2012; Claus et al., 2018; Narayan et al., 2009). Some retrospective studies (Adamczyk et al., 2017; Lipton et al., 2013; Y. H. Park et al., 2014) have confirmed that HER3 over-expression is predictive of poor outcome in HER2+ breast cancer patients who were treated with trastuzumab as a first-line therapy. However, HER3 has not been confirmed predictive of response to trastuzumab-based neoadjuvant chemotherapy (Yonemori et al., 2010). Other researchers (R. Giuliani et al., 2007; Gori et al., 2012; B. L. Smith et al., 2004) have reported contradictory results indicating that HER3 is not significantly associated with trastuzumab therapy response in metastatic HER2+ breast cancer cohorts. Similarly, two studies (Duchnowska et al., 2017; Nishimura et al., 2017) reported that HER3 is not predictive of OS in metastatic breast cancers that were progressed during trastuzumab therapy, and were consequently treated with lapatinib plus capecitabine. However, Han et al. (2012) found that low HER3 expression is predictive of reduced PFS using a similar study setting.

High-level expression of HER2:HER3 heterodimers has been shown to predict poor response to adjuvant trastuzumab therapy (Green et al., 2014). One mechanism that may confer trastuzumab resistance is increased HRGβ1 expression, which consequently drives HER3 activation and triggers HER2:HER3 heterodimerization (Nonagase et al., 2016), regardless of trastuzumab (Dey et al., 2015). On the other hand, HER2+ breast carcinomas expressing predominantly HER2 homodimers are

more susceptible to trastuzumab in comparison with carcinomas that express mostly HER2:HER3 heterodimers. One suggested explation is that HER2 homodimers are not able to trigger PI3K/Akt pathway signaling (Ghosh et al., 2011), but this has been recently questioned by Ruiz-Saenz et al. (2018). For these reasons, dual therapy approaches are preferred clinically relevant for HER2-driven breast cancers to circumvent induction of alternative HER3-mediated oncogenic signaling (Garrett et al., 2013; Lyu et al., 2015). On the contrary, abundancy of HER3 mRNA in HER2+ metastatic breast cancer was related to better prognosis in CLEOPATRA trial, in which patients were treated either with TPD or trastuzumab plus docetaxel regimen as a first-line therapy. However, no significant survival differences were observed between the treatment groups stratified for HER3, and therefore HER3 was not confirmed predictive of TPD therapy response. (Baselga et al., 2014). High HER3 protein expression was still found to predict response to TPD-based therapy in a minor HER2+ breast cancer cohort (Takada et al., 2018). HER3 has not been confirmed to predict response to neoadjuvantly administered trastuzumab plus pertuzumab therapy in NeoSphere (Bianchini et al., 2017) and TRYPHAENA (Schneeweiss et al., 2014) trials.

High HER3 expression has been suggested to determine endocrine therapy resistance through constitutive activation of PI3K/Akt and MAPK signaling cascades (Ghayad et al., 2010). Fulvestrant was shown to induce HER3 expression by sensitizing luminal breast cancer cells to HRGβ1 *in vitro* (Hutcheson et al., 2011). Another study demonstrated that HER3 blocking by anti-HER3 antibody abolished this cell growth promoting effect (Morrison et al., 2013). Combination of lumretuzumab (HER3 antibody), pertuzumab and fulvestrant was recently shown highly efficacious anti-tumour therapy *in vivo* using a mouse xenograft model of ER+ HER2 low (non-amplified) breast cancer (Collins et al., 2017). This study confirmed also that both HER2 and HER3 can directly interact with ER and mediate its phosphorylation in HRGβ1-induced breast cancer cells. HER3 over-expression has also been demonstrated to render HER2+ breast cancer cells resistant to tamoxifen (Liu et al., 2007) and letrozole (Curley et al., 2015). These studies demonstrated that HER3 inhibition delayed the onset of resistance and restored breast cancer cells sensitive to endocrine therapy. In contrast, retrospective analysis by Larsen et al. (2012) did not find any correlation between HER3 status and adjuvant endocrine therapy response in a large cohort comprising 1062 ER+ breast cancers. Co-expression of HER2 and HER3 has been shown to predict disease recurrence in ER+ breast cancer patients that were treated with tamoxifen (Tovey et al., 2005).

Table 5. Overview of earlier published studies clarifying the prevalence and prognostic role of HER3 expression in breast cancer.

Published by	Methodology	Cohort description	Prognostic role of HER3 expression (% of cells / cut-off)	
(Takada et al., 2018)	IHC (RTJ2) HER3-M/C	met-HER2+BCA (*n*=29) TPD	↓	Low HER3 (<33.9%) was demonstrated in 34.5% of carcinomas and was associated with shorter PFS (only in univariate analysis) but was not prognostic of OS. High HER3 was predictive of response to chemotherapy with TPD regimen. (F 497 days)
(Adamczyk et al., 2017)	IHC (SP71) HER3-M/C	HER2+BCA (*n*=97) Adj-T	= ↑	High HER3 (>10%) was demonstrated in 28.3% of carcinomas. High HER3 was associated with shorter 5-year MFS only in a PTEN negative subgroup.
(Duchnowska et al., 2017)	VeraTag®	met-HER2+BCA (*n*=157) LC after T	=	HER3 was not associated with OS in advanced stage HER2+BCA treated with LC therapy following progression on trastuzumab therapy.
(Nishimura et al., 2017)	VeraTag®	met-HER2+BCA (*n*=47) LC after T	=	HER3 was not prognostic of PFS in trastuzumab-refractory breast carcinomas treated with LC therapy.
(Koutras et al., 2015)	qRT-PCR	BCA (*n*=663, of which HER2+BCA *n*=143), HE10/00 trial (HeCOG)	= ↓	Low HER3 mRNA was associated with shorter DFS only in a subgroup displaying concurrently high EGFR, high HER2 and low HER4 mRNA. HER3 was not associated with OS (F 8.2y).
(Baselga et al., 2014)	qRT-PCR IHC (DAK-H3-IC) HER3-M	met-HER2+BCA (*n*=740) met-HER2+ BCA (*n*=497) TPD (CLEOPATRA study)	↓ =	Low HER3 mRNA level, demonstrated in 49.7% of carcinomas, was associated with worse prognosis. No prognostic value for HER3 protein expression level (high in 53.1% of carcinomas).
(Berghoff et al., 2014)	IHC (DAK-H3-IC) HER3-M	met-BCA (*n*=110, of which met-HER2+BCA *n*=34)	= ↑	High HER3 (≥10%), demonstrated in 20.9% of all carcinomas, was associated with shorter OS only in HER2+ metastatic breast cancer subgroup (F 4.3y).
(Y. H. Park et al., 2014)	IHC (DAK-H3-IC) HER3-M	met-HER2+BCA (*n*=125) T+CT	↑	High HER3 (>10%), demonstrated in 27.2% of all carcinomas, was associated with shorter PFS. Low HER3 status predicted response to T+CT therapy (F 3.0y).
(Schneeweiss et al., 2014)	IHC (DAK-H3-IC) HER3-M qRT-PCR	HER2+BCA (*n*=112) HER2+BCA (*n*=210) neoAdj-TP+CT, Adj-T TRYPHAENA study	=	HER3 was not associated with pCR and was not found predictive of response to neoadjuvant therapy with trastuzumab plus pertuzumab in combination with regular chemotherapy (followed by adjuvant trastuzumab after surgery).

Overview of earlier published studies clarifying the prevalence and prognostic role of HER3 expression in breast cancer (Continued).

Published by	Methodology	Cohort description		Prognostic role of HER3 expression (% of cells / cut-off)
(Bae et al., 2013)	IHC (DAK-H3-IC) HER3-M	BCA (*n*=886, of which HER2+BCA *n*=235, TNBC *n*=229)	↑	High HER3 (>10%) expression was associated with shorter DFS in HER2+BCA (72% displayed high HER3) and TNBC (41% displayed high HER3) subgroups. High HER3 was associated with shorter OS only in TNBC subgroup (F 9.1y)
(Czopek et al., 2013)	IHC (DAK-H3-IC) HER3-M/C	HER2+BCA (*n*=35)	=	HER3 was not associated with clinical outcome in terms of 5- and 10-year DFS and OS.
(Lipton et al., 2013)	VeraTag®	met-HER2+BCA (*n*=89) T	↑	High HER3 was associated with shorter PFS.
(Gori et al., 2012)	IHC (RTJ1) HER3-C	met-HER2+BCA (*n*=61) T	=	High HER3 (>50%) expression status, demonstrated in 50.8% of carcinomas, was not associated with clinical outcome on in terms of PFS and OS (F 2.3y).
(S. W. Han et al., 2012)	VeraTag®	met-HER2+BCA (*n*=50) LC after T	↓	Low HER3 was associated with shorter PFS in trastuzumab-refractory cancers. High co-expression of HER2 and HER3 was associated with longer PFS and OS (F 2.3y).
(Larsen et al., 2012)	IHC (DAK-H3-IC) HER3-M	ER+BCA (*n*=1062) Adj-ET, BIG 1-98 trial	=	High HER3 (≥10%) was demonstrated in 45.3% of carcinomas. HER3 status was not associated with DFS or response to adjuvant endocrine therapy.
(Chiu et al., 2010)	IHC (Ab-10 pAb) HER3-M/C	BCA (*n*=3123)	↑	High HER3 (>20%), demonstrated in 10% of carcinomas, was associated with reduced BCSS (F 12.5y).
(Yonemori et al., 2010)	IHC (DAK-H3-IC) HER3-M/C	HER2+BCA (*n*=44) neoAdj-T	=	HER3 expression was demonstrated in 27.3% of carcinomas (biopsy sampling before neoAdj-T therapy). HER3 status was not associated with pCR.
(Giltnane et al., 2009)	AQUA™	BCA (*n*=550)	↑	High HER3 expression alone, co-expression of HER3 and EGFR in TNBC subtype, and co-expression of HER2 and HER3 were associated with decreased 10-year BCSS (F 8.8y).
(Haas et al., 2009)	IHC (SGP1) HER3-C/N	HER2-BCA (*n*=171) GENICA study	=	High HER3, demonstrated in 39% of carcinomas, was not associated with clinical outcome as determined by DFS and OS (*n*=88).

Overview of earlier published studies clarifying the prevalence and prognostic role of HER3 expression in breast cancer (Continued).

Published by	Methodology	Cohort description		Prognostic role of HER3 expression (% of cells / cut-off)
(Sassen et al., 2008)	IHC (5A12) HER3-M/C	BCA (*n*=173)	= ↓	High HER3 protein expression, demonstrated in 75.1% of carcinomas, was not prognostic of survival. Presence of *HER3* amplification was related to decreased DFS (F 10.5y).
(R. Giuliani et al., 2007)	IHC (RTJ1) HER3-M	met-HER2+BCA (*n*=103) T (n=46), T+CT (n=57)	=	HER3 status was positive (>0%) in 67.8% of studied carcinomas (*n*=59 of 87) and was not associated with clinical outcome (PFS, OS) or therapy response.
(Y. Lee et al., 2007)	IHC HER3-M	IDC (*n*=378)	↓	HER3 expression (>10%) was demonstrated in 13.8% of carcinomas and was associated with longer DFS.
(Bianchi et al., 2006)	IHC (RTJ1) HER3-C	BCA (*n*=145)	= ↑	High HER3 (≥25%), demonstrated in 49.7% of carcinomas, was not associated with 15-year OS. High co-expression of HER2/HER3/HER4 receptors predicted worse clinical outcome (mean F 17.8 y).
(Fuchs et al., 2006)	IHC (C-17 pAb) HER3-M/C	BCA (*n*=48)	↑	High HER3 was demonstrated in 25% of carcinomas. High HER3 alone and high HER1/2/3 co-expression was associated with decreased OS (F 4.8y).
(Robinson et al., 2006)	IHC HER3-C	met-HER2+BCA (*n*=104) T	↑	High HER3, demonstrated in 9% of carcinomas, was associated with shorter OS.
(Wiseman et al., 2005)	IHC (2-18C9 pAb) HER3-C	BCA (*n*=242)	↑	High HER3 (>20%) expression was demonstrated in 9.1% of carcinomas. HER3 alone and co-expression of ≥2 (HER1, HER2, HER3) was associated with decreased BCSS (F 15.0y).
(Abd El-Rehim et al., 2004)	IHC (RTJ1) HER3-C	BCA (*n*=1499)	= ↑	HER3 expression, demonstrated in 45% of carcinomas, was not associated with clinical outcome. High co-expression of HER3 and HER2 was associated with shorter DFS and OS. Co-expression of HER3 and HER4 predicted better outcome. (F 4.8y)
(B. L. Smith et al., 2004)	IHC	met-HER2+BCA (*n*=77) T	=	HER3 expression, demonstrated in 90.9% of carcinomas, was not associated with patients' clinical outcome.

Overview of earlier published studies clarifying the prevalence and prognostic role of HER3 expression in breast cancer (Continued).

Published by	Methodology	Cohort description		Prognostic role of HER3 expression (% of cells / cut-off)
(Bieche et al., 2003)	qRT-PCR	BCA (*n*=130)	↑	High HER3 mRNA, demonstrated in 46.2% of carcinomas, was associated with shortened RFS (only in univariate analysis) (F 8.1y).
(Witton, Reeves, Going, Cooke, & Bartlett, 2003)	IHC-Fr (H3.105.5)	BCA (*n*=220)	↑	High HER3 was present in 17.5% of carcinomas. High HER3 alone and co-expression of HER1-3 were shown prognostic of decreased 10-year BCSS.
(Suo et al., 2002)	IHC (sc-415) HER3-C qRT-PCR	BCA (*n*=100)	= ↑	HER3 protein expression (>5%), demonstrated in 26% of carcinomas, was not associated with clinical outcome. High co-expression of HER3 and HER2, HER1-3, and HER1-4 associated with shorter 5-year DFS and BCSS.
(Pawlowski et al., 2000)	qRT-PCR	BCA (*n*=365)	↓	Low HER3 mRNA expression was associated with shorter OS (only in univariate analysis).
(Travis et al., 1996)	IHC (RTJ1) HER3-C	BCA (*n*=346) met-BCA (*n*=145)	=	High HER3 expression was shown in 15% of M0 carcinomas and in 35% of advanced breast carcinomas. HER3 was not associated with clinical outcome.
(Lemoine et al., 1992)	IHC (49.3 pAb) HER3-C	BCA (*n*=195)	=	HER3 expression, demonstrated in 22% of carcinomas, was not associated with clinical outcome.

Abbreviations: Adj-ET: adjuvant endocrine therapy; Adj-T: adjuvant trastuzumab therapy; AQUA: automated quantitative analysis; BCSS: breast cancer specific survival; DFS: disease-free survival; BCA: primary breast cancer (not specified for subtype); ER+BCA: estrogen receptor-positive breast cancer; F: follow-up (median, in years); HER2-BCA: HER2-negative primary breast cancer; HER2+BCA: HER2-positive primary breast cancer; IDC: invasive ductal carcinoma; IHC: immunohistochemistry (specified with mAb clone) on FFPE section; IHC-Fr: immunohistochemistry on frozen tissue section; LC: lapatinib plus capecitabine; met-: metastatic breast cancer; MFS: metastasis-free survival; neoAdj-TP+CT: neoadjuvant therapy with trastuzumab plus pertuzumab plus chemotherapy; OS: overall survival; pAb: polyclonal antibody; pCR: pathologically complete response; PFS: progression-free survival; T: trastuzumab therapy for advanced disease; T+CT=trastuzumab plus chemotherapy (mainly paclitaxel); TNBC: triple-negative breast cancer; TPD: trastuzumab, pertuzumab, docetaxel regimen; TTP: time to progression; ↓: low HER3 (mRNA/protein) is associated with worse clinical outcome; ↑: high HER3 (mRNA/protein) is associated with worse clinical outcome; = HER3 is not associated with breast cancer prognosis

IHC-based studies (Methodology-column): Subcellular localization of staining reaction considered positive for HER3 expression is marked by 'HER3-M' = only membranous, 'HER3-C' = only cytoplasmic, 'HER3-M/C' = membranous and/or cytoplasmic, 'HER3-C/N' = cytoplasmic and/or nuclear (if mentioned in the publication).

2.3.2.3 Genetic aberrations of the HER3 gene (*ERBB3*)

Activating mutations provide one oncogenic mechanism to alter protein expression. Cancer-related somatic mutations in *ERBB3* gene are rare, although such single nucleotide polymorphisms (SNPs) that could predictively disrupt HER3 structural conformation and function have been found *in silico* (Raghav & Sharma, 2013). A recent study (Verlingue et al., 2018) reported that *ERBB3* was mutated in 3.7% of 844 genotyped cancers of various origin. Colon and gastric adenocarcinomas have been associated with exceptionally high (11 to 12%) *ERBB3* mutation prevalence (Jaiswal et al., 2013). *In silico* analysis on breast cancer data revealed that the frequency of *ERBB3* point mutations is low, 2.13% (109 of 5122), as well as copy number variation (gain) that was shown in 0.13% (2 of 1544) of breast carcinomas (COSMIC Sanger Institute, 14th August, 2018). A study performed on clinical HER2+ breast cancers, found mutated *ERBB3* in a singular case (1 of 74) (Toomey et al., 2017). Another study found mutated *ERBB3* in 3.6% of ILC (Desmedt et al., 2016).

Interestingly, SKBR3 breast cancer cell line has been confirmed to carry E933Q gain-of-function mutation, which has been proposed to confer TK catalytic activity (McCabe Pryor et al., 2015). *In vitro* experiments on transfected ER+HER2- breast cancer cells showed that T355I mutation increased cell proliferation in comparison with non-transfected cells expressing normal *ERBB3*. Proliferation was induced via HER4/EGFR-dependent ERK1/2 and cyclin D-mediated signaling routes, providing evidence for the independency of HER2 signaling. (Mishra et al., 2018). Certain *ERBB3* point mutations (F94L, G284R, D297Y, T355I, E1261A) were recently shown to increase HER2:HER3 heterodimerization and predict lapatinib resistance *in vitro*. In addition, E952Q, F94L, G284R, D297Y, T355I and E1261A mutations were associated with neratinib sensitivity *in vitro* (Mishra et al., 2018). HER2+ breast cancer patients with minor *ERBB3* allele (refSNP: rs2229046 and rs773123, both in non-coding region) and who received adjuvant TCH (docetaxel, carboplatin, trastuzumab)-based treatment were shown to have increased risk of disease recurrence (Cote et al., 2018).

Oncogene amplifications are very common genetical alterations in cancers (Matsui, Ihara, Suda, Mikami, & Semba, 2013). Compared to *HER2*, *ERRB3* copy number alterations are relatively rare in breast cancer (M. R. Choi et al., 2014; Mishra, Hanker, & Garrett, 2017). Previous studies have demonstrated that *ERBB3* amplification rate varies from 0% (Guo et al., 2017) to 10% (Zaczek et al., 2008) in unspecified breast cancer cohorts. Comparison between the previous studies is

complicated because the frequency rate depends on applied methodology (*e.g.* PCR, ISH, MS, NGS -based assays) and cut-offs used to define amplification. Surprisingly, Zaczek et al. (2008) reported that *ERBB3* amplifications were more common in less aggressive breast carcinomas (grade ≤II or pN0). Berghoff et al. (2014) did not found correlation between amplified *ERBB3* and HER3 protein expression level. *ERBB3* amplification prevalences in cBioPortal (TCGA) and GENIE breast cancer datasets were 0.6% and 0.2%, respectively (Mishra et al., 2017). Based on these findings, *ERBB3* amplification does not mechanistically appear to account for the frequency of HER3 over-expression in breast carcinomas.

2.3.3 Insights into HER3-targeted therapy

Indications for therapeutic HER3 targeting in breast cancer

HER3 is considered as a fascinating target for breast cancer therapy, particularly in *HER2*-amplified subtype due to the close interaction between HER2 and HER3. HER family members are known for their compensatory mechanisms to activate alternative HER escape pathway if certain HER is blocked. Accordingly, HER3 targeting may be clinically relevant *e.g.* to overcome resistance to anti-HER2 therapies. (N. Zhang, Chang, Rios, & An, 2016). Treatment of trastuzumab-refractory breast cancer cells *in vitro* and tumour xenografts *in vivo* with HER3-specific antibody and trastuzumab was shown to significantly reduce cell growth and induce cell cycle arrest (J. Huang et al., 2013). Additionally, anti-HER3 therapy has been shown to synergistically increase the effect of HER2- and EGFR -targeting agents (Garner et al., 2013; Mirschberger et al., 2013). HER3-targeted therapy may be beneficial also for breast cancer patients who are resistant to endocrine therapy (Collins et al., 2017; Curley et al., 2015) or chemotherapy (S. Wang et al., 2013) since HER3 has been shown to mediate resistance to these therapies. Targeting of HER3 has been suggested also for HER3-dependent breast cancers carrying normal *HER2* to prevent growth-promoting signaling triggered *e.g.* by HER3-EGFR dimerization (Campbell & Moasser, 2015; Karamouzis et al., 2016). Karamouzis et al. (2016) identified a subgroup of HER2-low (IHC ≤2+, ISH negative) breast cancers that was characterized with high frequency of EGFR:HER3 dimers. Despite of low-level HER2 expression, some non-*HER2*-amplified breast cancers may still be driven by HRGβ1-induced HER2:HER3 interaction since have been shown to respond to trastuzumab therapy (Paik, Kim, & Wolmark, 2008). Compliancy to trastuzumab may originate *e.g.* from responsive CSC population (Y. G. Kim et al., 2017). HRGβ1

has been shown to induce stem cell properties in breast cancer cells by acting through HER3 receptors (Jeong et al., 2017). A recent study (Weitsman et al., 2016) supports this concept by reporting that presence of HER2:HER3 dimers in breast carcinomas does not correlate with HER2 expression level, but is still predictive of unfavorable outcome. Furthermore, high HER3 expression has been demonstrated as a negative prognostic factor in breast cancers displaying normal HER2 and EGFR expression (Chiu et al., 2010). On the other hand, a recent study (Schneeweiss, Park-Simon et al., 2018) did not confirm the effect of lumretuzumab (HER3 antibody), in combination with pertuzumab and paclitaxel, in patients with HER3+ HER2-low (non-amplified) metastatic breast cancer. Lumretuzumab was earlier demonstrated effective in HER2-low ER+ mouse xenograft model in combination with pertuzumab and fulvestrant (Collins et al., 2017), and also as monotherapy in TNBC tumour model (Mirschberger et al., 2013).

HER3-targeting drug molecules

Plenty of novel HER3-targeting drug molecules have been developed. Because HER3 is catalytically inactive, targeting of HER3 ECD by specific antibody has been the dominant approach to block HER3 activation. HER3-targeting drugs that have been studied in the clinical trials include monoclonal humanized HER3-specific antibodies (seribantumab, lumretuzumab, elgemtumab, patritumab, KTN3379), HER3-targeting antibody-drug conjugate (U3-1402) and bispecific antibodies against HER2 and HER3 (MM-111, MCLA-128). Currently, clinical data on these drugs is restricted to tolerability and preliminary efficacy (Phase I/II), mostly in advanced metastatic or refractory HER2+ breast cancer cohorts. None of these agents has been approved for the clinical use now (Jacob, James, Hasmann, & Weisser, 2018). HER3-specific antibodies bind to receptor ECD and exert their growth-inhibitory effects through diverse mechanisms (N. Zhang et al., 2016). Seribantumab and lumretuzumab block HRGβ1 binding to its receptor by adhering themselves to HER3 domain I and thus prevent ligand-dependent HER3 dimerization (Mirschberger et al., 2013; Schoeberl et al., 2009). Elgemtumab and KTN3379 prevent HER3 function by binding to its ECD domains II and IV and thus lock HER3 in inactive configuration rendering it unable to heterodimerize (Garner et al., 2013; S. Lee et al., 2015). Lumretuzumab, a glycoengineered humanized HER3 antibody, enhances also ADCC reaction (Mirschberger et al., 2013). Patritumab has been shown to trigger internalization of receptor-mAb complexes via endocytosis, which promotes HER3 receptor degradation (Mota et

al., 2017). Bispecific antibodies targeting both HER2 and HER3, *e.g.* MM-111, form a trimeric complex by docking onto these receptors and thus prevent HER2:HER3 dimerization and consequent downstream signaling (McDonagh et al., 2012). Seribantumab, in combination with paclitaxel, has also been shown to promote HER2+ breast cancer cells to undergo apoptosis via downregulation of survivin both *in vitro* and *in vivo* (S. Wang et al., 2013).

Concerning HER3-targeted therapy strategies, it is necessary to identify predictive biomarkers that can be used to identify patients who are most responsive to these drugs (Loi & Savas, 2016; N. Zhang et al., 2016). In some clinical trials, high HER3 and HRGβ1 expression levels (by IHC) have been used as inclusion criteria. Some studies have shown positive correlation between tumour HRGβ1 expression and tumour growth inhibition achieved by HER3 antibody therapy (Meetze et al., 2015; Schoeberl et al., 2017). Schneeweiss et al. (2018) did not find significant relationship between HER3 expression level and therapy response to lumretuzumab plus pertuzumab plus paclitaxel in HER2-low breast cancers. So far, neither HRGβ1 nor HER3 has been recognized as a clinically applicable biomarker for predicting response to HER3-targeting therapies. (Jacob et al., 2018).

2.3.4 HER3 degradation by ubiquitin-proteasome pathway

Membranous HER3 receptors are internalized via clathrin-dependent endocytosis, both in the presence and absence of HRGβ1 (Fosdahl et al., 2017; Sak et al., 2012), and are mainly degraded through ubiquitin-proteasome pathway (Foot, Henshall, & Kumar, 2017; Mujoo et al., 2014). Ubiquitination is a well-known post-translational process that crucially controls membrane protein quantity, function, and subcellular localization by involving the degradation process. Firstly, E1 ubiquitin-activating enzyme binds and activates ubiquitin, a 76 amino acid protein, by forming a thiol ester linkage, which is then transferred to E2 ubiquitin-conjugating enzyme. Subsequently, E3 ubiquitin ligase, in concert with E2, catalyzes attachment of ubiquitin tag(s) to specific protein substrate by forming a covalent bond between the COOH-terminal glycine (in ubiquitin) and ε-amino group in lysine residue (in substrate). Ubiquitin-moiety labels the substrate for 26S proteasome degradation. (Qiu & Goldberg, 2002; Zou, Levy-Cohen, & Blank, 2015). Defects in this process are critical leading *e.g.* to aberrant growth factor receptor -mediated signaling. Accordingly, altered ubiquitination of these receptors has been proposed to contribute to breast pathogenesis. (Carraway, 2010; Lipkowitz, 2003).

HER3 ubiquitination is a constitutive process and is increased upon HRGβ1 binding (Szymanska et al., 2016). Experiments with *ERBB2*-transgenic mice xenografts have demonstrated that HER3 over-expression occurs even during normal *ERBB3* transcription resulting in normal HER3 mRNA quantity (Siegel et al., 1999). One mechanism by which HER3 may abundantly accumulate in cancer cells relates to dysregulated post-transcriptional modifications or defects in signaling mechanisms that regulate HER3 membrane trafficking (Amin et al., 2012). For instance, aberrant expression of factors that regulate HER3 degradation may influence on the number of membranous HER3 receptors. Two E3 ubiquitin ligases, NEDD4-1 (*neural precursor cell expressed, developmentally downregulated 4-1*) and NRDP1 (*neuregulin receptor degradation protein 1*), are known necessary for HER receptor maintenance and quantity control by mediating their degradation via ubiquitin-proteasome pathway (Carraway, 2010). NEDD4-1 (Z. Huang et al., 2015) and NRDP1 (Cao, Wu, Yen, Sweeney, & Carraway, 2007; Diamonti et al., 2002; Qiu & Goldberg, 2002) have been shown to involve controlling the subcellular localization, membrane retention and signaling of HER3 receptors. These interactions result in HER3 ubiquitination, which prevents its constant recycling to the cell surface. Low-level expression of NEDD4-1 and NRDP1 may therefore be associated with HER3 over-expression. Hypothetically, NEDD4-1 and NRDP1 may be even more predictive of active HER3 signaling than HER3 receptor quantity itself due to constant trafficking.

2.3.4.1 NEDD4-1 mediated HER3 degradation

NEDD4-1 belongs to a family of HECT E3 ubiquitin ligases. NEDD4-1 is localized in cytoplasm, where it involves in the regulation of several intracellular signaling molecules by targeting them for degradation (Anan et al., 1998; Ingham, Gish, & Pawson, 2004). NEDD4-1 dysfunctions have been attributed to the pathogenesis of several human malignancies (Zou et al., 2015). NEDD4-1 over-expression has been related to adverse clinical outcome, *e.g.* in non-small cell lung cancer (Amodio et al., 2010), gastric adenocarcinoma (A. Sun et al., 2014), and hepatocellular carcinoma (Z. J. Huang, Zhu, Yang, & Biskup, 2017). Two studies (Y. Chen, van de Vijver, Hibshoosh, Parsons, & Saal, 2016; Jung et al., 2013) have confirmed that NEDD4-1 is over-expressed in approximately 50% of breast carcinomas, but its biological and clinical significance is unknown. Low NEDD4-1 expression has been demonstrated to activate HER3 signalling and result in increased proliferation both in MCF-7 (breast cancer) and DU145 (prostate cancer) cells *in vitro* and in xenotransplanted tumour mouse model *in vivo*. Conversely, NEDD4-1 over-

expression has been shown to decrease HER3 expression by increasing its degradation through ubiquitin-proteasome pathway. (Z. Huang et al., 2015). Interestingly, upregulated HER3 expression due to NEDD4-1 knockdown has been shown to sensitize MCF-7 cells for growth inhibitory effects of anti-HER3 therapy (Z. Huang et al., 2015). Huang et al. (2015) reported also their finding of inverse relationship between NEDD4-1 and HER3 expression in prostate cancer tissue. Altogether, these results strongly suggest that NEDD4-1 negatively regulates HER3 expression and function.

2.3.4.2 NRDP1 mediated HER3 degradation

NRDP1, known also as FLRF (*fetal liver ring finger protein 1*) and RNF41 (*ring finger protein 41*), belongs to a family of RING finger domain-containing E3 ubiquitin ligases. NRDP1 has been shown to regulate the quantity of steady-state HER3 receptors by ubiquitination leading to proteasomal HER3 degradation independent of HRGβ1. (Diamonti et al., 2002; Fry, Simion, Sweeney, & Carraway, 2011; Qiu & Goldberg, 2002). Upon HRGβ1 binding, HER3 downstream signaling molecule Akt recruits deubiquitination enzyme USP8 to stabilize NRDP1. Thus, USP8 negatively regulates cellular HER3 expression by enhancing its ubiquitination and degradation. (Cao et al., 2007). Activated androgen receptors are also involved in HER3 quantity control by promoting *NRDP1* transcription, which leads to increased HER3 ubiquitination (Gaborit, Lindzen, & Yarden, 2016). Cao et al. (2007) showed that loss of NRDP1 followed by *NRDP1* knockdown suppressed HRGβ1-induced HER3 ubiquitination and degradation in MCF7 breast cancer cells *in vitro*. Another study (Yen et al., 2006) demonstrated that NRDP1 over-expression results in decreased HER3 expression and consequently inhibit breast cancer cell growth and motility *in vitro*. Vice versa, NRDP1 loss was shown to enhance HER3 expression and induce HER2/HER3-dependent tumour cell growth and mammary tumour progression in *ERBB2*-transgenic mice *in vivo* (Yen et al., 2006). Later, another study (Ingalla et al., 2010) abrogated this finding by showing that transgenic human *NRDP1* expression in the mouse mammary gland was not sufficient to suppress HER3 expression and tumour growth in *ERBB2*-transgenic mice *in vivo*.

Thus far, only two studies have been published on NRDP1 expression in clinical breast cancer material. Firstly, Yen et al. (2006) reported their finding of inverse correlation between NRDP1 and HER3 expression in breast carcinomas and demonstrated that NRDP1 is suppressed or lost in half (57%, 20 of 35) of carcinomas. Among the HER2+ tumours (n=12), 58% were shown to display

decreased NRDP1 expression. Later, another retrospective study (Jiao et al., 2015) confirmed this finding, and found significant association between NRDP1 loss (in 42% of carcinomas) and reduced 10-year BCSS. Altogether, previous observations strongly suggest that NRDP1 loss influence HER3 over-expression and function in breast cancers. Nevertheless, the clinical significance of HER3-NRDP1 interaction, especially in HER2+ breast cancer subtype, remains unknown. Addtionally, Jiao et al. (2015) proposed that dysregulated NRDP1 do not singly contribute to HER3 expression because NRDP1 loss was shown to predict poor clinical outcome also in HER3-negative breast cancer cases.

2.4 MCM proteins in DNA replication and breast carcinoma

2.4.1 Mechanisms of DNA replication initiation in eukaryotics

DNA replication is a fundamental process in the growth control of eukaryotic cells displaying proliferation capacity. Replication takes place in the S-phase. This process is critical for the maintenance of cell genomic integrity and is therefore strictly regulated (Fei & Xu, 2018). MCM (*Minichromosome Maintenance*) protein family, especially proteins 2, 3, 4, 5, 6 and 7 (collectively called as MCM2-7), is known to critically involve replication. MCM2-7 proteins form the catalytic core of helicase, which is a key factor in the replication initiation and elongation as it promotes replication fork movement. (Li & O'Donnell, 2018; Neves & Kwok, 2017).

Firstly, two complexes consisting of MCM2-7 ('double hexamer') are recruited at replication origin (ORI), together with origin recognition complex (ORC, consists of Orc1-6), Cdc6 and Cdt1 proteins. These actors orchestrate the assembly of two DNA-encircled pre-replication complexes (pre-RC) during early G1 phase (Figure 3A, p.63). This process is called as DNA Replication Licensing. At the beginning of S phase, MCM2-7, Cdc6 and Cdt1 proteins are phosphorylated by S phase promoting Cdks, Clb5-Cdc28 and Dbf4-dependent Cdc7 kinase (DDK) (Figure 3B, p.63). Cdc6 and Cdt1 are subsequently released from the pre-RC and are replaced by Cdc45 and GINS (consists of Psf1-3 and Sld5) to form two replicative CMG (*Cdc45-MCMs-GINS*) complexes (Figure 3C, p.63). CMG complexes then form two replication forks that move in opposite directions and have helicase activity to unwind the parental double-stranded DNA bi-directionally. This enables building of complementary strands for original DNA strands. Recruitment of additional factors

(Sld2, Sld3, Sld7, Dpb11, Pol ε, Pol α, Pol δ, MCM10) results in formation of replisome progression complex that controls DNA replication fork movement. (Li & O'Donnell, 2018; Martinez, Wacker, Bruck, & Kaplan, 2017). CMG complexes are disassembled and unloaded from the chromatin during the late S phase. MCM proteins are prevented from re-binding to DNA at least by active cyclin A/Cdk2 and functional inactivation of Cdt1 and Cdc6 proteins that are needed to load MCM2-7 hexamers onto chromatin. These mechanisms ensure that genome is duplicated only once in each cell cycle. (Martinez et al., 2017; Tachibana, Gonzalez, & Coleman, 2005). MCM2-7 proteins are finally degraded in the late M and early G1 phases by ubiquitin-mediated proteolysis. (Braun & Breeden, 2007).

Assembly of pre-RC complex is crucially regulated by cyclin E1 and Cdk2, both of which are required for G1/S transition (Ekholm-Reed et al., 2004). In the deficiency of cyclin E1 and Cdk2 activity, MCM2-7 loading onto chromatin, pre-RC assembly, DNA replication and cellular regeneration were shown to be remarkably reduced both *in vitro* and *in vivo* (Hu et al., 2014; Powell et al., 2015; Teixeira et al., 2015). Vice versa, cyclin E1 over-expression has been shown to abnormally induce replication and impair replication fork progression, which triggers cancer-promoting mutations and genetic loss in cells that are under replication stress (Jones et al., 2013; Teixeira et al., 2015). Cyclin E1 has also been shown compulsory for promotion of DNA re-licensing and driving of DNA synthesis in quiescent cells before re-entering into the S-phase (Geng et al., 2003). The exact mechanisms by which cyclin E1 regulates replication initiation are mostly unknown. Experiments performed with quiescent mammalian cells re-entering the cell cycle suggest that cyclin E1/Cdk2 is needed to stabilize and accumulate *e.g.* Cdc6 protein that is required during pre-RC assembly process (Chuang et al., 2009). Cyclin E1 has also been suggested to contribute to induction of cyclin A expression which is necessary to drive S phase progression (Hu et al., 2014).

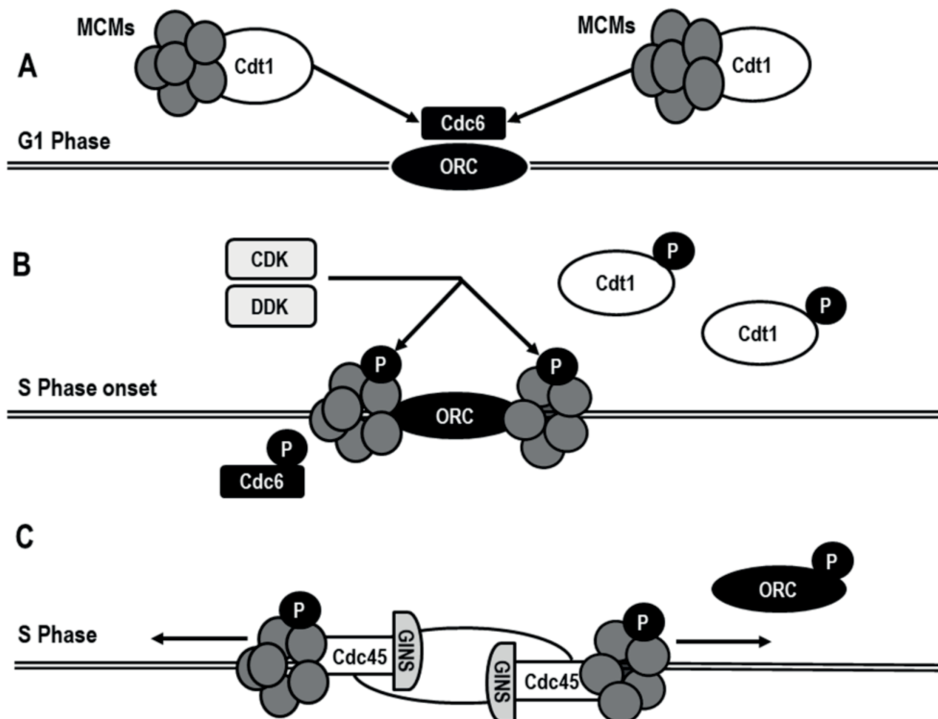

Figure 3. Assembly of pre-RC and CMG complexes in the DNA replication initiation. In early G1, two hexamer complexes (MCM2-7) with Cdt1, Cdc6 and ORC proteins are recruited at ORI region to form pre-RC complexes (**A**). During the S phase onset, MCM2-7, Cdc6 and Cdt1 are phosphorylated by CDK and DDK, which triggers dissociation of Cdc6 and Cdt1 from the complex (**B**). Next, GINS and Cdc45 are associated with MCMs to form replicative CMG complexes (**C**). (Martinez et al., 2017; Neves & Kwok, 2017)

2.4.2 MCM2 expression with clinicopathological associations

MCM2-7 proteins are expressed in proliferating cells throughout the active cell cycle, but are absent in quiescent cells resting in G0-phase (Stoeber et al., 2001). MCM2-7 proteins are often over-expressed in cancer cells, and are thus mechanistically associated with sustained cell growth and carcinogenesis (Neves & Kwok, 2017; Tachibana et al., 2005). A recent meta-analysis (Gou et al., 2018) demonstrated that especially MCM2, MCM5, and MCM7 proteins contribute to cancer progression and are associated with poor clinical outcome. Of MCM family, particularly MCM2 has been studied for its role in breast cancer biology (Gou et al., 2018). MCM2 protein

is expressed at relatively low level in histologically normal breast. Only luminal epithelial cells in the inner layer of breast glands display nuclear MCM2 expression, while myoepithelial cells bordering the basal lamina do not express MCM2. Breast cancer cells typically display high MCM2 expression, similarly to tumour-associated immune cells (TAICs) and proliferative cells in the adjacent stroma. (Reena et al., 2008; Stoeber et al., 2001; Yousef et al., 2017).

MCM2 has been recently demonstrated clinically applicable biomarker for the assessment of breast cancer cell proliferation activity as an alternative to Ki-67 (Joshi et al., 2015; Yousef et al., 2017). On the other hand, MCM2 has been criticized for its predominantly high expression, especially in poorly differentiated breast carcinomas (Shetty et al., 2005). Compared to Ki-67, MCM2 is more frequently expressed in breast cancers. In a study by Gonzalez et al. (2003), median Ki67-LI and MCM2-LI were 39% and 78%, respectively. Shetty et al. (2005) reported that median MCM2-LIs (*vs* Ki67-LI) were 55% (10%), 70% (17%), and 97% (44%) in grade I, II, and III carcinomas, respectively. In contrast, Yousef et al. (2017) reported remarkably lower MCM2 expression levels in breast cancers. They noticed high MCM2-expression (cut-off 40%) in 3%, 29% and 74% of grade I, II and III carcinomas, respectively. MCM protein expression is not restricted to actively proliferating cells, but is present also in cells that possess growth potential but are held in prolonged G1 phase 'in-cycle arrest' (Stoeber et al., 2001), which probably explains high MCM2 expression in breast cancers. Loddo et al. (2009) have demonstrated that MCM2 is over-expressed in 82% of breast carcinomas. Of these, 24% represented tumour phenotypes in which cells are predominantly arrested in G1-phase (low expression of S-G2-M progression markers). Nieto-Jimenez et al. (2016) analyzed cBioportal genomics data set on invasive breast cancers and found only few aberrations in *MCM2* gene. More precisely, *MCM2* amplifications, deletions and point mutations were found in 0.6%, 0.1%, and 0.3% of carcinomas, respectively. Thus, high MCM2 protein expression in breast carcinomas is not merely explained by the genetical alterations in *MCM2*.

MCM2 over-expression (mRNA/protein) has been shown to define aggressive breast cancer phenotype. High MCM2 has been shown to associate with high grade, high proliferation activity (Ki67-LI), basal-like phenotype, TNBC subtype (Abe et al., 2015; Bukholm, Bukholm, Holm, & Nesland, 2003; Gonzalez et al., 2003), HER2+ status (Ali et al., 2012; Yousef et al., 2017), luminal B subtype (Yousef et al., 2017), negative HR status (Joshi et al., 2015; Yousef et al., 2017) and large tumour size (Gonzalez et al., 2003; Wojnar et al., 2011). High MCM2 expression has been demonstrated also in breast cancer cells representing CSC population (Abe et al.,

2015). Over-expression of MCM2 has been shown to independently predict poor breast cancer outcome in retrospective studies (Table 6, p.66). However, the clinical relevance of MCM2 has not been comprehensively confirmed in biologically distinct breast cancer subtypes, *e.g.* in *HER2*-amplified breast carcinoma. Gonzalez et al. (2003) reported that MCM2 protein expression is a strong independent prognostic factor and even superior to histological grade, Ki-67, and lymph nodal status in predicting BCSS. In a study by Joshi et al. (2015), high MCM2 expression was associated with detrimental 15-year BCSS in univariate analysis but lost its significance in multivariate analysis. Yousef et al. (2017) reported that MCM2 expression is more sensitive than that of Ki-67 in predicting breast cancer recurrence, albeit both were shown clinically useful in predicting RFS. Kwok et al. (2014) demonstrated that MCM2-7 mRNA levels strongly correlate with each other, and concurrent over-expression of at least four MCM mRNA types is related to worse clinical outcome. This study suggests that transcription of *MCM2-7* genes may be closely co-regulated to promote breast carcinogenesis.

Table 6. Overview of previous studies on MCM2 expression and its prognostic significance in breast cancer.

Published by	Methodology	Sample material	Prognostic role of MCM2 expression (% of cells / cut-off)	
(Yousef et al., 2017)	*In silico* data analysis (mRNA) IHC	*In silico* data (*n*=2652) BCA FFPE (*n*=364 of which HER2+ *n*=144)	↑	High MCM2 mRNA and protein expression (≥40%, detected in 58.2% of carcinomas) was associated with reduced RFS (*n*=200).
(Nieto-Jimenez et al., 2016)	*In silico* data analysis (mRNA)	*In silico* data (*n*=3500)	↑	High MCM2 mRNA was associated with shorter RFS and OS in luminal A and B breast cancer types. The prognosis was worst when *MCM2* was upregulated with *TRIP13*, *RAD51* and *GINS1*.
(Tokes et al., 2016)	IHC	BCA FFPE (*n*=57)	↑	Low MCM2 (<47.5% VA, <42.8% CA) was predictive of pCR on primary systemic therapy.
(Joshi et al., 2015)	IHC	BCA FFPE (*n*=309)	↑	High MCM2 (>12%) was associated with a decreased 15-year BCSS (only in univariate analysis).
(Kwok et al., 2014)	*In silico* data analysis (mRNA)	*In silico* data (*n*=1441)	↑	High MCM2 mRNA was associated with decreased BCSS alone and in combination with other simultaneously upregulated MCMs.
(Ali et al., 2012)	IHC	BCA FFPE (*n*=2206 of which ER+ *n*=1485)	↑	MCM2 expression was high (≥34%) in 23% of all carcinomas and was associated with reduced 10-year BCSS in ER+ subgroup (only in univariate analysis).
(Wojnar et al., 2011)	IHC	IDC FFPE (*n*=117)	=	High MCM2 (>25%) was demonstrated in 25.6% of carcinomas. MCM2 was not associated with clinical outcome (mean follow-up 4.7 years).
(Loddo et al., 2009)	IHC	BCA FFPE (*n*=182)	↑	High MCM2 (≥30%) was demonstrated in 82% of all carcinomas. Of these actively cycling tumours (58%) were associated with shorter DFS compared to tumours arrested in G1 (24%) and those resting in G0 (18%) (median follow-up 47 months, *n*=167).
(Bukholm et al., 2003)	IHC	BCA FFPE (*n*=147)	↑	High MCM2 (≥10%) was demonstrated in 77.6% of carcinomas. MCM2 was associated with decreased BCSS but if adjusted for cyclin A, MCM2 lost its prognostic value in multivariate analysis.

Overview of previous studies on MCM2 expression and its prognostic significance in breast cancer (Continued).

Published by	Methodology	Sample material	Prognostic role of MCM2 expression (% of cells / cut-off)
(Gonzalez et al., 2003)	IHC	BCA FFPE (*n*=221)	↑ High MCM2 (≥50%) was demonstrated in 69.2% of carcinomas and was associated with shorter RFS and OS (median follow-up 9.3 years).

Abbreviations: BCA: primary breast cancer (subtype not specified); BCSS: breast cancer-specific survival; CA: computational analysis; DFS: disease-free survival; ER+: estrogen receptor-positive; HER2+: HER2-positive breast cancer; IDC: invasive ductal carcinoma; OS: overall survival; pCR: pathologically complete remission; RFS: relapse-free survival; VA: visual analysis (conventional microscopy); ↓: low MCM2 (mRNA/protein) is associated with worse clinical outcome; ↑: high MCM2 (mRNA/protein) is associated with worse clinical outcome; = MCM2 is not associated with breast cancer prognosis

3 AIMS OF THE STUDY

The aim of this study was to retrospectively clarify histopathological associations and clinical relevance of cyclin E1, HER3, NEDD4-1, NRDP1 and MCM2 in relation to the first disease recurrence and short-term (9-wk) adjuvant trastuzumab therapy. The focus was primarily on *HER2*-amplified primary breast cancer subtype, in which these biomarkers were not comprehensively studied before. The detailed aims were as follows:

I. To determine prevalence, predictive and prognostic implications of cyclin E1 expression and *CCNE1* amplification in comparison with conventional histopathological characteristics of *HER2*-amplified breast carcinoma. (**Study I**)

II. To validate immunohistochemical staining method for reliable and accurate detection of HER3 expression in breast cancer tissue. (**Study II**)

III. To demonstrate expression, histopathological and clinical associations of HER3, NEDD4-1 and NRDP1 in *HER2*-amplified breast carcinoma. To clarify association between HER3 expression and HER2 status. (**Study III**)

IV. To implement a novel fluoro-chromogenic double labelling technique and digital image analysis for the assessment of breast cancer cell proliferation activity by MCM2 expression. To determine prevalence of MCM2 expression, its histopathological and clinical associations in comparison with Ki-67 in *HER2*-amplified breast carcinomas. (**Study IV**)

4 MATERIALS AND METHODS

4.1 Breast cancer cell lines (I, II)

MDA-MB-157 breast cancer cell line known to carry *CCNE1* amplification was used as a positive control in CISH experiments in **Study I**. MCF7 (breast cancer) and MKN7 (gastric adenocarcinoma) cell lines were used as positive controls in Ki-67 IHC assay in **Study I**. Eight *HER2*-amplified breast cancer cell lines (MDA-MB-361, MDA-MB-453, SKBR3, BT-474, HCC1419, JIMT-1, UACC-812, and EFM-192A), one HER2- breast cancer cell line (MDA-MB-231), and one *HER2*-amplified esophageal adenocarcinoma cell line (OE-19) were used to analyze HER3 expression in **Study II**. Of these, EFM-192A was purchased from Leibniz Institute DSMZ-German Collection of Microorganism and Cell Cultures (Braunschweig, Germany). OE-19 cell line was purchased from Health Protection Agency Culture Collections HPACC (Porton Down, Salisbury, England). MDA-MB-157, MDA-MB-231, MDA-MB-361, MDA-MB-453, SKBR3, BT-474, HCC1419, MCF7 and MKN7 cell lines were acquired from American Type Culture Collection ATCC (Manassas, VA, USA). JIMT-1 cell line was earlier established and characterized in our laboratory (Tanner et al., 2004), and is available to supply from the Leibniz Institute DSMZ-German Collection of Microorganism and Cell Cultures.

Cells were cultured at +37ºC in humidified 5% carbon dioxide atmosphere with 5% (v/v) fetal bovine serum and L-Glutamin (2 mM) supplementation in the growth media, as was recommended by the cell line suppliers. The media was changed twice a week and cells were passaged once per week (70 to 80% confluency) until achieved constant growth rate. Cell morphology and growth were regularly inspected with inverter microscope. Cells were periodically indicated mycoplasma free. Before deployment for this study, cells were stored in liquid nitrogen at -191°C. For IHC assay, FFPE cellblocks were prepared using plasma-thrombin method that is widely applied in diagnostic cytology. Briefly, cells harvested from culture flasks were centrifuged as pellets following wash with sterile 0.9% NaCl and coagulation with human blood plasma and thrombin. Once a cohesive cell pellet was formed, it was fixed with 10% neutral-buffered formalin (NBF), processed and embedded in paraffin.

4.2 Clinical breast carcinoma samples (I-IV)

Two archival breast cancer tissue collections (referred to hereafter as Sample Cohort I and II) with derived clinicopathological data were used for retrospective biomarker analyses performed by IHC. Sample Cohort I was used in Studies I-IV and Sample Cohort II exclusively in Study III. Studies were conducted in compliance with the REMARK guidelines (McShane et al., 2005).

4.2.1 Sample Cohort I

Sample Cohort I consisted of 202 consecutive invasive *HER2*-amplified breast carcinomas that were surgically resected and diagnosed at Tampere University Hospital, Tampere, Finland, between December 23rd in 2002 and December 12th in 2007. Of these, 193 were diagnosed as early primary carcinomas (pT1-T3, pN0-3, M0) and were included in survival analyses, while cases diagnosed as *de novo* metastatic disease (M1, *n*=9) were mainly discarded from the analyses (except for Study I). All carcinomas were resected from female patients. 8.4% (*n*=16 of 190) of patients had family history of breast or ovarian carcinoma but were not confirmed as inherited disease. Clinicopathological characteristics of Sample Cohort I are presented in Table 7 (p.72). Approximately half of the patients (*n*=87, 45.1%), diagnosed after September 2005, were treated with a combination of adjuvant trastuzumab (Herceptin®) and chemotherapy during a 9-wk period as a first-line therapy (Joensuu et al., 2006). The other half of the cohort (*n*=106, 54.9%) did not receive any HER2-targeted therapy. Of all primary M0 breast cancer patients, 144 received adjuvant chemotherapy, 121 post-operative radiotherapy, and 113 endocrine therapy as a primary treatment (Table 8, p.73). None of the patients received any neoadjuvant therapy.

Breast cancer tissues were originally fixed overnight with 10% NBF, processed and embedded in paraffin (FFPE) according to the routine procedure. *HER2* amplification status was determined by CISH technique with a digoxigenin (DIG) labelled in-house probe (BAC clone RP11-94 L15) during the diagnostic procedure. HER2 positivity was determined by the presence of *HER2* gene amplification on basis of ASCO/CAP guidelines (≥6 gene copies/cell or clusters). Carcinomas with equivocal HER2 status were not included. HR status was also determined earlier by IHC with mouse monoclonal antibodies (dilution 1:500) specific for ER (clone 6F11) and PR (clone 312), both from Leica Biosystems Novocastra Laboratories Ltd.,

Newcastle Upon Tyne, UK. HR status was considered positive if ≥10% of cancer cells displayed nuclear staining for ER or PR. Clinicopathological patient data was retrospectively collected from the medical records. Saved data included: tumour sample ID, patient age, tumour histological type, ER and PR statuses, *HER2* amplification status, proliferation index (Ki67-LI), pTNM, histological grade, and primary therapies. For the clinical follow-up, the dates of breast surgery, recurrence, death, and data filing were retrieved. Patients were followed-up until the onset of disease recurrence or the last date of data filing (February to April 2012). The mean follow-up period was 5.3 years, ranging from one month to 9.1 years.

4.2.2 Sample Cohort II

Sample Cohort II consisted of 308 primary breast carcinomas that were surgically resected and diagnosed at Tampere University Hospital between March 18th in 1990 and December 17th in 1999. Cases that were available for this study represented a sub-cohort of larger sample collection established originally for another study purpose (Korhonen, Huhtala, & Holli, 2004). This cohort was enriched of ILC cases. In total, 40 patients received adjuvant chemotherapy, 198 post-operative radiotherapy, and 97 endocrine therapy (Table 8, p.73). Patients did not receive any neoadjuvant therapy or HER2-targeted therapies.

Breast cancer samples were fixed with 10% NBF and were processed into paraffin blocks following the routine practice. One histologically representative tumour region was selected from each FFPE block, punched (2 mm core diameter) and implanted into tissue microarray (TMA) block. The whole sample cohort was represented in six TMA blocks. Clinicopathological patient data was received from Professor Anne Kallioniemi, MD. Data included the following parameters: patient age, tumour histological type, ER and PR statuses, HER2 status, pTNM, histological grade, primary treatments, and follow-up information with anonymized ID. Sample Cohort II was unselected for HER2 status; 15.3% (*n*=47 of 308) of carcinomas were classified as HER2+, and 84.7% (*n*=261 of 308) were HER2- according to HER2 protein expression level by IHC. *HER2* amplification status was not available. TNBC phenotype was defined by concurrent HER2 and HR negativity (ER-PR-HER2-) using the clinically relevant 10% cut-off for HR (Fujii et al., 2017). Detailed clinicopathological characteristics of Sample Cohort II are shown in Table 7 (p.72). The mean follow-up period was 10.4 years, ranging from one month to 22 years.

Table 7. Description of clinicopathological characteristics of primary breast carcinomas included in Sample Cohorts I and II. Number of carcinomas with available data is marked in the column *n*.

Clinicopathological parameters	*n*	Sample Cohort I (*n*=193), *n* (%)	*n*	Sample Cohort II (*n*=308), *n* (%)
Age	193		308	
<50 years		39 (20.2)		64 (20.8)
≥50 years		154 (79.8)		244 (79.2)
ER status	193		307	
Negative (<10%)		68 (35.2)		59 (19.2)
Positive (≥10%)		125 (64.8)		248 (80.8)
PR status	193		307	
Negative (<10%)		113 (58.5)		106 (34.5)
Positive (≥10%)		80 (41.5)		201 (65.5)
HER2 status*	193		308	
Negative		0 (0.0)		261 (84.7)
Positive		193 (100.0)		47 (15.3)
Triple-negativity	193		307	
Other type		193 (100.0)		277 (90.2)
TNBC (HER2-ER-PR-)		0 (0.0)		30 (9.8)
Ki-67 index	193		230	
Low (<20%)		39 (20.2)		165 (71.7)
High (≥20%)		154 (79.8)		65 (28.3)
Histological type	193		304	
Ductal		172 (89.1)		173 (56.9)
Lobular		12 (6.2)		131 (43.1)
Other		9 (4.7)		
Histological grade	190		232	
I-II		46 (24.2)		179 (77.2)
III		144 (75.8)		53 (22.8)
Tumour size (cm)	188		177	
<2 cm		78 (41.5)		57 (32.2)
≥2 cm		77 (41.0)		120 (67.8)
multifocal		33 (17.5)		
Tumour size (pT)	189		308	
pT1-2		177 (93.7)		282 (91.6)
pT3-4		12 (6.3)		26 (8.4)
Lymph nodal status	185		286	
Negative (pN0)		107 (57.8)		172 (60.1)
Positive (pN+)		78 (42.2)		114 (39.9)

*) Positive HER2 status was defined by the presence of *HER2* amplification (Sample Cohort I) or HER2 protein over-expression (Sample Cohort II).

Table 8. Description of primary treatments for breast cancer patients in Sample Cohorts I and II.

Primary treatment	Sample Cohort I (*n*=193)		Sample Cohort II (*n*=308)	
	n	%	*n*	%
Breast surgery				
Mastectomy	110	57.0	161	52.4
Breast-conserving surgery	79	40.9	146	47.6
No surgery	4	2.1		
Unknown			1	
Post-operative radiotherapy				
Yes	121	62.7	198	65.3
No	72	37.3	105	34.7
Unknown			5	
Adjuvant endocrine therapy				
Yes	113	58.5	97	32.1
No	80	41.5	205	67.9
Unknown			6	
Adjuvant chemotherapy [1]				
Yes	144	74.6	40	13.4
No	49	25.4	259	86.6
Unknown			9	
Adjuvant trastuzumab therapy [2]				
Yes	87	45.1		
No	106	54.9	308	100.0

1) Sample Cohort I: mostly CEF (cyklofosfamide-epirubicin-5-fluorouracil), taxanes
Sample Cohort II: mostly CMF (cyklofosfamide-metothrexate-5-fluorouracil)

2) 9-wk regimen

4.2.3 Ethical considerations

The study protocol and use of clinical samples and related clinicopathological data was approved by the Ethics Committee of Pirkanmaa Hospital District (references no. R07082 [Sample Cohort I], R00143 [Sample Cohort II]), the Ministry of Social Affairs and Health, and the National Authority for Medicolegal Affairs in Finland (References no. 2441/04/044/06, 3042/32/300/02). The need for informed consent was waived by the aforementioned authorities owing to the large number of individual samples and the verity that a sizeable quantity of the patients was already deceased. Individual patient data has not been made available and the dataset has been processed anonymized during the study. Patient names and social security numbers were available only during the data retrieval from the medical records.

4.3 Immunohistochemistry and staining interpretation (I-III)

Biomarker expression studies (I-III) were performed using indirect IHC stainings with horseradish peroxidase (HRP)-conjugated polymer and 3'3-diaminobentzidine tetrahydrochloride (DAB) visualization. Samples were represented as consecutive 3-4 µm thick whole tissue sections (WTS) that were placed on SuperFrostPlus® slides. Tissue sections were deparaffinized and pretreated using heat-induced epitope retrieval (HIER) in TE buffer (50mM Tris-HCl, 1 mM EDTA pH 9) at 98°C for 15 minutes. Stainings were conducted automatically using LabVision™ Autostainer 480S platform (Thermo Scientific/Lab Vision Corporation, Fremont, CA, USA). Mayer's hematoxylin was used for light counterstaining against brown DAB precipitation. Positive and negative controls (primary antibody omitted) were included in each staining batch. Used primary antibodies are listed in Table 9 (p.80). Detailed staining procedures are described in the Original Publications I-III.

4.3.1 Cyclin E1 (I)

Immunohistochemical staining

Cyclin E1 expression was studied in 202 *HER2*-amplified breast carcinomas representing Sample Cohort I (**Study I**). Normal placenta was used as a positive control tissue for its known cyclin E1 expression in basal trophoblastic cells (Olvera et al., 2001). Cyclin E antibody clone 13A3 (Leica Novocastra, New Castle Upon Tyne, UK) was used at a dilution of 1:100 with Power Vision Plus Poly-HRP detection kit (ImmunoLogic, AD Duiven, Netherlands) and ImmPACT™ DAB (Vector Laboratories Inc., Peterborough, UK) according to the manufacturers' instructions. Antibody clone 13A3 detects only full-length cyclin E1 (EL), while cytoplasmical LMW isoforms remain undetectable (Karakas et al., 2016).

Microscopy and staining interpretation

Sample slides were analyzed with Olympus BX43 microscope (Olympus, Tokyo, Japan) using ×20 magnification. Photomicrographs from five fields showing the most intensive nuclear DAB reaction were captured with a CCD microscope camera using automated image acquisition controlled by the Surveyor Imaging System (Objective Imaging, Cambridge, UK). Image J software (NIH) with Cell Counter plug-in was used to calculate the percentage of stained cancer cells. Cytoplasmically

localized staining without nuclear labelling was regarded as a negative result. One hundred cells were counted from each five images (by S.L.). Sample slides were also scanned with Aperio ScanScope XT virtual microscope (Aperio Technologies, Vista, USA) into digital whole slide images (WSI) and were subjected to visual inspection on computer screen with JVSview JPEG2000 version 1.2 (downloadable at [http://jvsmicroscope.uta.fi/?q=jvsview]). A cut-off value of 50% was used to determine low (<50%) and high (≥50%) cyclin E1 expression level.

4.3.2 Ki-67 (I, III)

Immunohistochemical staining

Ki-67 expression was studied in 193 *HER2*-amplified primary breast carcinomas representing Sample Cohort I (**Study I**) and in 230 breast carcinomas of Sample Cohort II (**Study III**). MCF7 and MKN7 cells were used as positive control samples in each staining batch for their known Ki-67 staining pattern. Ki-67 antibody clone MIB-1 (Dako, Glostrup, Denmark), diluted at 1:500, and Ki67 antibody clone BS4 (Nordic BioSite, Täby, Sweden), diluted at 1:100, were used with Histofine® Simple Stain MAX PO (Nichirei Biosciences Inc., Tokyo, Japan) and ImmPACT™ DAB (Vector Laboratories Inc.) according to the manufacturers' instructions.

Image acquisition and staining interpretation

Ki-67 IHC stainings of Sample Cohort I (WTSs) were analyzed with Olympus BX43 microscope (Olympus, Tokyo, Japan) using ×20 magnification. Photomicrographs from at least three fields of hotspot area (>1000 cancer cells) displaying the most intensive nuclear DAB reaction were captured with a CCD camera using automated image acquisition controlled by the Surveyor Imaging System (Objective Imaging). The proportion of stained cancer cells (Ki-67 labelling index, Ki67-LI) was calculated using ImmunoRatio version 1.0c, an open source plug-in for Image J (downloadable at [http://153.1.200.58:8080/immunoratio/]) (by S.L.). ImmunoRatio uses colour deconvolution algorithm for the separation of staining components (brown DAB, blue hematoxylin counterstain) and adaptive thresholding algorithm for nuclear area segmentation (Tuominen et al., 2010). Ki-67 stainings of Sample Cohort II (TMAs) were scanned with Slide Strider (Jilab Inc., Tampere, Finland) into digital WSIs that were viewed with SlideVantage 1.2 (Jilab Inc.) and analyzed semi-automatically using ImmunoRatio version 2.5 plug-in tool. Ki67-LI was determined by calculating at

least 100 cancer cells from the hot spot area (by S.L). A cut-off value of 20% was selected to determine low (<20%) and high (≥20%) proliferation activity by Ki67-LI. This cut-off is recommended for clinical use (Dowsett et al., 2011), and has also been shown to yield in excellent prognostication with ImmunoRatio (Tuominen et al., 2010).

4.3.3 HER3 (II, III)

Immunohistochemical staining

HER3 expression was studied in 177 *HER2*-amplified primary breast carcinomas representing Sample Cohort I (**Studies II, III**) and in 308 breast carcinomas of Sample Cohort II (**Study III**). HER3 expression was studied also in the following human cancer cells lines: MDA-MB-231, MDA-MB-361, MDA-MB-453, SKBR3, BT-474, HCC1419, JIMT-1, UACC-812, EFM-192A (breast cancers), and OE-19 (esophageal adenocarcinoma). HER3 antibody clone DAK-H3-IC (Dako) was used at a dilution of 1:100 with Bright Vision+ Poly-HRP detection kit (ImmunoLogic) and ImmPACT™ DAB (Vector Laboratories Inc.) according to the manufacturers' instructions. Normal human prostate (Koumakpayi et al., 2006) and MDA-MB-453 cells (Lemoine et al., 1992; Xia et al., 1999) were used as positive control samples for their known HER3 expression. Breast cancer sample of known staining pattern was also included in each staining batch to confirm assay reproducibility.

Detailed description of HER3 IHC validation procedure is presented in the Original Publication II. Briefly, staining performance was tested with the following HER3 antibodies: clone DAK-H3-IC (Dako), clone SP71 (Spring Bioscience Inc., Pleasanton, CA, USA), clone RTJ1 (Leica Novocastra), and polyclonal antibody SAB4500793 (Sigma-Aldrich, Steinheim, Germany) (Table 9, p.80). Following detection kits were tested: BrightVision+ Poly-HRP (ImmunoLogic), Histofine Simple Stain MAX PO (Nichirei Biosciences Inc), UltraVision™ Quanto Detection System HRP DAB (Thermo Fisher Scientific, Fremont, CA, USA) and CSA II Biotin-free Tyramide Signal Amplification System (Dako). To assess the effect of fixation on HER3 antigen preservation, an additional breast carcinoma set (n=13), collaterally fixed with 10% NBF and alcohol-based PAXgene Tissue FIX (PreAnalytiX GmbH, Hombrechtikon, Switzerland), was stained with the validated IHC protocol.

Image acquisition and staining interpretation

Stained sample slides were scanned with Aperio ScanScope XT virtual microscope (Aperio Technologies, Vista, USA) into digital WSIs and were analyzed on computer screen with JVSview. For HER3 appearance, both membranous and cytoplasmic staining patterns were evaluated and classified separately according to the staining intensity and proportion of stained cancer cells (by S.L.). Membranous HER3 staining (HER3-M) was scored followingly: [0] absent/low (<10% of cells) staining, [1+] intermediate circumferential staining (10-30% of cells), and [2+] strong circumferential staining (>30% of cells). Cytoplasmic HER3 staining (HER3-C) was categorized using the following criteria: [0] absent/faint staining, [1+] overall low-intensity staining, and [2+] predominant high-intensity staining in most of the cancer cells. Score 1+ was set as a threshold to define HER3 positivity in accordance with the corresponding studies published earlier (Table 5, p.51). For total cellular HER3 staining (HER3-T), negative HER3-T status was determined as [0] or [1+] HER3-M concurrently with [0] or [1+] HER3-C. Positive HER3-T status was given if either HER3-M or HER3-C or both were classified as [2+].

For testing the performance of digital image analysis (DIA) for HER3, photomicrographs taken with a CCD camera (×20 magnification) from the most representative area were analyzed using ImmunoMembrane software version 1.0i (downloadable at [http://153.1.200.58/?q=software]). ImmunoMembrane utilizes colour deconvolution for the separation of brown DAB and blue hematoxylin and a customized algorithm for cell membrane segmentation. ImmunoMembrane analyzes completeness (0-10 points) and intensity (0-10 points) of cell membrane staining, and by summing these values forms quantitative IM-Score (0 to 20 points). (Tuominen, Tolonen, & Isola, 2012). Only IM Intensity Score value was used in Study II.

4.3.4 NEDD4-1 (III)

Immunohistochemical staining

NEDD4-1 expression was studied in 145 *HER2*-amplified breast carcinomas of Sample Cohort I (**Study III**). Normal human kidney was used as a positive control tissue for its known NEDD4-1 expression in proximal tubule cells (Xu et al., 2016). Polyclonal NEDD4-1 antibody against the WW2 domain (Merck KGaA, Darmstadt, Germany) was used at a dilution of 1:750 with BrightVision+ Poly-HRP detection

kit (ImmunoLogic) and Histofine DAB-2V kit (Nichirei Biosciences Inc.) according to the manufacturers' instructions.

Image acquisition and staining interpretation

Stained sample slides were scanned with Slide Strider (Jilab Inc.) into digital WSIs and were inspected with JVSview on computer screen. Staining pattern was analyzed within the invasive carcinoma area displaying the most intensive cytoplasmic DAB reaction (by S.L.). The staining reaction intensity was scored with the following criteria: [0] no staining, [1+] weak, [2+] intermediate, and [3+] strong. Score [3+] was set as a threshold to define high NEDD4-1 expression level. The percentage of stained cells was not calculated due to homogenous staining pattern in cancerous areas.

4.3.5 NRDP1 (III)

Immunohistochemical staining

NRDP1 expression was studied in 145 *HER2*-amplified breast carcinomas of Sample Cohort I (**Study III**). Normal human testis was used as a positive control tissue. Testicular cells in the seminiferous tubules (Sertoli cells) and mononuclear blood cells have been confirmed to display high NRPD1 expression (Qiu & Goldberg, 2002; The Human Protein Atlas, 2018). NRDP1 (FLRF/RNF41) antibody (Bethyl Laboratories Inc., Montgomery, Texas, USA) was used at a dilution of 1:3000 with EnVision™FLEX High pH HRP and DAB+ kit (Dako) according to the manufacturers' instructions.

Image acquisition and staining interpretation

Stained sample slides were scanned with Slide Strider (Jilab Inc.) into digital WSIs that were analyzed with Slide Vantage 1.2 (Jilab Inc.). Staining pattern was analyzed within the invasive carcinoma area displaying the most intensive DAB reaction (by S.L.). Staining intensity and proportion of stained cells displaying nuclear staining reaction were evaluated. Staining intensity was classified accordingly: [0] no staining, [1+] weak, [2+] moderate, and [3+] strong. ImmunoRatio 2.5 was used for calculation of cancer cells displaying nuclear NRDP1 expression and was graded followingly: [0] <1% of cells, [1] 1-24% of cells, [2] 25-49% of cells, [3] 50-74% of

cells, and [4] 75-100% of cells displaying nuclear staining reaction. To get a final grade for nuclear NRDP1 (NRDP1-N) expression, scores were multiplied. Sample scores ≤3 were determined low and samples with score ≥4 as high for NRDP1-N. When the staining reaction was localized in cytoplasm, samples were classified high for cytoplasmic NRDP1 (NRDP1-C) expression if the staining intensity was graded as [2+] (moderate) or [3+] (strong).

4.3.6 CK5/14 (III)

Immunohistochemical staining

Expression of basal cytokeratins 5 and 14 was studied in 167 *HER2*-amplified breast carcinomas of Sample Cohort I (**Study III**). Basal breast carcinoma that was confirmed with high CK5/14 expression was used as a positive control. CK5 (clone XM26) and CK14 (clone LL0022) antibodies (both from Leica Novocastra) were used at a dilution of 1:150 (cocktail) with Bright Vision+ Poly-HRP detection kit (ImmunoLogic) and ImmPACT™ DAB (Vector Laboratories Inc.) according to the manufacturers' instructions.

Microscopy and scoring

Stained slides were analyzed with Olympus BX43 microscope (Olympus Corporation) using 20-40× magnification (by S.L.) and were defined CK5/14 positive if >20% of cancer cells displayed clear cytoplasmic staining (Laakso, Loman, Borg, & Isola, 2005). Basal-like cancer phenotype (of basal myoepithelial cell origin) was defined by concurrent CK5/14 expression and ER negativity (Laakso et al., 2005).

Table 9. Details of primary antibodies used in IHC staining protocols for biomarker detection (Studies I-IV).

Antibody for	Catalogue code	Host/Clonality	Dilution/Incubation	Manufacturer/Provider
Cyclin E	NCL-CYCLINE	mouse mAb 13A3	1:100, RT 1h	Leica Biosystems/Novocastra Laboratories Ltd.
Ki-67*	M7240	mouse mAb MIB-1	1:500, RT 30 min	Dako A/S
Ki-67**	BSH-7302	mouse mAb BS4	1:100, RT 30 min	Nordic BioSite Ab
HER3	M7297	mouse mAb DAK-H3-IC	1:100, RT 30 min	Dako A/S
HER3†	NCL-c-erbB-3	mouse mAb RTJ1	various tested	Leica Biosystems/Novocastra Laboratories Ltd.
HER3†	M3712	rabbit mAb SP71	various tested	Spring Bioscience Inc.
HER3†	SAB4500793	rabbit pAb	various tested	Sigma-Aldrich
FLRF/RNF41	A300-049A	rabbit pAb	1:3000, RT 30 min	Bethyl Laboratories Inc.
NEDD4 WW2 domain	#07-049	rabbit pAb	1:750, RT 30 min	Merck KGaA
CK5	NCL-L-CK5	mouse mAb XM26	1:150, RT 30 min	Leica Biosystems/Novocastra Laboratories Ltd.
CK14	NCL-L-LL0022	mouse mAb LL002	1:150, RT 30 min	Leica Biosystems/Novocastra Laboratories Ltd.
Digoxigenin	200-002-156	mouse mAb HY-A.1	1:10000, RT 30 min	Jackson ImmunoResearch Laboratories Inc.
MCM2	BSH-7698-100	mouse mAb BS18	1:200, RT 30 min	Nordic BioSite Ab
CYFRA21-1 (CK19)≠	100221	mouse mAb 1603	10 µg/ml, RT 30 min	Medix Biochemica Ab
CYFRA21-1 (CK19)≠	100223	mouse mAb 1605	10 µg/ml, RT 30 min	Medix Biochemica Ab
pan-CK≠	10-108-BULK	mouse mAb C11	10 µg/ml, RT 30 min	EXBIO

Abbreviations: mAb: monoclonal antibody; pAb: polyclonal antibody; RT: room temperature

* used for Sample Cohort I; ** used for Sample Cohort II, † used in HER3 IHC assay validation, ≠ used for pan-CK cocktail

4.4 Chromogenic *in situ* hybridization (I)

CCNE1 amplification status was studied in 185 *HER2*-amplified breast cancers (Sample Cohort I) in **Study I** by applying CISH technique (Isola et al., 2004; Tanner et al., 2000) with *CCNE1*-specific bacterial artificial chromosome (BAC) probe. MDA-MB-157 breast cancer cell line was previously confirmed to carry amplified *CCNE1* (Keyomarsi & Pardee, 1993; Natrajan et al., 2012) and was used as a positive control.

4.4.1 Design and preparation of CCNE1 probe

Culturing of BAC clone harboring probe DNA

CCNE1 probe was generated from a human BAC clone RP11-104J24 transformed in *Escherichia coli* bacteria (Invitrogen Ltd., Paisley, UK). To replicate the BAC probe, bacteria were first cultured on agar plates containing Luria-Bertani (LB) medium with chloramphenicol antibiotic (25 μg/ml), and were grown in incubator at 37°C. Individual colonies were isolated from the plates and were suspended into liquid LB (chloramphenicol added), and were gradually scaled-up to get sufficient amount of bacteria for DNA extraction. Bacteria were harvested from the cultures by centrifuging them into pellets and were re-suspended in LB (chloramphenicol added) and were frozen (-20°C) before proceeding to DNA extraction. Bacterial glycerol stocks were also established as back-ups for long-term storage (-70°C).

Probe DNA extraction, amplification and DIG-labelling

Probe DNA was extracted with Qiagen® Large-Construct Kit according to the manufacturer's protocol (Qiagen Inc., Valencia, CA). After extraction, probe DNA consentration and purity (A_{260}/A_{280}) was measured with Qubit® 2.0 Fluorometer (Invitrogen) using Qubit® dsDNA BR Assay Kit (Invitrogen). Probe DNA was amplified using illustra GenomiPhi™ HY DNA Amplification Kit (GE Healthcare) and PCR cycling following manufacturer's instructions. Concentration of amplified probe DNA was measured with Qubit fluorometer, and the probe DNA length was checked by agarose gel electrophoresis (AGE) before and after the amplification

step. Amplified probe DNA was directly labelled with digoxigen (DIG)-11-dUTP (Roche Biochemicals, Mannheim, Germany) using nick-translation protocol (Hyytinen, Visakorpi, Kallioniemi, Kallioniemi, & Isola, 1994). After the labelling, the DNA fragment length was again confirmed by AGE.

Preparation of probe mixture

Ready-to-use probe mixture was prepared by combining 2 µl labelled CCNE1 DNA probe [20 ng/µl], 0.5 µl human Cot-1 DNA [1 µg/µl] (Roche Biochemicals, Mannheim, Germany), 1 µl placental DNA [1 µg/µl] (Sigma), and 6.5 µl hybridization buffer pH 7.0 [containing 15% (w/v) dextran sulphate and 70% (w/v) formamide in 20× standard saline citrate (SSC)] per slide. Probe mixture was stored frozen (-20°C) and was taken at RT just before use.

4.4.2 Pretreatment and hybridization reaction

For CISH, 4 µm thick FFPE tissue sections were deparaffinized followed by absolute ethanol bath and air-drying. Slides were then boiled in TE buffer (50mM Tris-HCl 1mM EDTA, pH9) at +98°C for 15 minutes using PT Module (LabVision Corporation, Fremont, CA, USA). After cooling into +65°C, slides were washed with distilled water and immersed in 0.2M HCl for 5 minutes enabling protein denaturation. After that, slides were rinsed with distilled water. Enzymatic digestion was performed by applying Digest-All™ 3 Pepsin solution (Invitrogen, UK) onto slides for 20 minutes at RT to enable probe penetration into tissue. The slides were washed with 1×PBS and post-fixed with 5% formalin for 10 minutes. After a rinse with distilled water, slides were dehydrated with graded ethanols and air-drying. Probe mixture (10-20 µl) was applied onto slides, covered with coverslips and sealed with rubber cement to prevent them from drying. Slides were denatured (+95°C for 5 minutes) to unwind the ds-DNA followed by hybridization at +42°C for 48 hours in humidified chamber in StatSpin® ThermoBrite slide hybridizer (IRIS International Inc., CA, USA). After hybridization, coverslip and rubber cement were removed and slides were washed to remove undetached probes. Stringency washes were performed by immersing slides sequentially in pre-warmed (+75°C) Wash Buffer I (0.4× SSC/0.3% NP-40) for 3 minutes and Wash Buffer II (2× SSC/0.1% NP-40) for 1 minute at RT. After washes, slides were immersed into 1×TBS-Tween (0.05%) until proceeding to the detection step.

4.4.3 Detection of hybridized probes by immunohistochemistry

Probe detection was performed by IHC with anti-DIG antibody (Jackson ImmunoResearch Laboratories Inc., PA, USA) at a dilution of 1:10 000, Histofine® Simple Stain MAX PO (Nichirei Biosciences Inc.) and ImmPACT™ DAB (Vector Laboratories Inc.) according to the manufacturers' instructions. IHC was conducted with LabVision™ Autostainer 480S (Thermo Scientific/LabVision Corporation).

4.4.4 Microscopy and interpretation of hybridization result

Sample slides were analyzed with Olympus BX43 light microscope (Olympus Corporation) using 40-60× magnification to calculate cellular *CCNE1* copies from the most representative cancer areas. Photomicrographs from these areas were captured with a CCD microscope camera using automated image acquisition controlled by the Surveyor Imaging System (Objective Imaging) to enable analysis on computer screen (by S.L.). *CCNE1* was considered amplified when CISH revealed the presence of ≥6 gene copy signals per cell or appearance of gene copy clusters. Presence of at least two *CCNE1* copies per cell (normal *CCNE1* status) was required to technically acceptable result.

4.5 Immunofluorescence labelling and flow cytometry (II)

4.5.1 Immunofluorescence labelling for HER3 and HER2

HER3 expression in breast cancer cell lines was demonstrated using indirect immunofluorescence (IF) labelling and fluorescence activated cell sorting (FACS) by flow cytometry (**Study II**). The aim was to compare visually evaluated staining intensity and scoring of HER3 expression (by IHC) to quantitated HER3 expression level (by FACS). Nine breast cancer cell lines (MDA-MB-231, MDA-MB-361, MDA-MB-453, SKBR3, BT-474, HCC1419, JIMT-1, UACC812 and EFM-192A) and one esophageal adenocarcinoma cell line (OE-19) were analyzed. For comparison of HER3 and HER2 expression levels, HER2 expression was also quantified by FACS in breast cancer cell lines.

Preparation of cells for IF-labelling and FACS

For IF, adherent cells from two T-75 culture flasks (at 70 to 90% confluency) were trypsinized, suspended into growth medium and were pelleted by centrifugation (800 rpm for 6 min at +4°C). Cells for HER3 labelling were washed with phosphate-buffered saline (PBS), and blocked with 3% (w/v) bovine serum albumin (BSA) in PBS for 15 minutes. After that, cells were pelleted by centrifugation (800 rpm for 6 min at +4°C) and were reconcentrated into 250 µl of 3% BSA-PBS. Then cells were divided into three Eppendorf-tubes followingly: 1) only cells (intrinsic fluorescence), 2) cells + goat anti-mouse IgG (background fluorescence), and 3) cells + anti-HER3 antibody + goat anti-mouse IgG (HER3 labelling reaction). The total volume in each tube was 50 µl, and the procedure was similar for each cell line. Cells for HER2 labelling were fixed with freshly prepared 4% (w/v) paraformaldehyde (PFA) for 15 minutes followed by subsequent PBS wash and centrifugation. Pelleted cells were incubated with 0.5% (w/v) saponin in PBS for 15 minutes to permeabilize cell membranes enabling binding of HER2-specific primary antibody to the intracellular HER2 domain. After that, cells were washed with PBS, pelleted by centrifugation, and were reconcentrated into 250 µl of 3% BSA-PBS. Then cells were pipetted into three Eppendorf-tubes followingly: 1) only cells (intrinsic fluorescence), 2) cells + goat anti-rabbit IgG (background fluorescence), and 3) cells + anti-HER2 antibody + goat anti-rabbit IgG (HER2 labelling reaction). The total volume in each tube was 50 µl, and the procedure was similar for each cell line.

IF-labelling procedure

To demonstrate HER3-M expression, mouse monoclonal c-erbB-3 Ab-4 (clone H3.90.6) antibody (Thermo Scientific/Lab Vision Corporation, Fremont, CA, USA) against the HER3 ECD was used at a concentration of 20 µg/ml. Unpermeabilized cells were incubated with antibody dilution for 20 minutes followed by washes with PBS and 3% BSA-PBS to remove unbound antibodies by centrifugation (800 rpm for 6 min at +4°C). Alexa Fluor® 488-conjugated AffiniPure Goat Anti-Mouse IgG (Jackson ImmunoResearch Laboratories Inc., PA, USA) was used as a secondary antibody at a concentration of 30 µg/ml. After 40 minutes incubation, labelled cells were washed with PBS and fixed with 1% (w/v) PFA in PBS. During the labelling, cells were kept on ice, and washed with ice-cold PBS to prevent internalization of membranous HER3 receptors. Labelled cells were kept under low-light condition to prevent the fluorochorome from fading. HER2 labelling procedure was performed

similarly with ErbB2/Her2 Rabbit Monoclonal Antibody clone EP1045Y (Epitomics Inc., Burlingame, CA, USA) at a dilution of 1:30. Alexa Fluor® 488-conjugated AffiniPure Goat Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories Inc.) was used as a secondary antibody at a concentration of 30 μg/ml. Both antibodies were diluted in 0.1% (w/v) saponin in 3% BSA-PBS.

4.5.2 Flow cytometry analysis

Labelled cells were analyzed using BD Accuri™ C6 Flow Cytometer with BD Accuri C6 Software (BD Biosciences, Franklin Lakes, New Jersey, USA) under identical equipment settings. Fluoresence intensity data was acquired by analyzing 20 000 events (cells). Mean fluorescence intensity (MFI) values were determined on FL1 (533/30 nm filter) detector. Non-labelled cells were measured to set the negative value. Data analysis was performed with cells gated on a dot-plot FSC (forward scatter) against SSC (side scatter). To obtain the exact MFI, background fluorescence (cells + secondary antibody) value was subtracted from the value measured for labelled cells. Labelled cells were kept at +4ºC until analyzed with flow cytometry. HER3/HER2 labelling procedure followed by FACS analysis was reproduced twice for each cell line, and each FACS measurement was carried out twice during the run to confirm assay reproducibility.

4.6 Fluoro-chromogenic double labelling method (IV)

4.6.1 Fluoro-chromogenic staining for MCM2 and CK

For studying MCM2 expression in *HER2*-amplified breast cancers (Sample Cohort I, *n*=142), a novel fluoro-chromogenic IHC staining method was implemented in **Study IV**. This method was firstly introduced for quantification of ER, PR and Ki-67 in breast cancer tissue (Isola J, Heinonen S, Tuominen V, 2013). In this method, chromogenic IHC and IF are sequentially performed to demonstrate the presence of two distinct antigens on a single tissue section. For the staining, 3-4 μm thick FFPE WTSs that were placed on Super Frost Plus® slides were deparaffinized and rehydrated. HIER was performed by boiling the slides in TE buffer (50 mM Tris-HCl 1 mM EDTA, pH 9) at +98°C for 15 minutes. MCM2 protein was detected with MCM2-specific mAb clone BS18 (Nordic BioSite, Täby, Sweden), used at a

dilution of 1:200, Histofine® Simple Stain MAX PO and Histofine DAB-2V kits (Nichirei Biosciences Inc.) according to the manufacturers' instructions. Followingly, breast cancer cells were detected with pan-cytokeratin (pan-CK) antibody cocktail consisting of clones 1603 and 1605 (Medix Biochemica Ab, Espoo, Finland) and C11 (EXBIO, Vestec, Czech Republic), each used at a concentration of 10 μg/ml. Cy2-conjugated AffiniPure Goat Anti-Mouse IgG (Jackson ImmunoResearch Laboratories Inc.) was used as a secondary antibody at a dilution of 1:100. Tonsil was used as a control tissue for its known intense MCM2 expression both in germinal center cells and squamous epithelial cells, and weak expression in the cells of mantle zones (The Human Protein Atlas, 2018). Staining was conducted automatically using LabVision™ Autostainer 480S platform (Thermo Scientific / LabVision Corporation). Detailed staining procedure is described in the Original Publication IV, and used primary antibodies are specified in Table 9 (p.80).

4.6.2 Image acquisition and digital image analysis

Multispectral imaging principle was applied to digitize stained slides into WSIs. Image acquisition was conducted with SlideStrider appliance (Jilab Inc.) by scanning the sample slides sequentially under bright field (for MCM2) and fluorescence (for CK) illumination with a ×20 magnification lens. WSIs were stacked and saved as multilayer images (JPEG2000 format) and were stored on a centralized server. WSIs were inspected on computer screen with SlideVantage 1.2 viewer (Jilab Inc.), by exploiting its bright field and fluorescence image-blending mode to display concurrently MCM2 and CK stainings. Percentage of MCM2 expressing cancer cells (MCM2 labelling index, MCM2-LI) was determined using semi-automatic DIA method with ImmunoRatio 2.5. Analysis was performed within the region of interest (ROI) comprising a minimum of 1000 CK-expressing cancer cells displaying the most intensive DAB reaction in their nuclei. MCM2-LI was calculated using the algorithm based on the following formulae:

$$\text{MCM2-LI} = \frac{\text{No. of MCM2-CK-stained cell in ROI}}{\text{Total no. of CK-stained cell in ROI}} \times 100$$

By using this method, MCM2-LI is determined only in CK-positive cancer cells (=IF correction) with no interference of MCM2 staining caused by proliferating normal cells in the adjacent stromal tissue or by TAICs. Cancer cell recognition is based on pan-CK expression demonstrated by IF labelling. MCM2 analysis by ImmunoRatio 2.5 premises on color deconvolution algorithm for separating cell nuclei that display brown DAB precipitate (MCM2+) and blue hematoxylin (MCM2-), and on adaptive thresholding for nuclear area segmentation (Tuominen et al., 2010) in CK-positive cancer cells (=IF-correction). Fluorescence parameters "maskSensitivity" and "maskSimplicity" were adjusted for the intensity and completeness of IF staining. DAB ('DABthres') and hematoxylin ('Hthres') threshold values were set to 120 and 200, respectively. Samples were analyzed independently by two experienced cell biologists (S.L. & T.H). For MCM2-LI, a cut-off value of 75% was used to determine low (<75%) and high (≥75%) proliferation activity based on Receiver Operator Characteristics (ROC) analysis.

4.7 Statistical analyses (I-IV)

The produced data was analyzed with BMDP Version 4.0 (BMDP Statistical Software Inc., Los Angeles, CA, USA), SPSS® Statistics for Windows Version 23 (IBM Corporation, Armonk, NY, USA) and Graph Pad Prism for Windows Versions 4.0 and 5.02 (Graph Pad Software Inc., San Diego, CA, USA). Contingency tables with Fisher's Exact test and Pearson's Chi-Square test (two-tailed) were used to analyze associations between dichotomic variables. Two-tailed Mann-Whitney *U* test (two groups) and Kruskal-Wallis *H* test (>two groups) were used to determine associations with continuous variables. Pearson's Correlation test and Spearman's rho test were used to test correlations between continuous variables and to test inter-rater consistency.

Kaplan-Meier survival analysis and univariate analysis with log-rank test (Mantel-Cox) were used to compare patients' survival according to dichomatous parameters. Statistically significant variables were included in multivariate analysis (Cox Regression). The primary end-point in survival analyses was the first breast cancer recurrence. Appearance of contralateral breast cancer or other malignancy was not considered as a recurrent disease. To determine recurrence-free survival (RFS), patients were followed-up from the date of breast surgery (diagnosis) to the date of disease progression manifested as a local recurrence or distant metastasis. Patients who did not experience recurrence during the follow-up were censored at the time

of death for unspecified reason or on the last day of data filing for this study. Receiver Operator Characteristics (ROC) analysis was used to set the cut-off values for certain variables. Overall, *p*-values <0.05 were considered statistically significant for any relationship being considered. Levels of statistical significance are marked with asterisk symbols as follow: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, or ns (no statistical significance).

5 SUMMARY OF RESULTS

5.1 Cyclin E1 expression and *CCNE1* amplification (I)

Study I demonstrated cyclin E1 expression in *HER2*-amplified breast carcinomas (Sample Cohort I). Majority of carcinomas displayed detectable cyclin E1 expression by IHC, although prominent inter-tumoral variability appeared in expression levels. Approximately one third of carcinomas (36.6%, *n*=74 of 202) were confirmed with high (LI≥50%) and rest (*n*=128) with low (LI<50%) cyclin E1 expression. Of these, only 3.5% (*n*=7 of 202) exhibited particularly weak staining reaction (LI<5%). Figure 4 (below) illustrates cyclin E1 expression in *HER2*-amplified primary breast carcinomas; 36.8% (*n*=71 of 193) of them were characterized with high cyclin E1 expression. Compared to Ki-67, cyclin E1 expression showed more inter-tumoral variation (Figure 4). Cyclin E1 was predominantly expressed in cancer cell nuclei, as was demonstrated by IHC with antibody specific to the full-length cyclin E1 (Figure 5, p. 90).

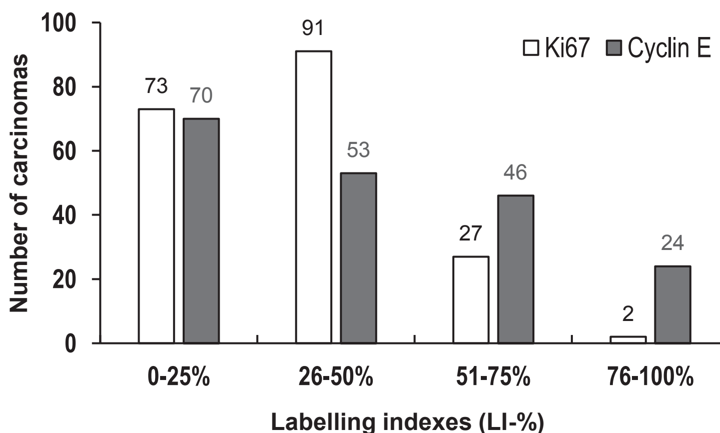

Figure 4. Cyclin E1 and Ki-67 expression, as demonstrated by IHC, in primary *HER2*-amplified breast carcinomas (*n*=193). Median cyclin E1-LI was 39% (ranging from 2 to 96%) and median Ki67-LI was 31% (ranging from 5 to 82%).

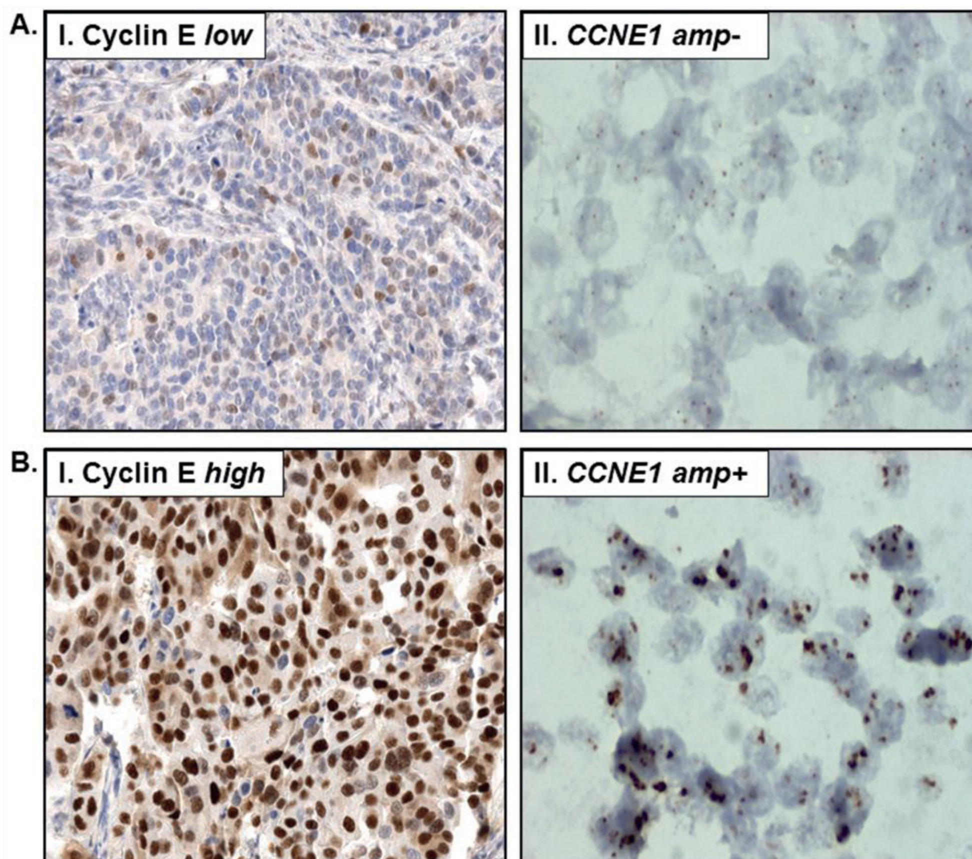

Figure 5. Examples of the corresponding IHC staining for cyclin E1 protein (**I**) and CISH for *CCNE1* gene copies (**II**) on adjacent breast cancer tissue (FFPE) sections. Low cyclin E1 expression (LI 20%) and normal *CCNE1* status (panel **A**). High cyclin E1 expression (LI 97%) and *CCNE1* amplification (panel **B**). Counterstaining with Mayer's hematoxylin. Magnifications: ×200 (**I**) and ×400 (**II**). Figure modified from the Original Publication I by Luhtala et al.

Study I revealed a small fraction of breast carcinomas carrying co-amplification of *CCNE1* and *HER2* genes. Prevalence of *CCNE1* amplification was 8.1% (*n*= 15 of 185) in the whole cohort, and 7.3% (*n*=13 of 177) in primary M0 carcinomas. *CCNE1* amplification was common in breast carcinomas displaying cyclin E1 over-expression; 10 of 13 *CCNE1*-amplified breast carcinomas showed high cyclin E1 level (Figure 5B). Interestingly, *CCNE1*/*HER2* co-amplification with cyclin E1 over-expression was confirmed in trastuzumab-resistant breast cancer cell line JIMT-1, that was established in our laboratory (Tanner et al., 2004).

5.2 Validation of HER3 IHC assay (II)

An IHC assay for the detection of HER3 was established in **Study II**. Of tested antibodies, clear circumferential membranous HER3 (HER3-M) staining was accomplished only with DAK-H3-IC clone. This antibody stained also cytoplasmically localized HER3 receptors, but nuclear HER3 staining was not observed. Other antibodies (RTJ1, SP71, SAB4500793) yielded in uncertain and nonreproducible staining results in comparison with DAK-H3-IC. Only faint – if any - HER3-M staining was detected with RTJ1 and SP71 antibodies. SAB4500793 caused strong non-specific staining reaction in stromal white blood cells. (Figure 6, p.92) ImmunoMembrane 1.0i was found useful in scoring of HER3-M expression on digitized sample slides when compared to visual assessment (VA) with conventional microscopy (Figure 14, p.99).

HER3 expression was detected also by FACS in breast cancer cell lines with known HER3 IHC staining pattern (DAK-H3-IC antibody). For quantification of HER3-M expression by FACS, unpermeabilized cells were labelled with antibody that binds to HER3 ECD (clone H3.90.6). MFI values were plotted against IM Intensity Scores and showed trend for interdependence, although did not show statistically significant correlation (Figure 8, p.94). Study II demonstrated that FACS is useful method for quantification of HER receptor expression in breast cancer cell suspensions.

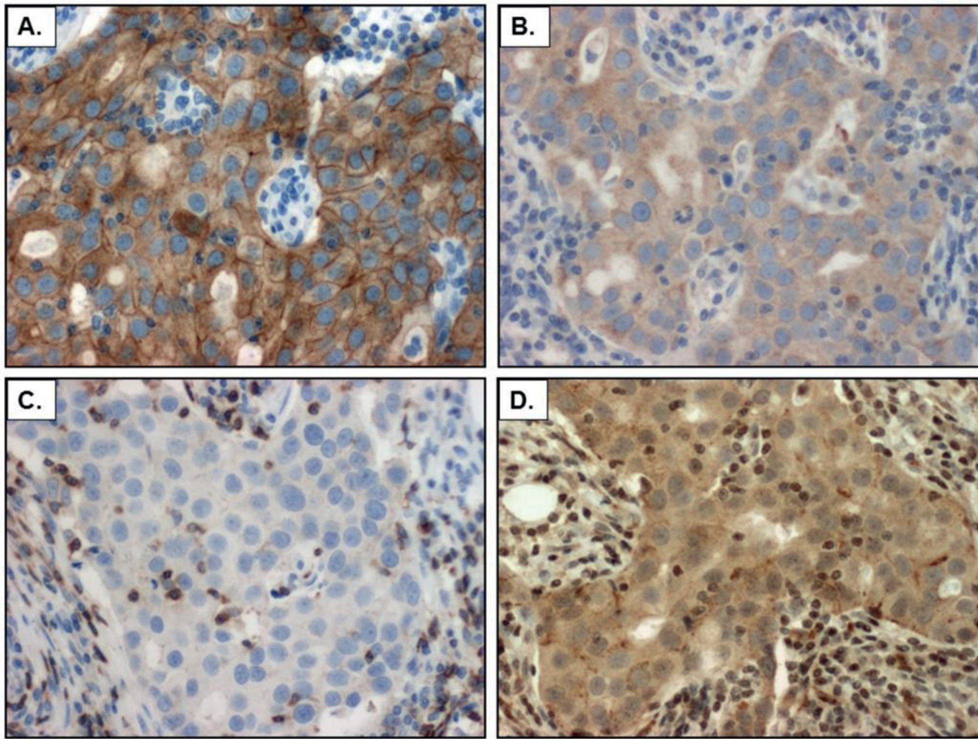

Figure 6. Comparison of HER3 IHC stainings on consecutive breast cancer tissue (FFPE) sections with the following antibodies: **A.** clone DAK-H3-IC (Dako), **B.** clone SP71 (Spring Biosciences), **C.** clone RTJ1 (Leica Biosystems Novocastra), and **D.** polyclonal SAB4500793 (Sigma-Aldrich). Mayer's hematoxylin as a counterstain. Magnification ×200. Figure modified from the Original Publication II by Luhtala et al.

5.3 HER3 expression (II, III)

5.3.1 HER3 in breast cancer cell lines (II)

Study II showed that breast cancer cell lines display differing HER3 expression levels, as was demonstrated by IHC and FACS. Of *HER2*-amplified breast cancer cell lines, MDA-MB-453, EFM-192A, and HCC1419 were shown to display the most intense HER3-M expression (IM Intensity Score ≥7), whereas JIMT-1 and SKBR3 were observed with notably weaker staining (IM Intensity Score ≤6) by IHC (Figure 7, p.93).

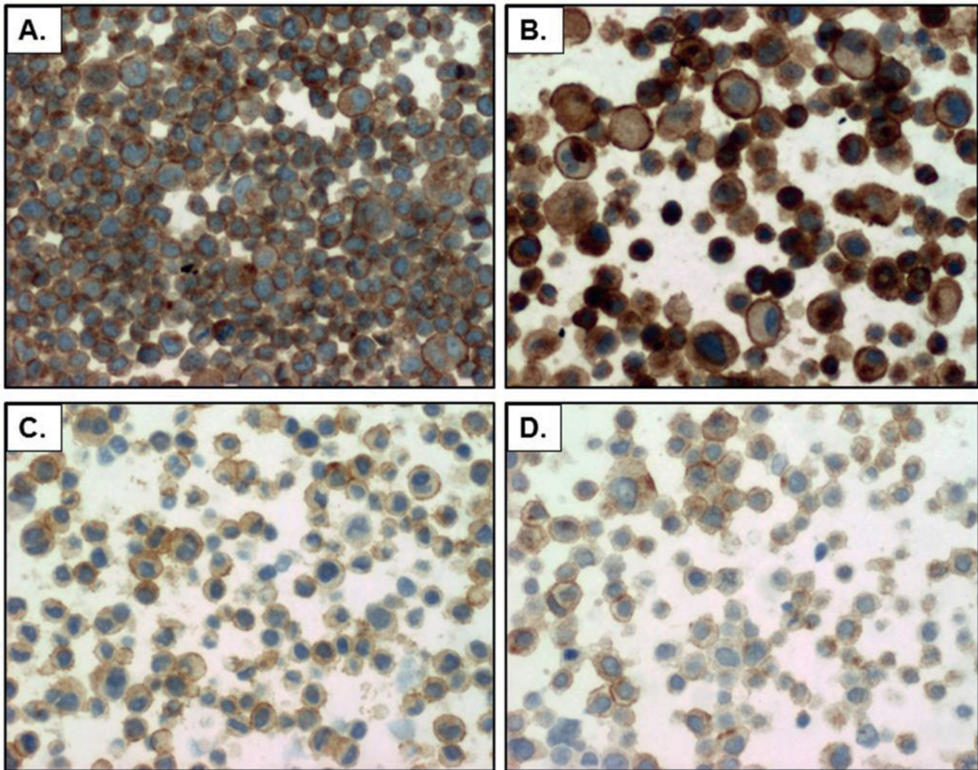

Figure 7. HER3 expression, as demonstrated by IHC (DAK-H3-IC antibody), in *HER2*-amplified breast cancer cell lines. High HER3 expression was shown in MDA-MB-453 (**A.**, IM Intensity Score 10) and HCC1419 (**B.**, IM Intensity Score 7) cells. Intermediate HER3 expression was shown in SKBR3 (**C.**, IM Intensity Score 6), and low HER3 expression in JIMT-1 (**D.**, IM Intensity Score 3) cells. Mayer's hematoxylin as a counterstain. Magnification ×200. Figure modified from the Original Publication II by Luhtala et al.

FACS analysis showed that HER3 expression was remarkably lower than that of HER2 in breast cancer cell lines, as was compared by MFI values (*unpublished data*, Table 10, p.94). Additionally, HER2 and HER3 expression levels did not show statistically significant correlation (*unpublished data*, Figure 9, p.95). However, these preliminary results are approximate due to technical discrepancies in HER2 and HER3 IF-labellings. HER2 labelling detected both membranous and cytoplasmic receptors (permeabilized cells), while HER3 labelling detected only membranous receptors (unpermeabilized cells).

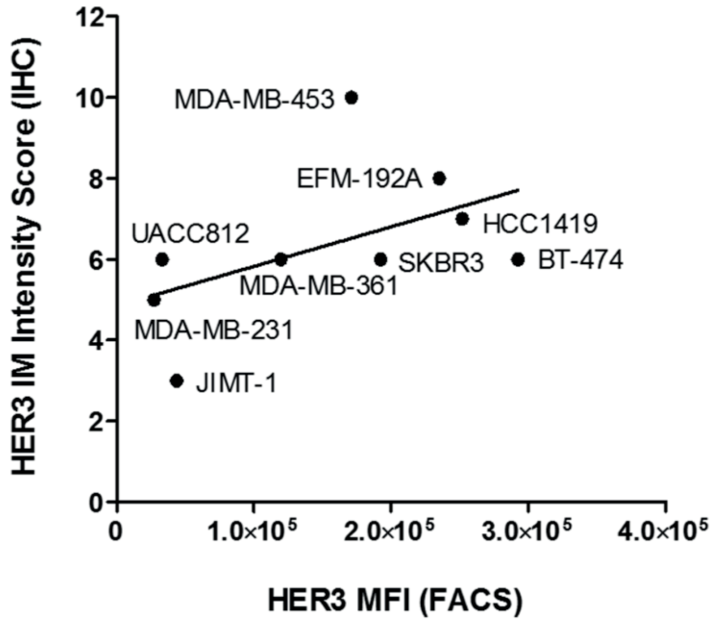

Figure 8. Correlation between HER3-M expression determined by ImmunoMembrane 1.0i DIA method for HER3 IHC (IM Intensity Score, *y-axis*) and by FACS (MFI, *x-axis*) in nine breast cancer cell lines (Pearson's r=0.5028, p=0.1624). MFI=mean fluorescence intensity. Figure modified from the Original Publication II by Luhtala et al.

Table 10. Membranous HER3 (HER3-M) and total cellular HER2 (HER2-T) expression in a panel of breast cancer cell lines quantified by FACS and expressed as mean fluorescence intensity (MFI).

Cell line	HER3 MFI	HER2 MFI
BT-474	2.9×10^5	8.2×10^6
HCC1419	2.5×10^5	10.3×10^6
EFM-192A	2.3×10^5	7.3×10^6
SKBR3	1.9×10^5	4.0×10^6
MDA-MB-453	1.7×10^5	1.5×10^6
MDA-MB-361	1.2×10^5	2.1×10^6
JIMT-1	0.4×10^5	1.1×10^6
UACC-812	0.3×10^5	9.5×10^6
MDA-MB-231	0.3×10^5	0.1×10^6

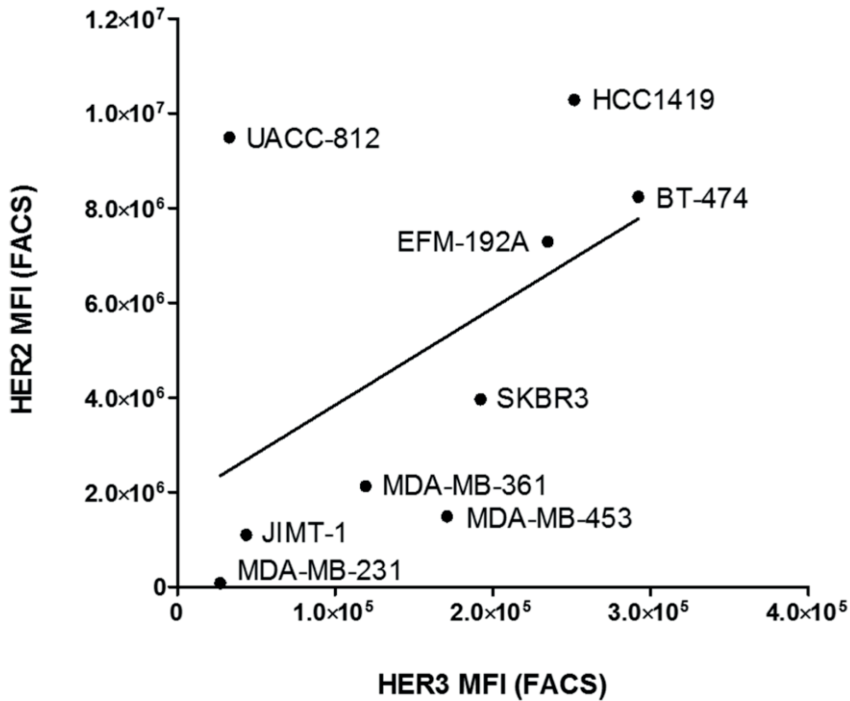

Figure 9. Correlation between HER2 (*y-axis*) and HER3 (*x-axis*) expression determined by FACS (MFI=mean fluorescence intensity) in nine breast cancer cell lines (Pearson's r=0.5217, p=0.1497).

5.3.2 HER3 in clinical breast carcinomas (II, III)

Studies II and **III** indicated that HER3 is frequently expressed in breast carcinomas and is localized in both cell membrane and cytoplasm. Considerable intra-tumoral heterogeneity was observed and therefore samples were consistently analyzed on hot spot region for HER3 status. High HER3-M expression (scored as [1+] or [2+]) was demonstrated in 80.2% of *HER2*-amplified breast carcinomas (Sample Cohort I). Similarly, HER3-C expression was demonstrated in a majority (91.5%) of carcinomas. Totally 75.7% of *HER2*-amplified breast carcinomas were defined positive for HER3-T expression. (Table 11, p.96). High HER3 expression was confirmed in histologically normal prostate and breast glandular epithelium (Figure 10, p.96) that were used as positive control samples in the staining batches. Examples of HER3 IHC staining patterns in breast cancers are shown in Figure 11, p.97.

Table 11. HER3 expression with subcellular localization, as demonstrated by IHC, in *HER2*-amplified breast carcinomas (*n*=177).

	HER3-M *n* (%)	**HER3-C** *n* (%)	**HER3-T** *n* (%)
Low [0]	35 (19.8)	15 (8.5)	
Intermediate [1+]	55 (31.1)	51 (28.8)	
High [2+]	87 (49.1)	111 (62.7)	
Low/negative (total)	35 (19.8)	15 (8.5)	43 (24.3)
High/positive (total)	142 (80.2)	162 (91.5)	134 (75.7)

Study III confirmed high HER3-M expression in a half of carcinomas (51.9%, 160 of 308) representing Sample Cohort II. Almost all carcinomas (95.8%) showed HER3-C expression, and 75.3% of carcinomas were defined positive for HER3-T expression. A subgroup of HER2+ carcinomas (*n*=47) showed similar HER3-T expression profile. Accordingly, Study III showed that HER3 is frequently expressed in breast cancers irrespective of HER2 status.

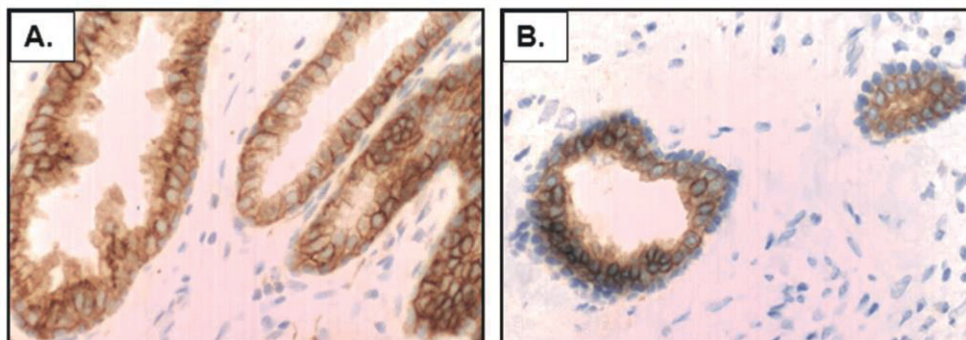

Figure 10. HER3 expression, as demonstrated by IHC (DAK-H3-IC antibody), in prostate glandular epithelium (**A**) and breast luminal epithelial cells (**B**). Mayer's hematoxylin as a counterstain. Magnification ×200. Figure modified from the Original Publications II and III by Luhtala et al.

A small set of positive axillary lymph nodes (FFPE) from breast cancer patients (*n*=18) were stained for HER3. HER3 staining patterns were comparable both in breast tumour and the corresponding lymph node metastasis (Figure 12, p.98). HER3 stainings were comparable also in breast cancer tissues (*n*=13) fixed collaterally with 10% NBF and PAXgene Tissue FIX. Accordingly, fixation method was not shown to considerably influence on preservation of HER3 antigen, although

the staining intensity was slightly more intense in NBF-fixed samples. (Figure 13, p.98).

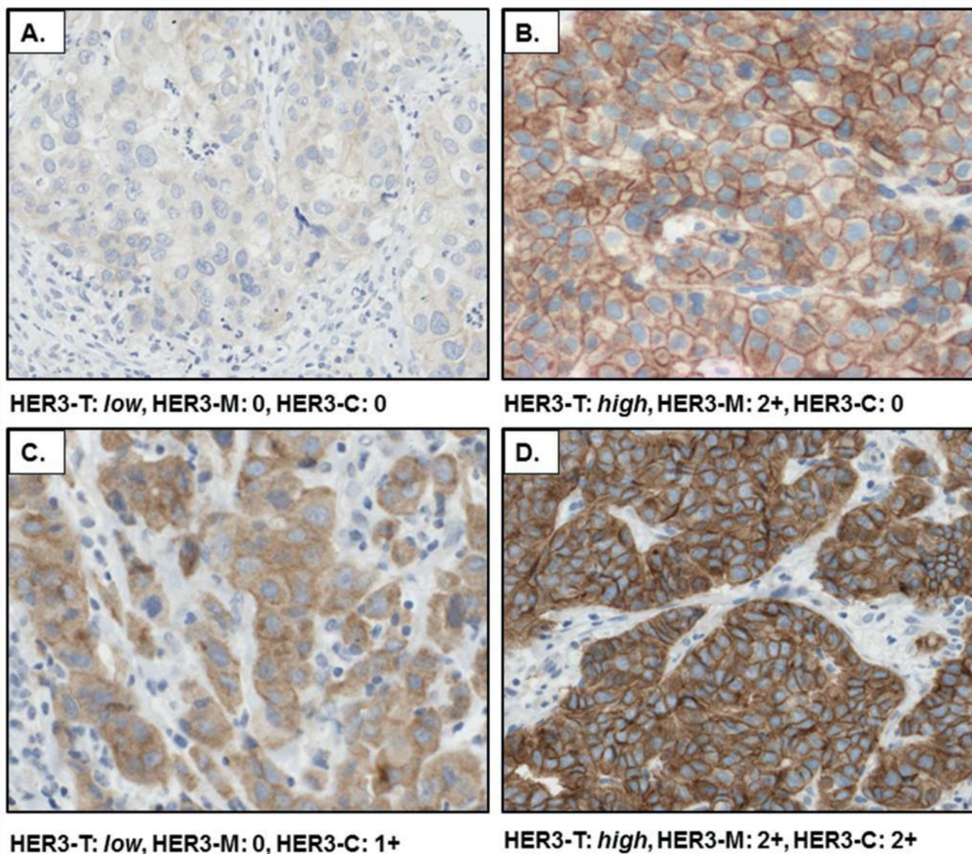

Figure 11. Examples of HER3 expression patterns in breast carcinomas (**A-D**), demonstrated by IHC (DAK-H3-IC antibody). HER3-T=total cellular HER3, HER3-M=membranous HER3, HER3-C=cytoplasmic HER3. Mayer's hematoxylin as a counterstain. Magnification ×200.

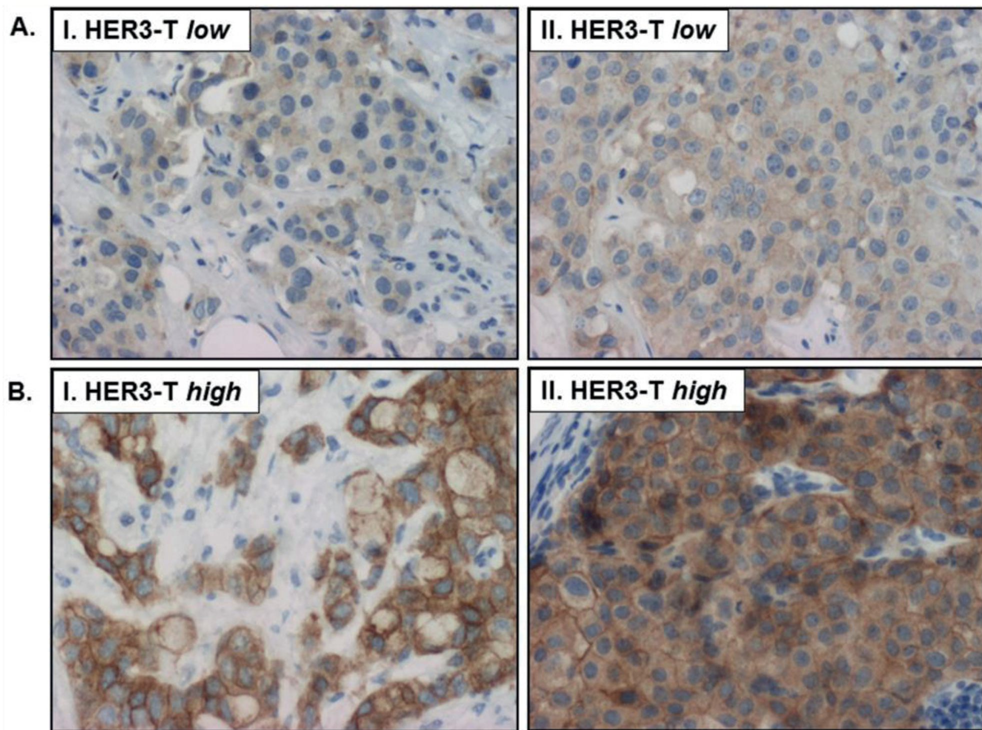

Figure 12. Comparable HER3-T expression pattern in breast carcinoma (**I**) and the corresponding axillary lymph node metastasis (**II**) by IHC (DAK-H3-IC antibody). Low [0] HER3-T (**A**), high [2+] HER3-T (**B**) expression. Magnification ×200.

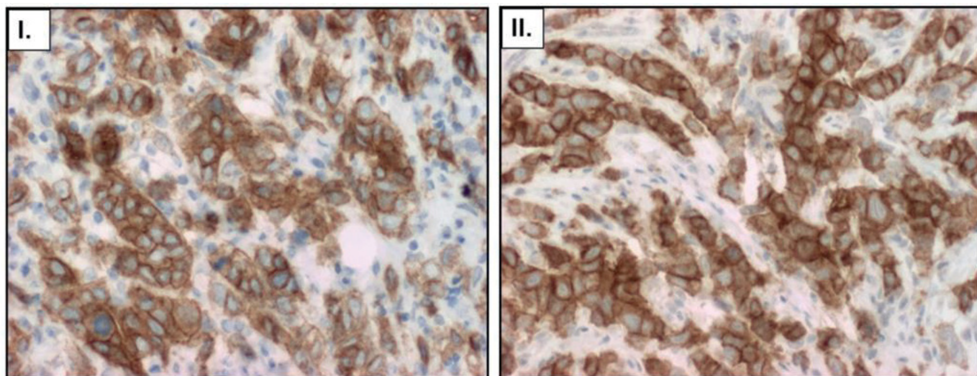

Figure 13. Comparable HER3 expression pattern in breast cancer tissue doublet fixed with 10% NBF (**I**) and PAXgene Tissue FIX (**II**). High [2+] HER3-T expression. Magnification ×200.

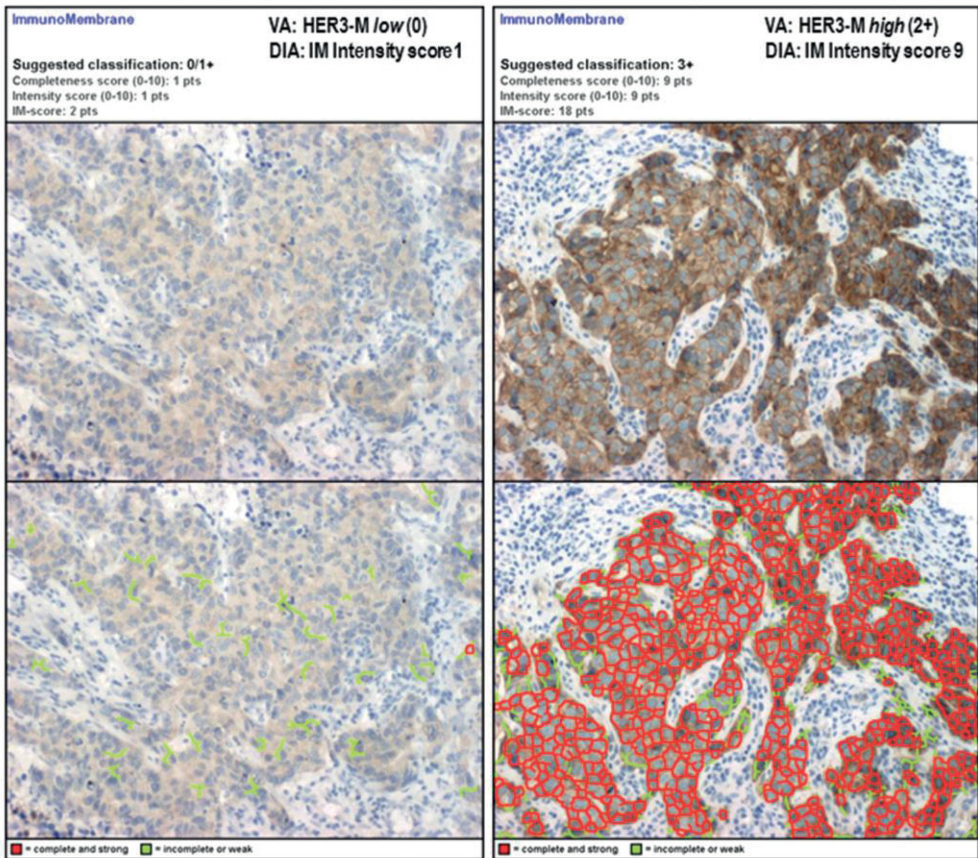

Figure 14. ImmunoMembrane 1.0i software (Jilab Inc., Tampere, Finland) for HER3-M expression analysis. **Left:** Breast carcinoma exhibiting low [0] HER3-M (VA) *vs* IM Intensity Score 1 pts (DIA). **Right:** Breast carcinoma exhibiting high [2+] HER3-M (VA) *vs* IM Intensity Score 9 pts (DIA). Red colour marks complete and strong circumferential HER3 membrane staining and green colour incomplete or weak membranous staining pattern.

5.4 NEDD4-1 and NRDP1 expression (III)

Study III demonstrated the predominance of NEDD4-1 expression in *HER2*-amplified breast carcinomas (Sample Cohort I). Positive staining was observed also in the adjacent histologically normal breast epithelium. Majority of carcinomas (82.8%, 120 of 145) showed intense [3+] cytoplasmic staining and were considered to over-express NEDD4-1 protein. Nuclear NEDD4-staining was not detected. No intra-tumoral variation was observed in the staining patterns (Figure 15).

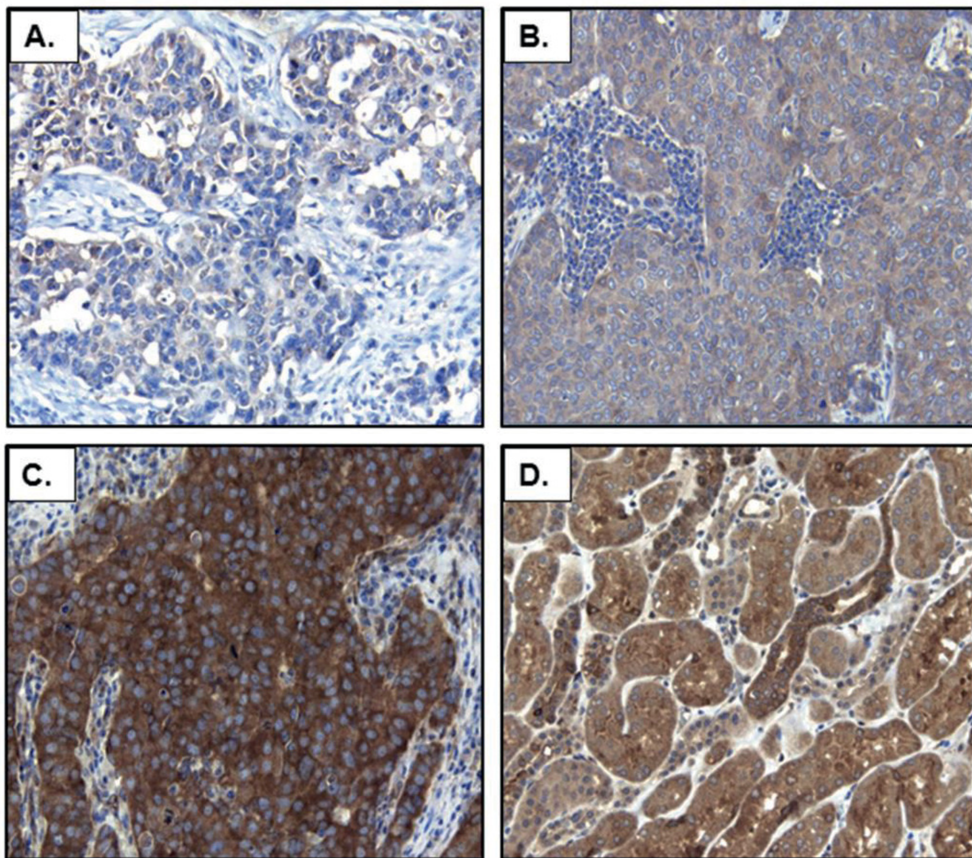

Figure 15. NEDD4-1 expression demonstrated by IHC. Breast carcinomas displayed low (**A**), moderate (**B**) and strong (**C**) cytoplasmically localized NEDD4-1 staining. Kidney (**D**) was used as a positive control. Figure modified from the Original Publication **III** by Luhtala et al.

Contrary to NEDD4-1, only a small fraction (8.3%, 12 of 145) of *HER2*-amplified breast carcinomas (Sample Cohort I) was characterized with NRDP1 protein expression. Highly expressed NRDP1 was localized either in nucleus (NRDP1-N, score ≥4) or cytoplasm (NRDP1-C, score ≥2), as shown in Figure 16.

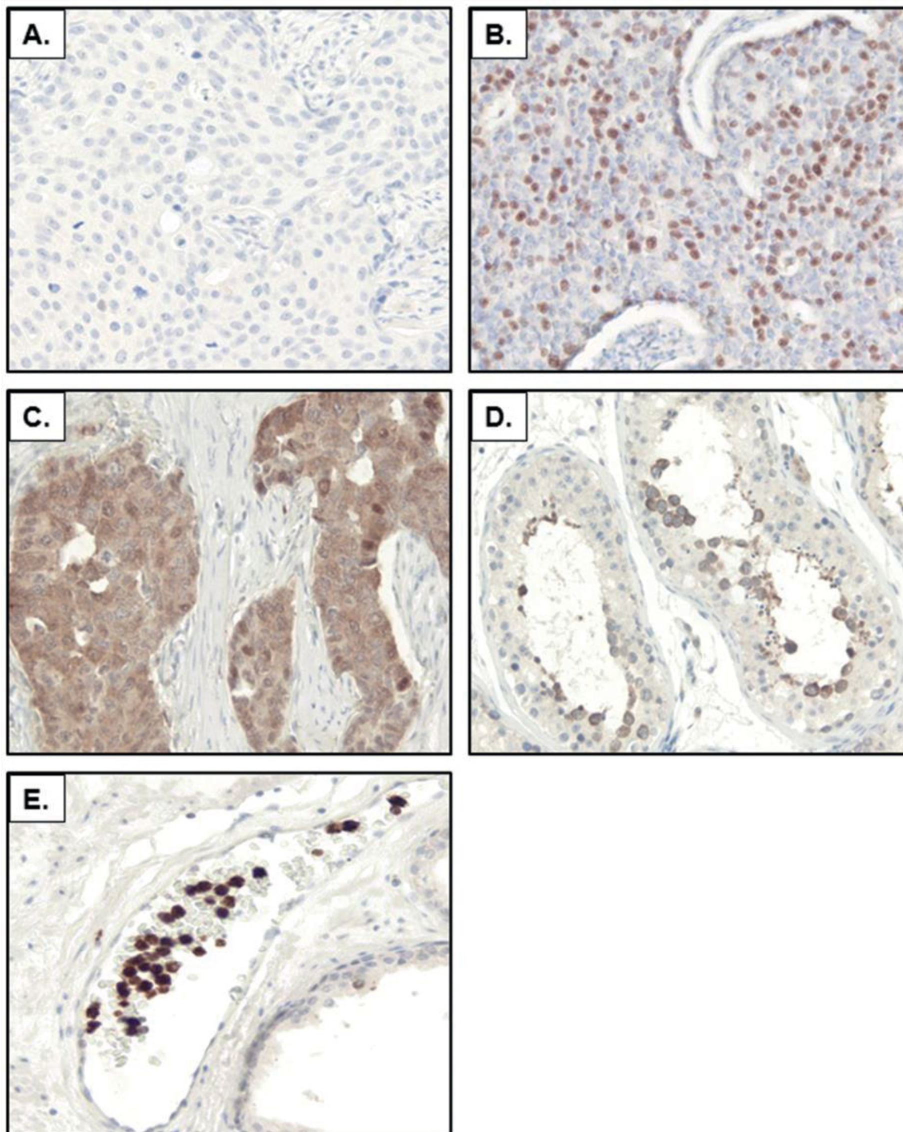

Figure 16. NRDP1 expression demonstrated by IHC. Breast carcinomas displayed low/absent staining (**A**), nuclear (**B**) or cytoplasmic (**C**) NRDP1 expression. Testicular cells in the seminiferous ducts (**D**) and white blood cells (**E**) were used as positive controls. Figure modified from the Original Publication III by Luhtala et al.

5.5 MCM2 expression (IV)

A novel fluoro-chromogenic double labelling IHC and DIA with ImmunoRatio 2.5 were used in **Study IV** for the assessment of MCM2 expression in breast cancers. This technique was shown to be feasible and accurate in the determination of cell proliferation activity (Figure 17, p.103 & Figure 18, p.104). Excellent inter-rater consistency was shown when MCM2-LI was determined independently by two raters using ImmunoRatio 2.5 on WSI self-defined ROIs (Figure 19, p.105).

Study IV confirmed strong MCM2 protein expression in *HER2*-amplified breast carcinomas (Sample Cohort I). Only few cells in histologically normal breast glandular epithelium were demonstrated to display MCM2 expression in contrast to cancerous areas and DCIS. MCM2 was shown to localize predominantly in cell nuclei, but singular cells at mitotic karyokinesis displayed only cytoplasmic MCM2. MCM2 expression was shown to vary inter-tumorally, and the staining pattern was mostly heterogeneous which for samples were consistently analyzed focusing on the hot spot regions. In general, the most intense staining reaction was present in the tumour boundary areas rich of actively proliferating cells. MCM2 expression (median LI 63.5%) was considerably more frequent than that of Ki-67 (median LI 33.0%) and cyclin E1 (median LI 45%) in *HER2*-amplified breast carcinomas (Figure 20, p.105). Mean MCM2-LIs were 43% and 66% in carcinomas displaying either low or high Ki-67 expression, respectively.

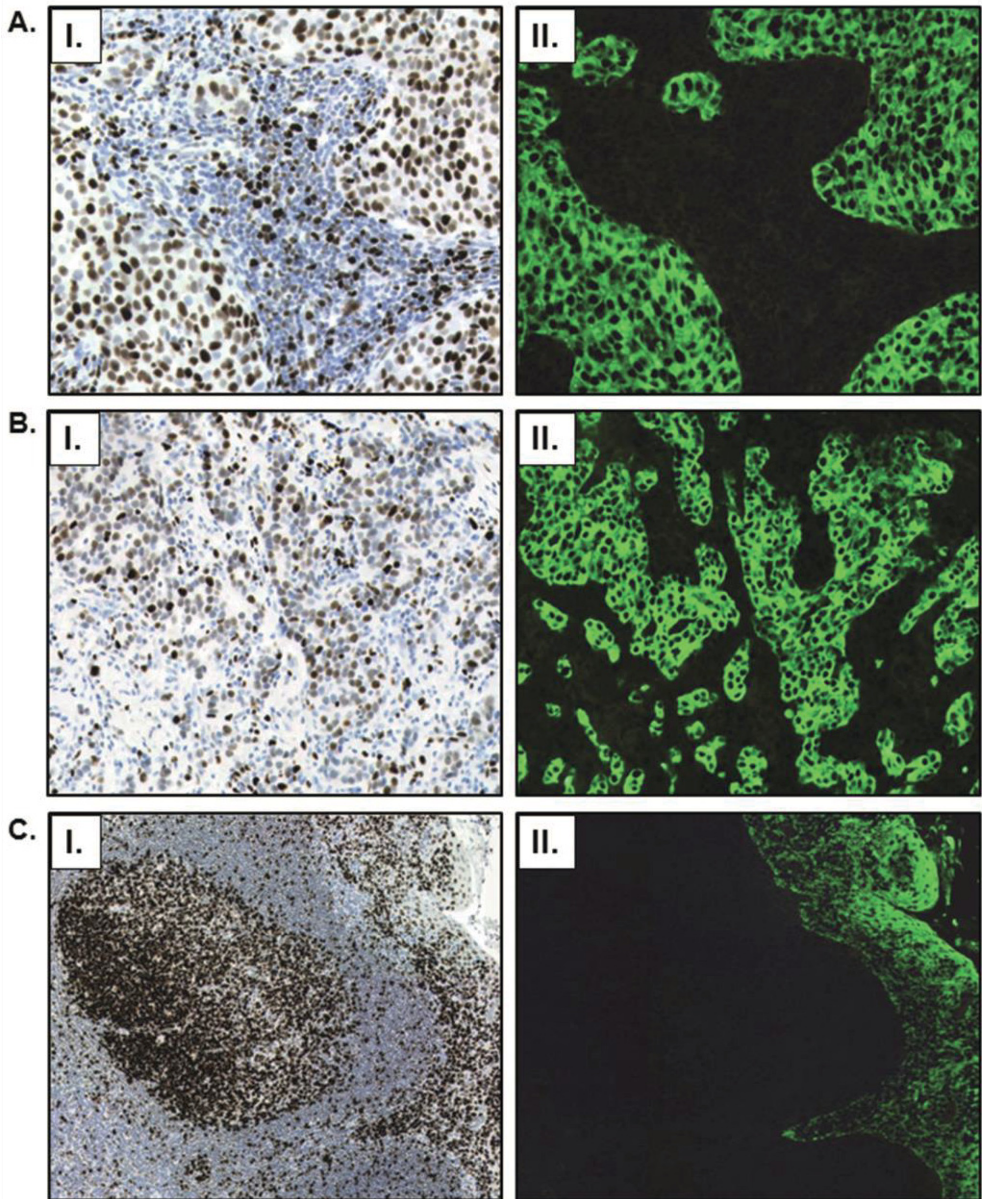

Figure 17. Fluoro-chromogenic double labelling for demonstration of proliferative MCM2-expressing cells (brown DAB precipitate in the cell nuclei) and CK-expressing epithelial cells (green fluorescence by Cy2) in breast carcinomas (**A, B**) and in tonsil control tissue (**C**). Distinct from cancer cells, non-epithelial cells in the adjacent stroma and TAICs do not show CK expression (absent fluorescence signal). Blue counterstaining with Mayer's hematoxylin. Bright field (**I**) and fluorescence (**II**) illumination. Figure modified from the Original Publication IV by Luhtala et al.

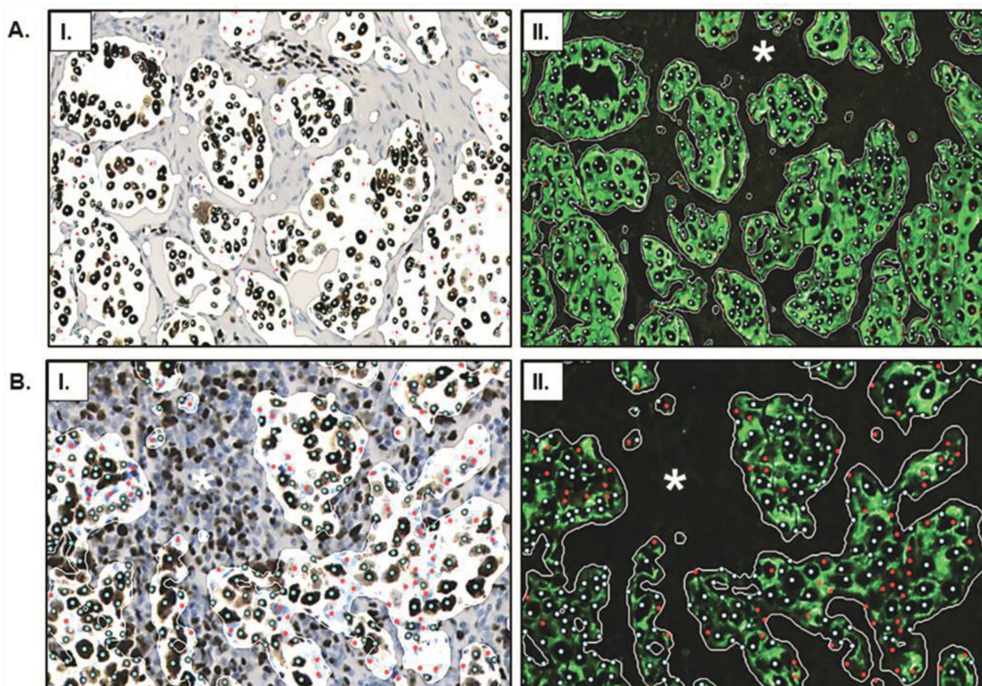

Figure 18. ImmunoRatio 2.5 with IF-correction for semi-automatic determination of cell proliferation activity by MCM2 expression in breast cancers (**A**, **B**) stained with fluoro-chromogenic double labelling for MCM2 and CK. In practice, the corresponding WSIs acquired under bright field (MCM2, **I**) and fluorescence illumination (CK, **II**) were virtually stacked into double-layer WSI in which the analyzable region (ROI) is marked with drawing tool. Using the IF correction, only immunofluorescent CK+ cancer cells (circumscribed cell populations) were counted for MCM2-LI. Normal adjacent stromal cells and TAICs (areas marked with *) were discarded from the analysis on basis of absent fluorescence signal. ImmunoRatio 2.5 marks CK-expressing MCM2+ cells (brown DAB in nuclei) with green dots and MCM2- cells (blue hematoxylin stain in nuclei) with red dots. Figure modified from the Original Publication IV by Luhtala et al.

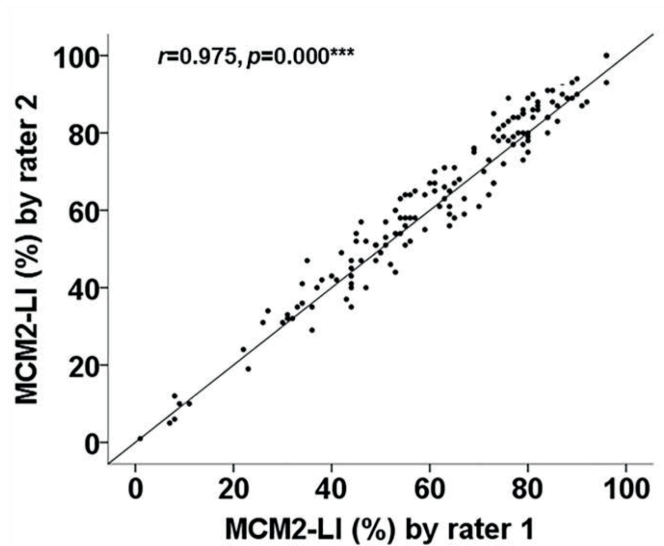

Figure 19. Excellent inter-rater consistency was found when MCM2-LI was determined with DIA tool (ImmunoRatio 2.5 with IF correction) in MCM2-CK labelled breast carcinomas (*n*=142) by two independent raters. Statistically significant concordance between the measurements was confirmed by Pearson's Correlation test (*r*=0.975, *p*=0.000), Spearman's rho test (R_S=0.968, *p*=0.000) and Intra Class Correlation test (coefficient=0.986).

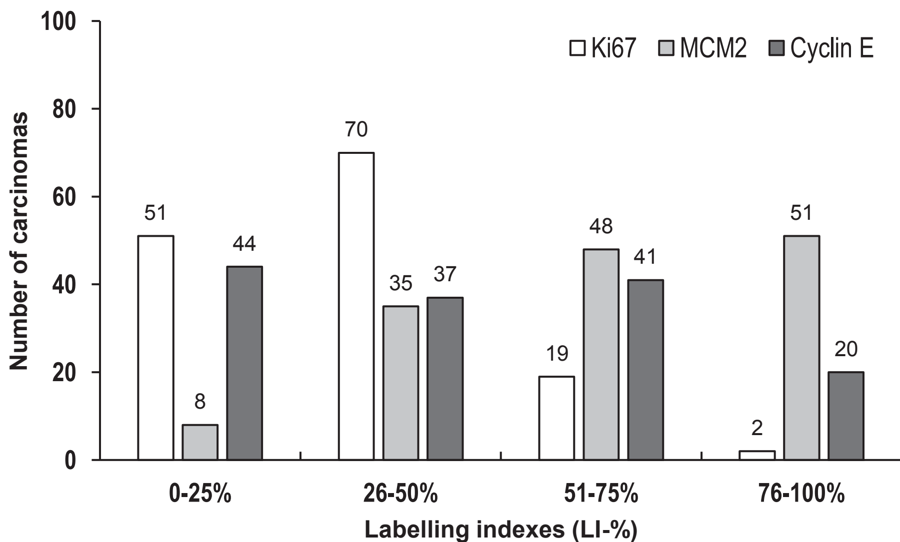

Figure 20. MCM2 expression in relation to Ki-67 and cyclin E1 in *HER2*-amplified breast carcinomas (*n*=142). Median LIs were 63.5% (ranging from 1 to 98%) for MCM2, 33.0% (ranging from 5 to 82%) for Ki-67, and 45% (ranging from 2 to 97%) for cyclin E1.

5.6 Histopathological associations (I, III, IV)

5.6.1 Associations with cyclin E1 and *CCNE1* amplification (I)

Study I showed that high cyclin E1 expression is associated with clinicopathological features that typically define more aggressive breast cancer type. In a group of primary M0 carcinomas (*n*=193, Sample Cohort I), high cyclin E1 was significantly associated with HR negativity, high Ki67-LI, high histological grade, CK5/14 expression, basal-like phenotype, large tumour size and young patient age (Table 12, p.107 & Figure 21, p.108). Results in the whole Sample Cohort I (*n*=202, M1 cases included) were similar. Compared to Ki-67, cyclin E1 was confirmed non-inferior biomarker for defining aggressive *HER2*-amplified breast cancer subtypes according to its associations with diagnostic histopathological parameters. Study I demonstrated also statistically significant associations between *CCNE1* amplification, high cyclin E1 and Ki67 expression levels and PR negativity (p=0.009) in *HER2*-amplified breast carcinomas (Sample Cohort I). (Figure 21, p.108).

Table 12. Clinicopathological characteristics of *HER2*-amplified primary M0 breast cancers (*n*=193, Sample Cohort I) in relation to cyclin E1 expression level. *p*-values from Pearson's Chi-Square test. Number of carcinomas with available data is marked in the column *n*.

Clinicopathological characteristic	***n***	**Cyclin E1 low (<50%)** *n* (%)	**Cyclin E1 high (≥50%)** *n* (%)	***p*-value**
Age	193			0.018*
<50 years		31 (79.5)	8 (20.5)	
≥50 years		91 (59.1)	63 (40.9)	
ER status	193			0.000***
Negative (<10%)		31 (45.6)	37 (54.4)	
Positive (≥10%)		91 (72.8)	34 (27.2)	
PR status	193			0.002**
Negative (<10%)		61 (54.0)	52 (46.0)	
Positive (≥10%)		61 (76.3)	19 (23.7)	
Ki-67 proliferation index	193			0.002**
Low (<20%)		33 (84.6)	6 (15.4)	
High (≥20%)		89 (57.8)	65 (42.2)	
Histological type	184			ns
Ductal		106 (61.6)	66 (38.4)	
Lobular		9 (75.0)	3 (25.0)	
Histological grade	190			0.000***
I-II		39 (84.8)	7 (15.2)	
III		80 (55.6)	64 (44.4)	
Tumour size (cm)	188			ns
<2 cm		53 (67.9)	25 (32.1)	
≥2 cm		46 (59.7)	31 (40.3)	
Tumour size (pT)	187			ns
pT1-pT2		111 (63.4)	64 (36.6)	
pT3-pT4		6 (50.0)	6 (50.0)	
Lymph nodal status	185			ns
Negative (pN0)		62 (57.9)	45 (42.1)	
Positive (pN+)		54 (69.2)	24 (30.8)	
CCNE1 status	177			0.003**
Normal (CCNE1-)		107 (65.2)	57 (34.8)	
Amplified (CCNE1+)		3 (23.1)	10 (76.9)	
CK5/14 expression	167			0.004**
Negative (CK5/14-)		93 (63.3)	54 (36.7)	
Positive (CK5/14+)		6 (30.0)	14 (70.0)	
Basal phenotype	167			0.032*
Non-basal		94 (61.8)	58 (38.2)	
Basal (CK5/14+ ER-)		5 (33.3)	10 (66.7)	

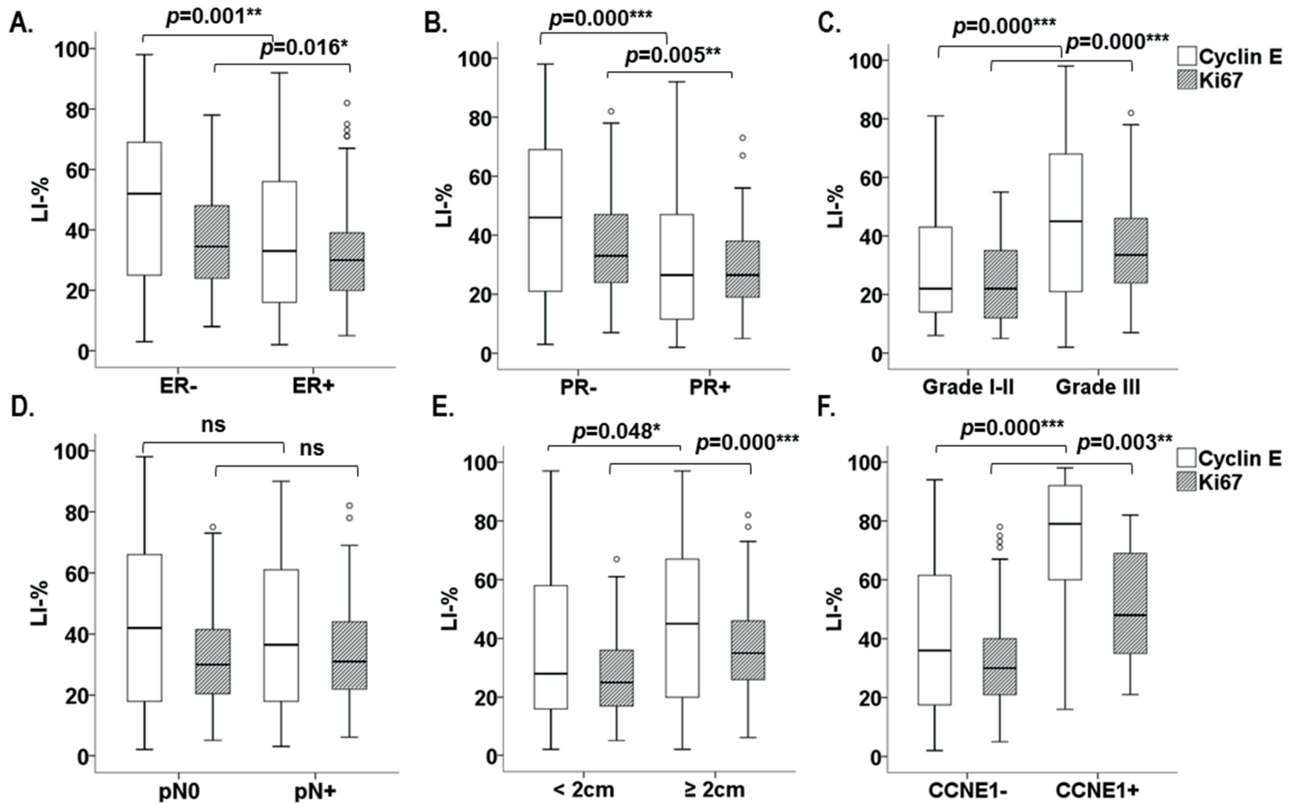

Figure 21. Associations between cyclin E1 and Ki-67 expression and histopathological characteristics in *HER2*-amplified primary M0 breast carcinomas for HR status; **A.** ER (n=193) and **B.** PR (n=193), **C.** histological grade (n=190), **D.** lymph node status (n=185), **E.** tumour size (n=155), and **F.** *CCNE1* amplification status (n=177). p-values from Mann-Whitney U test. Lines within the columns correspond to median LI-%, boxes to inter-quartile range, and bars to overall range. Outlying values are marked by circles (º).

5.6.2 Associations with HER3 (III)

Study III demonstrated that absent/low HER3 protein expression associated significantly with histopathological features specifying aggressive and therapeutically unfavourable breast cancer type. Low HER3 was related to large tumour size, positive lymph nodal status, ER negativity, TNBC subtype, and basal cell origin, depending on how the HER3 status was defined (HER3-M, HER3-C, HER3-T).

HER3 associations in Sample Cohort I

In *HER2*-amplified breast cancer group, low HER3-M associated with ER negativity and low HER3-C with large tumour size, young age at diagnosis (p=0.000) and premenopausal status (p=0.000). Low HER3-T was found to significantly associate with axillary lymph node invasion, CK 5/14 expression, and basal-like phenotype. (Figure 22, p.110).

HER3 associations in Sample Cohort II

In Sample Cohort II, low HER3-M was significantly related to TNBC subtype (p=0.000), PR negativity (p=0.002) and large tumour size (≥2 cm p=0.003). HER3 was not shown to significantly associate with HER2 status, irrespective of its subcellular localization (HER3-M p=0.615, HER3-C p=0.990) or total cellular expression (HER3-T p=0.882). In a subgroup of HER2- breast carcinomas (n=261), low HER3-M was more common in carcinomas characterized with HR negativity (p=0.003 for ER, p=0.002 for PR), grade III (p=0.008) and tumour size ≥2 cm (p=0.006). HER3-C and HER3-T did not show statistically significant associations with clinicopathological characteristics. HER2+ subgroup (n=47) was not separately analyzed as was considered unrepresentative with respect to the number of cases.

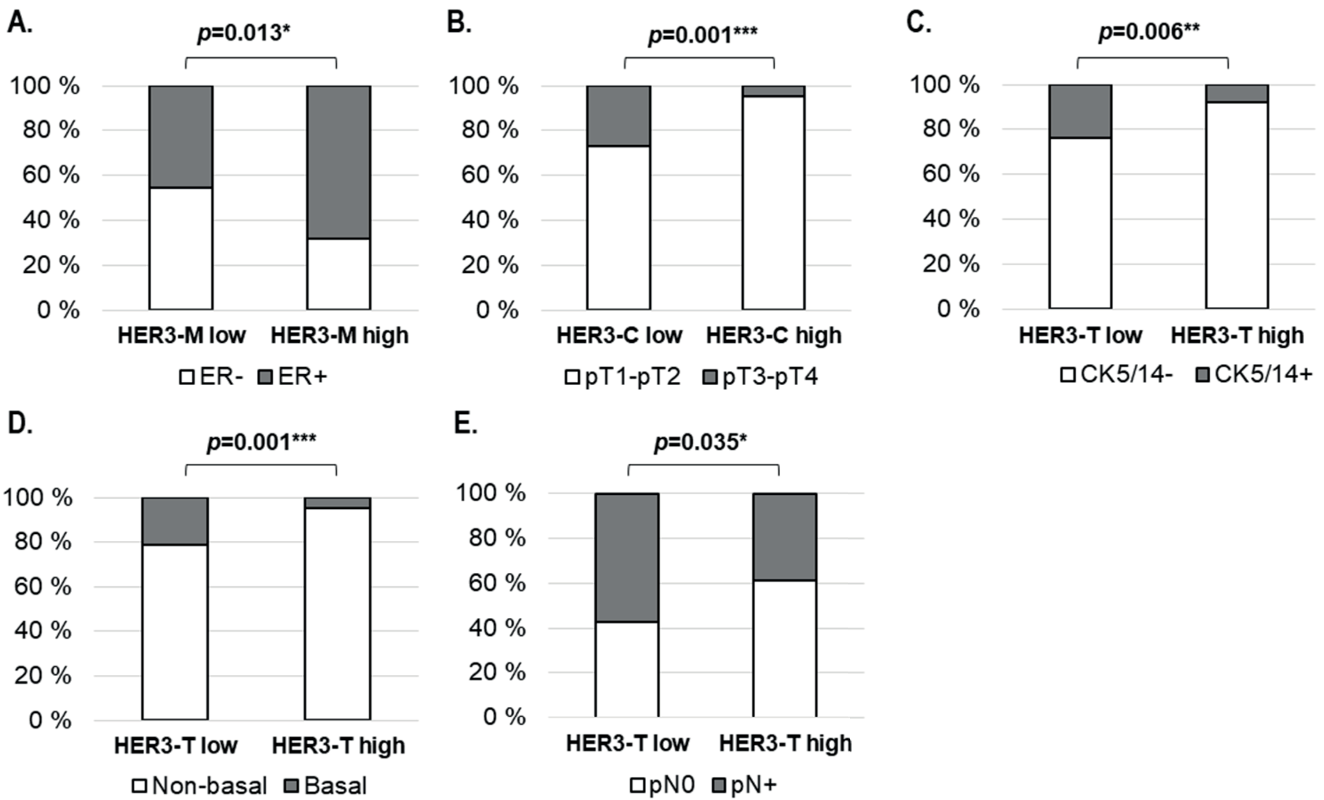

Figure 22. Statistically significant associations between HER3 expression (M=membranous, C=cytoplasmic, T=total) and histopathological characteristics in *HER2*-amplified primary breast carcinomas (n=177) for **A.** ER status (n=177), **B.** tumour size (n=172), **C.** cytokeratin 5/14 expression (n=156), **D.** basal-like phenotype (n=156), and **E.** lymph nodal status (n=169). p-values from Pearson Chi-Square test.

5.6.3 Associations with NEDD4-1 and NRDP1 (III)

Study III demonstrated clinicopathological associations of NEDD4-1 and NRDP1 proteins in *HER2*-amplified primary breast cancer (Sample Cohort I). High NEDD4-1 expression associated significantly with high HER3-M expression (p=0.002); even 87.4% (104 of 119) of carcinomas displayed concurrent NEDD4-1 and HER3-M over-expression. Low NRDP1-N expression was related to high patient age (p=0.004), and high NRDP1-C to positive PR status (p=0.006) and high HER3-T expression (p=0.041). NEDD4-1 and NRDP1 levels did not show correlation. Based on Study III, NEDD4-1 and NRDP1 were not considered useful in subcatecorizing *HER2*-amplified breast carcinomas in a clinically relevant manner.

5.6.4 Associations with MCM2 (IV)

Study IV demonstrated statistically significant parallel correlation between MCM2, Ki-67 (Figure 23, p.112) and cyclin E1 (Figure 24, p.112) expression in *HER2*-amplified breast carcinomas (Sample Cohort I). MCM2 expression was more frequent than that of Ki-67 and cyclin E1. Median LIs for MCM2, Ki-67 and cyclin E1 were 63.5%, 33% and 45%, respectively. High MCM2 was shown to significantly associate with HR negativity, high histological grade, and *CCNE1* amplification (p=0.036). MCM2 did not associate with lymph nodal status or tumour size. MCM2 over-expressing breast carcinomas were often positive for CK5/14 expression (p=0.025) but did not represent true basal-like phenotype. Compared to Ki-67, Study IV confirmed that MCM2 is non-inferior biomarker for defining more aggressive *HER2*-amplified breast cancer subtypes according to its associations with diagnostic histopathological parameters (Figure 25, p.113).

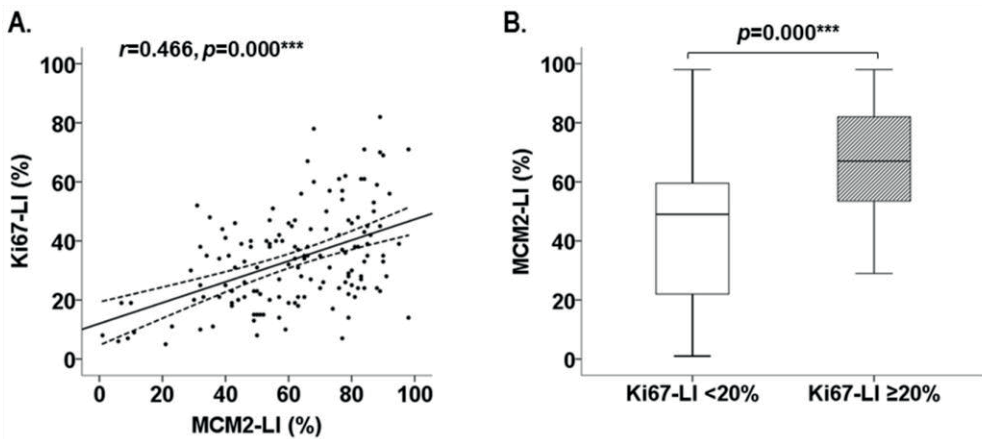

Figure 23. **A.** Scatter-plot showing statistically significant correlation between MCM2 and Ki67 labelling indeces in *HER2*-amplified breast carcinomas (*n*=142), Pearson's test *r*=0.466, *p*=0.000. Mean LI (-) with 95% CI (- -) is marked in the plot. **B.** Association between MCM2-LI and Ki67-LI when 20% cut-off was used to determine low and high Ki67 expression, *p*-value from Mann-Whitney *U* test. Lines within the columns correspond to median MCM2-LI, boxes to inter-quartile range, and bars to overall range. Figure modified from the Original Publication IV by Luhtala et al.

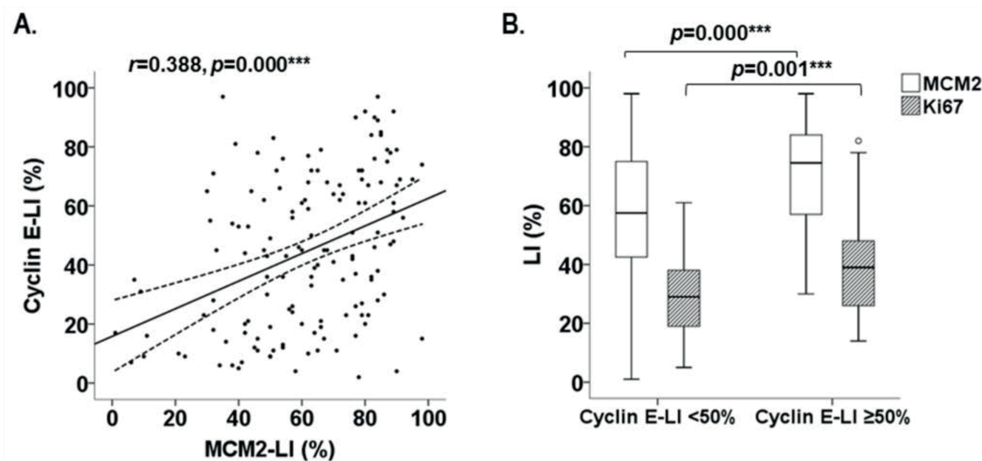

Figure 24. **A.** Scatter-plot showing statistically significant correlation between MCM2 and cyclin E1 labelling indeces in *HER2*-amplified breast carcinomas (*n*=142), Pearson's test *r*=0.388, *p*=0.000. Mean LI (-) with 95% CI (- -) is marked in the plot. **B.** Associations between MCM2-LI, Ki67-LI and cyclin E1-LI when 50% cut-off was used to determine low and high cyclin E1 expression level, *p*-values from Mann-Whitney *U* test. Lines within the columns correspond to median LI-%, boxes to inter-quartile range, and bars to overall range. Outlying value is marked by circle (º). Figure modified from the Original Publication IV by Luhtala et al.

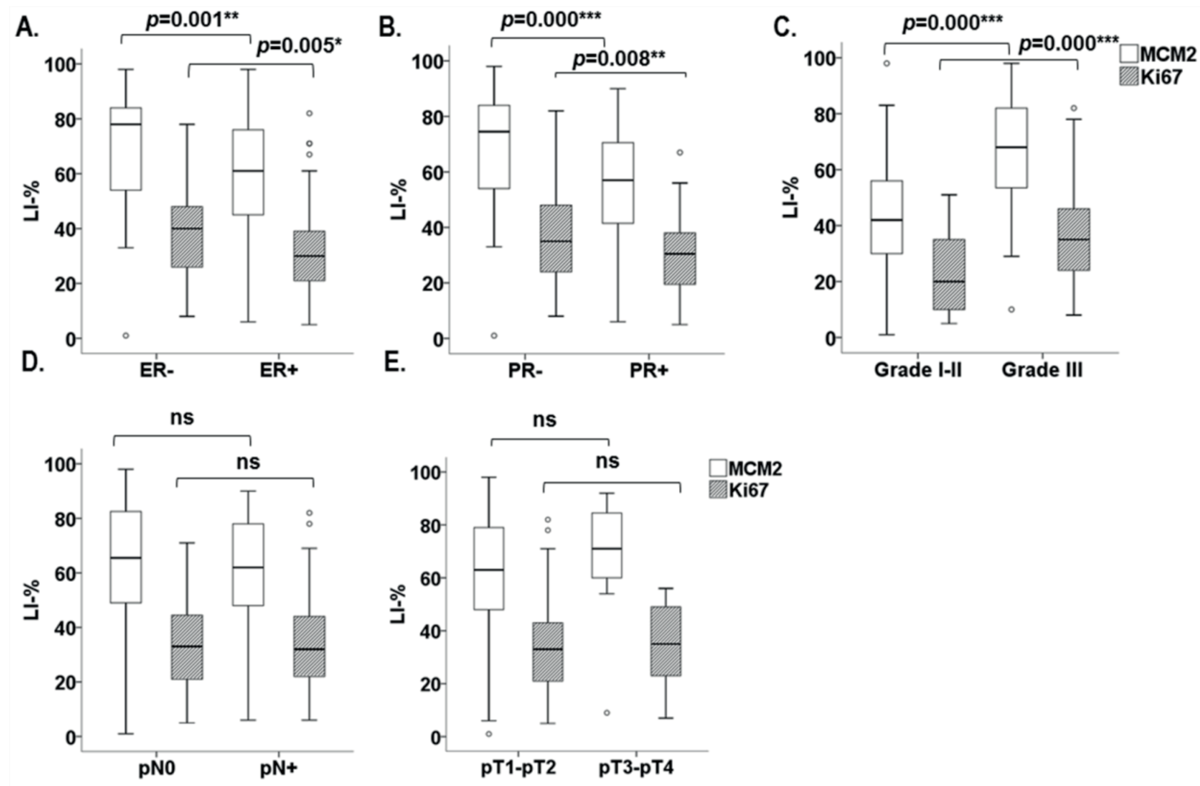

Figure 25. Associations between MCM2-LI, Ki67-LI and histopathological characteristics in *HER2*-amplified primary M0 breast carcinomas (*n*=142) for HR status; **A.** ER (*n*=142) and **B.** PR (*n*=142), **C.** histological grade (*n*=141), **D.** lymph nodal status (*n*=137), and **E.** tumour size pT (*n*=140). *p*-values from Mann-Whitney *U* test. Lines within the columns correspond to median LI-%, boxes to inter-quartile range, and bars to overall range. Outlying values are marked by circles (º).

5.7 Prognostic and predictive implications (I, III, IV)

Studies I, III and IV aimed at clarifying the clinical relevance of selected markers in predicting the first breast cancer recurrence. In *HER2*-amplified primary breast cancer group (Sample Cohort I), 38 recurrences were diagnosed during the mean follow-up of 5.3 years. The recurrence rate was 19.7% (*n*=38 of 193). Of these, 15 were local and 23 distantly localized metastases. Mean RFS for relapsed patients was 2.3 years (range: 1 month to 5.8 years). 9-wk adjuvant trastuzumab therapy did not significantly associate with RFS (*p*=0.573). Mean RFS was 6.2 years (95% CI 5.8-6.6 years) for adjuvant trastuzumab-treated (Adj-T) patients, and 7.5 years (95% CI 6.9-8.1 years) for patients who were not given trastuzumab. The recurrence rate was slightly lower in Adj-T group (17.2%, *n*=15 of 87) in comparison with non-trastuzumab treated group (21.7%, *n*=23 of 106). The proportion of ER- breast carcinomas (*n*=40 of 68) was higher in Adj-T group. Breast cancer patients with family history of breast or ovarian carcinoma (not confirmed as hereditary disease) were also mainly included in the AdjT-group (*n*=13 of 16). In Sample Cohort II, totally 112 disease-specific recurrences were diagnosed during the long-lasting follow-up period (mean 10.4 years). The recurrence rate was 36.4% (*n*=112 of 308). Mean RFS for patients with recurrent HER2+ carcinoma (*n*=14 of 47) was 1.7 years (range: 1 month to 5.5 years), and 5.3 years (range: 1 month to 19.3 years) for those having recurrent HER2- cancer (*n*=98 of 261). Results of this study confirm early-onset relapsing pattern in HER2+breast carcinomas.

5.7.1 Cyclin E1 expression and *CCNE1* amplification (I)

In **Study I**, neither cyclin E1 expression level (*p*=0.490) nor *CCNE1* amplification status (*p*=0.243) was confirmed predictive of early recurrence in *HER2*-amplified breast cancer type (Sample Cohort I). Cyclin E1 was studied in a group of 193 M0 breast cancer patients (mean follow-up 5.3 years), of whom 38 experienced recurrence. In a group of 177 M0 breast cancers with *CCNE1* amplification status, 34 disease recurrences appeared during the follow-up. Kaplan-Meier analysis with Mantel-Cox log-rank test did not confirm survival differences in adjuvant trastuzumab treated and untreated patient groups stratified for cyclin E1 expression level (*p*=0.336 *vs* *p*=0.933, respectively) or *CCNE1* amplification status (*p*=0.718 *vs* *p*=0.283).

5.7.2 HER3 expression (III)

Prognostic value of HER3 in Sample Cohort I

In **Study III**, the prognostic role of HER3 was clarified in a group of 177 *HER2*-amplified breast cancer patients during the mean follow-up of 5.3 years. Totally 36 patients experienced disease-specific recurrence. Kaplan-Meier analysis with univariate Mantel-Cox log-rank test confirmed that low HER3-T expression is a strong indicator of reduced RFS in *HER2*-amplified breast cancer (Figure 26A, p.116). Almost half (41.7%, 15 of 36) of relapsed carcinomas were characterized with low HER3-T. Estimated mean RFS periods for patients having HER3-T low *vs* high carcinomas were 6.3 years and 8.0 years, respectively (Table 13, p.118). HER3-T was tested also for its prognostic relevance in Multivariate Cox regression analysis with tumour size and axillary lymph node status. All these three variables were demonstrated to independently predict reduced survival in *HER2*-amplified breast cancers. Low HER3-T was associated with 2.3-fold risk of breast cancer recurrence, while lymph nodal involvement and large tumour size related to 3.5-fold and four-fold relapsing risk, respectively. Low HER3-M and HER3-C expression levels were also shown to significantly predict disease recurrence (Figure 26B-C, p.116 & Table 13, p.118). One third (33.3%, 12 of 36) of relapsing carcinomas displayed low HER3-M and one fifth (19.4%, 7 of 36) low HER3-C expression. HER3-T was not shown to predict the effect of 9-wk adjuvant trastuzumab therapy. When survival analyses were performed separately in adjuvant trastuzumab treated and untreated groups, high HER3-T was significantly associated with reduced RFS only in patients treated without trastuzumab therapy (p=0.073 vs p=0.028, respectively).

Prognostic value of HER3 in Sample Cohort II

Study III clarified the prognostic role of HER3 also in Sample Cohort II. During the mean follow-up of 10.4 years, totally 36.4% (n=112 of 308) of patients experienced disease recurrence. According to Kaplan-Meir analysis performed with Mantel-Cox log-rank test, HER3 was not found to significantly associate with RFS. The result was confirmed in both HER2- and HER2+ breast cancer subgroups, but the number of HER2+ carcinomas (n=47) was considered too small for survival analysis. In HER2- subgroup, HER3 was not shown to predict disease recurrence, irrespective of its subcellular localization (p=0.277 for HER3-T, p=0.678 for HER3-M, p=0.400 for HER3-C).

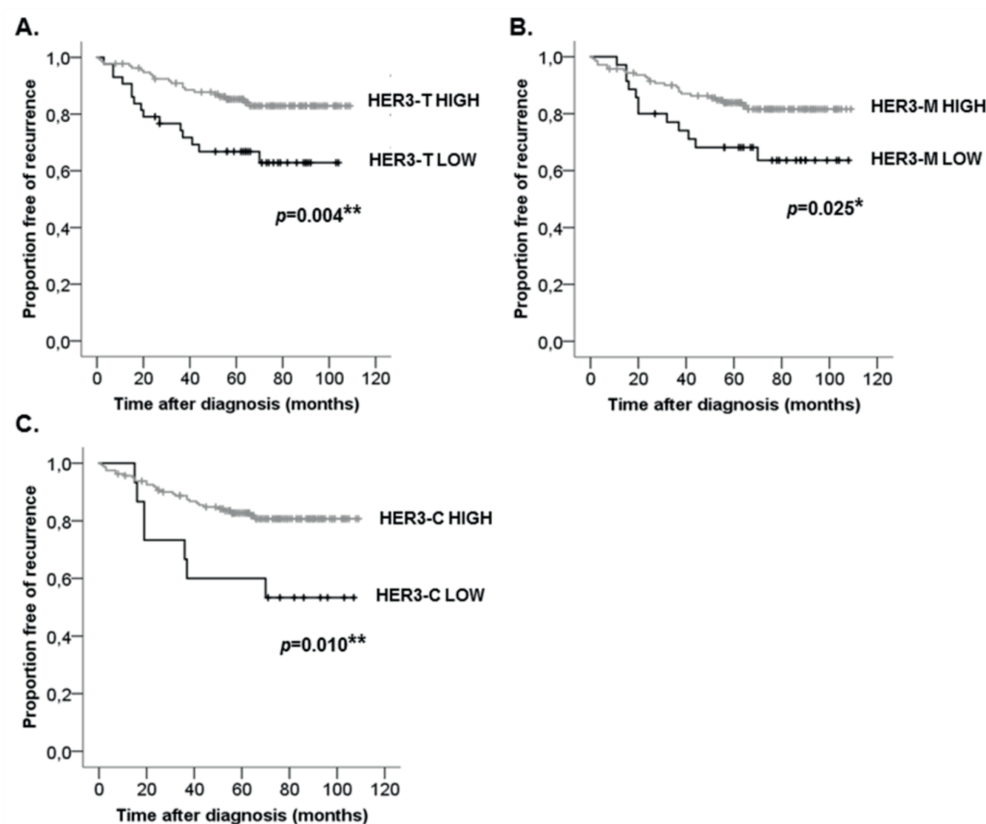

Figure 26. Kaplan-Meier survival curves for patient groups stratified for **A.** total cellular HER3 (HER3-T), **B.** membranous HER3 (HER3-M), and **C.** cytoplasmic HER3 (HER3-C) expression in *HER2*-amplified primary breast carcinomas (*n*=177, Sample Cohort I). *p*-values from univariate Mantel-Cox log-rank test. Figure modified from the Original Publication III by Luhtala et al.

5.7.3 NEDD4-1 and NRDP1 expression (III)

In **Study III**, the prognostic roles of NEDD4-1 and NRDP1 were studied in a group of 145 *HER2*-amplified breast carcinomas (Sample Cohort I). During the mean follow-up of 5.3 years, totally 29 patients experienced relapse. NEDD4-1 expression was not shown to significantly associate with patients' RFS (*p*=0.261). Survival differences were neither seen when Kaplan-Meier analysis was performed separately in adjuvant trastuzumab treated (*p*=0.962) and untreated (*p*=0.267) patient groups. Neither NRDP1-N (*p*=0.689) nor NRDP1-C (*p*=0.711) expression was

demonstrated predictive of breast cancer recurrence, irrespective of adjuvant trastuzumab therapy.

5.7.4 MCM2 and Ki-67 expression (IV)

In **Study IV**, the prognostic role of MCM2 was studied in a group of 142 *HER2*-amplified breast cancers (Sample Cohort I). During the mean follow-up of 5.3 years, totally 28 patients experienced disease recurrence. MCM2-LI was not shown to be clinically applicable biomarker to distinguish recurrent carcinomas from non-recurrent ones (AUC=0.540 ≈0.500, ROC analysis). By applying 75% cut-off for MCM2-LI, assay specificity and sensitivity for predicting the recurrence were 66% and 43%, respectively. Kaplan-Meier analysis performed with 75% cut-off showed that MCM2 expression does not significantly associate with RFS (p=0.331), irrespective of adjuvant trastuzumab therapy. Instead, Ki-67 was demonstrated to significantly predict recurrence when 34% was used as a cut-off to define low and high expression level (Figure 27 below) in *HER2*-amplified breast cancers. Survival differences were not statistically significant when Kaplan-Meier analysis was performed using generally applied 20% as a cut-off to define Ki-67 expression level (p=0.478).

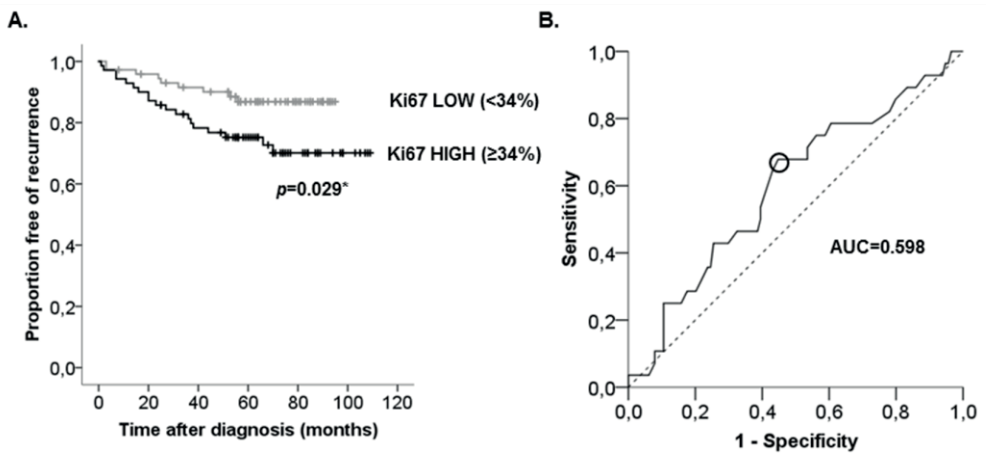

Figure 27. Kaplan-Meier survival curve for RFS in *HER2*-amplified breast cancers (n=142) stratified for Ki-67 expression using 34% cut-off value, p-value from Mantel-Cox log-rank test (**A**). The cut-off value was set on basis of ROC Analysis showing 68% sensitivity and 55% specificity for the assay with this cut-off point (marked with circle) (**B**).

Table 13. Clinical relevance of studied biomarkers in predicting early recurrence in *HER2*-amplified breast cancer type (Sample Cohort I). Survival analyses were conducted with Kaplan-Meier method, *p*-values from Mantel-Cox log-rank test (univariate). Mean RFS was 5.3 years. Number of cases with available data is marked in the column *n*.

Histopathological parameter	**RFS**			
	RFS ± SE	**95% CI**	***p***	***n***
Cyclin E1-LI			ns	193
low (<50%)	7.4 ± 0.3	6.9 – 8.0		
high (≥50%)	7.9 ± 0.3	7.2 – 8.5		
***CCNE1* status**			ns	177
normal	7.6 ± 0.2	7.2 – 8.1		
amplified	6.9 ± 0.9	5.0 – 8.7		
HER3-T			0.004**	177
low	6.3 ± 0.5	5.3 – 7.3		
high	8.0 ± 0.2	7.6 – 8.4		
HER3-M			0.025*	177
low	6.6 ± 0.6	5.6 – 7.7		
high	7.9 ± 0.2	7.4 – 8.3		
HER3-C			0.010**	177
low	5.9 ± 0.9	4.2 – 7.6		
high	7.8 ± 0.2	7.4 – 8.2		
NEDD4-1			ns	145
negative	6.8 ± 0.6	5.6 – 7.9		
positive	7.8 ± 0.3	7.3 – 8.3		
NRDP1-N			ns	145
negative	7.7 ± 0.2	7.2 – 8.2		
positive	6.6 ± 0.8	5.0 – 8.2		
NRDP1-C			ns	145
negative	7.6 ± 0.3	7.2 – 8.1		
positive	7.7 ± 0.7	6.3 – 9.0		
MCM2-LI			ns	142
low (<75%)	7.8 ± 0.3	7.2 – 8.3		
high (≥75%)	7.4 ± 0.4	6.6 – 8.3		

5.7.5 Conventional prognostic biomarkers in breast cancer

Clinicopathological parameters predicting RFS in Sample Cohort I

Kaplan-Meier analysis with univariate Mantel-Cox log-rank test confirmed the statistical significance of axillary lymph node invasion and large tumour size in predicting early recurrence in *HER2*-amplified primary breast cancers. During the follow-up, estimated mean RFS periods were 6.5 years (pN+) *vs* 8.4 years (pN0), and 4.3 years (pT≥3) *vs* 7.8 years (pT<3) for patients grouped on basis of pN and pT statuses (Table 14, p.120).

Clinicopathological parameters predicting RFS in Sample Cohort II

In HER2- primary breast cancers, the following parameters were shown to significantly predict disease recurrence: lymph nodal spread, large tumour size, high histological grade, high Ki67-LI, HR negativity, and TNBC subtype. Positive lymph nodal status was confirmed to predict disease recurrence in a small subgroup of HER2+ breast cancers. (Table 14, p.120). Estimated mean RFS periods were 15.3 years (95% CI 14.1-16.4 years) and 15.0 years (95% CI 12.4-17.6 years) for HER2- and HER2+ breast cancer patient groups. HER2 status was not shown to predict disease recurrence in this cohort (p=0.386). Multivariate Cox regression analysis was performed with parameters that were statistically significant in univariate analysis concerning the whole Sample Cohort II. Of these (PR, TNBC, grade, pN, pT), only pN+ status was shown to independently predict disease recurrence (p=0.002). The recurrence risk in pN+ primary breast carcinomas was two-fold during the long-term follow-up.

Table 14. Mean estimated RFS (in years) in relation to conventional prognostic biomarkers in Sample Cohorts I and II. Mean follow-up periods were 5.3 years and 10.4 years for Sample Cohorts I and II, respectively. *p*-values from Mantel-Cox log-rank test.

Histopathological parameter	***HER2*-amplified** (*n*=193, **Sample Cohort I**)			**HER2-positive** (*n*=47, **Sample Cohort II**)			**HER2-negative** (*n*=261, **Sample Cohort II**)		
	RFS ± SE	**95% CI**	***p***	**RFS ± SE**	**95% CI**	***p***	**RFS ± SE**	**95% CI**	***p***
ER status			ns			ns			0.041*
negative (<10%)	7.0 ± 0.4	6.2 – 7.8		14.0 ± 1.8	10.6 – 17.5		12.4 ± 1.8	8.8 – 16.0	
positive (≥10%)	7.0 ± 0.2	6.6 – 7.4		14.6 ± 1.9	11.0 – 18.3		15.2 ± 0.6	14.0 – 16.3	
PR status			ns			ns			0.021*
negative (<10%)	7.2 ± 0.3	6.6 – 7.8		13.2 ± 1.5	10.2 – 16.2		12.6 ± 1.2	10.2 – 14.9	
positive (≥10%)	7.2 ± 0.2	6.7 – 7.6		18.0 ± 1.9	14.3 – 21.7		16.1 ± 0.7	14.8 – 17.4	
Triple-negativity									0.006**
Other type							15.8 ± 0.6	14.6 – 17.0	
TNBC							10.2 ± 1.7	6.9 – 13.5	
Ki67-LI			ns			ns			0.042*
low (<20%)	7.1 ± 0.4	6.4 – 7.8		11.6 ± 2.6	6.6 – 17.0		15.7 ± 0.7	14.4 – 17.1	
high (≥20%)	7.5 ± 0.2	7.0 – 7.9		15.0 ± 1.8	11.5 – 18.4		12.8 ± 1.6	9.7 – 15.9	
Tumour grade			ns			ns			0.000***
I-II	6.9 ± 0.3	6.3 – 7.6		13.6 ± 1.8	10.0 – 17.2		17.0 ± 0.7	15.6 – 18.4	
III	7.6 ± 0.3	7.1 – 8.1		13.8 ± 2.1	9.6 – 17.9		9.0 ± 1.4	6.3 – 11.7	
Tumour size			0.010**			ns			0.003**
<2 cm	7.4 ± 0.2	6.9 – 7.8		15.5 ± 2.3	11.0 – 19.9		17.0 ± 1.1	14.8 – 19.1	
≥2 cm	7.1 ± 0.4	6.4 – 7.8		14.3 ± 1.7	11.0 – 17.6		12.1 ± 0.9	10.4 – 13.9	
Tumour size			0.000***			ns			0.007**
pT1-pT2	7.8 ± 0.2	7.4 – 8.2		14.4 ± 1.3	11.8 – 17.0		15.7 ± 0.6	14.5 – 16.8	
pT3-pT4	4.3 ± 1.0	2.3 – 6.2		12.0 ± 4.9	2.4 – 21.6		8.4 ± 1.6	5.3 – 11.6	
Lymph node status			0.000***			0.002**			0.000***
pN0	8.4 ± 0.2	8.0 – 8.8		17.6 ± 1.3	15.2 – 20.1		17.2 ± 0.7	15.9 – 18.6	
pN+	6.5 ± 0.4	5.7 – 7.3		9.3 ± 2.2	5.1 – 13.6		11.6 ± 0.9	9.8 – 13.4	

Abbreviations: CI: confidence interval; ns: no statistical significance; RFS: relapse-free survival; SE: standard error

6 DISCUSSION

6.1 Study setting and methodological approaches

Cyclin E1, HER3, NEDD4-1, NRDP1, and MCM2 were decided to study in this thesis for their proposed or confirmed role in the breast carcinogenesis and clinical relevance as prognostic or predictive biomarkers. Still, previous studies were not comprehensively characterized these factors in HER2+ breast carcinoma.

Studies I-IV concentrated predominantly on *HER2*-amplified primary breast cancer (Sample Cohort I). The number of carcinomas in this cohort (*n*=193) was seen adequate for the retrospective biomarker studies by considering the prevalence of HER2+ breast cancer type in general. All carcinomas were confirmed to carry *HER2* amplification by ISH, equivocal cases were not included. Only samples that fulfilled the criteria of representative tumour histology and technical quality were analyzed, and therefore the total number of included cases (*n*=142 to 193) varied in Studies I-IV. The mean follow-up period (5.3 years) was seen appropriate for the assessment of biomarkers' prognostic applicability for early disease recurrence in this particularly aggressive breast cancer subtype. A recent study (Geurts et al., 2017) demonstrated that the risk of first recurrence is highest one year after the initial breast cancer diagnosis. Studies on HER2+ primary breast cancer type have confirmed that disease recurrence takes place for the most part at 12 (S. Park et al., 2012) to 20 months (Ribelles et al., 2013) post-diagnosis. Overall, HER2+ breast cancers relapse primarily within the first 5 years after the diagnosis in contrast to HR+ and HER2- breast carcinomas that have higher risk of relapsing later than 5 years post-surgery (Esserman et al., 2011; Strasser-Weippl et al., 2015; Wangchinda & Ithimakin, 2016). Sample Cohort II was exclusively used in Study III for the characterization of HER3 expression and related clinicopathological associations. This cohort consisted mainly of HER2- breast carcinomas (*n*=261 of 308), and thus enabled studying of association between HER3 expression profile and HER2 status. Additionally, extended follow-up (mean 10.4 years) allowed long-term survival analysis.

The current study aimed also at clarifying biomarkers' clinical relevance in predicting response to 9-wk adjuvant trastuzumab administered as a first-line therapy

according to a schema described by Joensuu et al. (2006). Studies I-IV did not find any statistically significant associations between biomarkers' expression and RFS in comparison of adjuvant trastuzumab treated and untreated patient groups within Sample Cohort I. Currently, one-year administration of adjuvant trastuzumab plus chemotherapy is the standard of care for HER2+ primary breast carcinoma (Denduluri et al., 2016). Recently published results of Short-HER (Conte et al., 2018) and SOLD (Joensuu et al., 2018) trials confirm that one-year therapy is preferable to 9-wk regimen. This aspect may have influenced on patients' survival in the present study and must be taken into consideration because survival differences among adjuvant trastuzumab treated and untreated patient groups were not observed during the follow-up. Additionally, these two therapy groups were not fully balanced for ER status; ER+ carcinomas were more frequently represented in non-trastuzumab treated group, which may have attributed to favorable survival. In general, ER+HER2+ breast cancer patients have better prognosis (Bagaria et al., 2012); they are less likely to experience disease recurrence (H. J. Lee et al., 2014; Vaz-Luis et al., 2012), and are more responsive to anti-HER2 therapy than those with ER-HER2+ tumours (Zhao, Zhao, & Zhao, 2018).

IHC is a widely applied routine methodology in clinical practice, *e.g.* in breast cancer diagnostics, because most of biological factors determined for tumour characterization are proteins (Duffy et al., 2017). IHC techniques were principally applied also in Studies I-IV for biomarkers' expression profiling with optimized protocols and appropriate controls to confirm assay proficiency and reproducibility. IHC stainings were analyzed using digitized WSIs and DIA, whenever possible. In general, computer-aided analysis 'digital pathology' is considered as a state-of-the-art practice in histopathology due to improved diagnostic accuracy (Cheng & Tan, 2017). Sample Cohort I was represented as WTSs which enabled comprehensive analysis of biomarkers' staining pattern considering *e.g.* intra-tumoral variation/heterogeneity. This is crucial especially when the analysis is restricted to hot spots or otherwise specified ROIs. Instead, cases of Sample Cohort II were represented as singular TMA spots. This restricted analysis on minor tissue area and may therefore have caused false interpretation of biomarkers' expression pattern (Besusparis et al., 2016). However, TMAs are widely used in research and have been confirmed applicable for the detection of diagnostic breast cancer markers in comparison with WTSs (Chavan, Ravindra, & Prasad, 2017).

6.2 Cyclin E1 in *HER2*-amplified breast carcinoma

Deregulation of cyclin E1 has been confirmed to mechanistically enable breast malignant transformation by facilitating G1/S transition and cell cycle progression (Liang et al., 2010; Shaye et al., 2009). Accordingly, cyclin E1 is often highly expressed in breast malignancies (Scott & Walker, 1997). The clinical relevance of cyclin E1 remains still controversial and has not been profoundly studied in *HER2*-amplified breast cancer type. Mittendorf et al. (2010) demonstrated that cyclin E1 is commonly over-expressed in HER2+ breast carcinomas and associates with reduced 5-year BCSS, probably due to vicarious interaction between HER2 and cyclin E1. Vice versa, cyclin E1 expression was shown to decrease upon HER2 downregulation by trastuzumab in breast cancer cells *in vitro* and in mice tumour xenografts *in vivo* (Mittendorf et al., 2010). Another study by Scaltriti et al. (2011) suggested that high cyclin E1 expression/*CCNE1* amplification confers trastuzumab resistance in HER2+ breast cancers. These two studies awoke the interest to retrospectively study cyclin E1 and its clinical significance in *HER2*-amplified breast cancers.

Study I confirmed cyclin E1 over-expression in approximately 40% of *HER2*-amplified primary breast carcinomas. Likewise, Mittendorf et al. (2010) reported that 50% of HER2+ breast cancers display total cyclin E1 over-expression. Consistently with Study I, several studies have previously found that high cyclin E1 associates with features defining aggressive breast cancer type; HER2+ status, high grade, HR negativity, lymph node invasion, high Ki67-LI, and large tumour size (Lindahl et al., 2004; Potemski et al., 2006; Zagouri et al., 2017). Nonetheless, Study I did not find RFS differences between HER2+ breast cancer patients having either low or high cyclin E1 expressing tumours, irrespective of 9-wk adjuvant trastuzumab therapy. Accordingly, Study I did not confirm previously published results by Mittendorf et al. (2010) and Duchnowska et al. (2016) who found association between high cyclin E1 and reduced survival in HER2+ breast cancer cohorts.

Cyclin E1 seems to have differing roles in biologically different breast cancer subtypes (Agarwal et al., 2009), and therefore its prognostic applicability may be subtype-dependent. Additionally, comparison of earlier studies is complicated due to various technical and analytical differences (detection method, scoring/cut-off value) and divergent treatments within and between the patient cohorts. Study I was based on determination of full-length cyclin E1 expression with antibody (clone 13A3) that does not bind to LMW-E isoforms. In contrast, several previous studies have used antibodies that detect both full-length nuclear cyclin E1 and cytoplasmic LMW-E isoforms (total cyclin E1), as clarified in Table 3 (p.35). The most recent

studies have demonstrated that reduced BCSS is particularly associated with cytoplasmic cyclin E1 expression (Hunt et al., 2017; Karakas et al., 2016). Mittendorf et al. (2010) have shown that LMW-E isoforms are highly expressed also in HER2+ breast cancers and are predictive of reduced survival in patients who were not treated with adjuvant trastuzumab. No data was found concerning the prognostic value of LMW-E expression in adjuvant trastuzumab treated HER2+ breast carcinomas and remains therefore interesting subject to study in the future. Doostan et al. (2017) reported that cytoplasmic cyclin E1 is predictive of endocrine therapy resistance and may thus explain inferior survival in HR+ breast cancers. All these perspectives provide explanations to contradictive results achieved in the past. Conclusively, although cyclin E1 has been recognized as an oncogene and was confirmed frequently over-expressed in *HER2*-amplified breast cancers, its clinical applicability as a prognostic biomarker remains uncertain.

Study I confirmed *CCNE1*/*HER2* co-amplification in a small proportion (7.3%) of primary M0 breast carcinomas. Neither earlier studies, albeit few, have reported high *CCNE1* amplification frequency in HER2+ breast cancer type. However, the number of HER2+ carcinomas has been rather small in these studies. Natrajan et al. (2012) reported that only 1.6% (1 of 64) of HER2+ breast carcinomas carry *CCNE1* amplification. Moelans et al. (2010) found *CCNE1* amplifications in 7% (2 of 27) of HER2+ breast carcinomas. Vice versa, Scaltriti et al. (2011) reported that *CCNE1* amplification frequency was 20% (13 of 64) in aCGH dataset of HER2+ carcinomas. Additionally, 35% of HER2+ breast carcinomas (another sample set) were characterized with cyclin E1 over-expression or *CCNE1* amplification, but the study does not report the exact prevalence of *CCNE1* aberrations. Study I showed that *CCNE1* amplification associates with high cyclin E1 expression, as has been reported previously (Callagy et al., 2005; Keyomarsi & Pardee, 1993; Natrajan et al., 2012), but cyclin E1 over-expression was more frequently present than *CCNE1* amplification. Consequently, cyclin E1 over-expression do not predominantly stem from *CCNE1* amplification but is related to other oncogenic alterations as well. For instance, dysfunction in post-transcriptional regulatory mechanisms may contribute to cellular cyclin E1 expression (Siu et al., 2012). In Study I, *CCNE1*-amplified breast carcinomas were shown to display high proliferation activity (Ki67-LI) but were not associated with significantly reduced clinical outcome. Scaltriti et al. (2011) have proposed earlier that dysregulated cyclin E1 is predictive of disease progression in a small cohort of HER2+ breast cancer patiens. However, this study did not report precisely whether the tumours were defined with *CCNE1* amplification and/or cyclin E1 over-expression.

6.3 HER3 in breast carcinoma

Validation of HER3 IHC assay

HER3 protein expression has been widely studied in breast cancers with varying detection methods and scoring systems. In addition, criteria for defining biologically deviant HER3 expression have not been presented. (Nuciforo et al., 2015). Consequently, comparison between the previously published results is complicated due to these discrepancies. Compared with HER2, IHC-based detection of HER3 is technically demanding due to its relatively low and unstable expression. HER2 receptors detach tightly into the cell membrane and are resistant to constitutive trafficking (Bertelsen & Stang, 2014; Jeong et al., 2017). This membrane retention enables HER2 detection in its putative localization by IHC. Contrarily, HER3 receptors are unstable and constitutively internalized from the cell membrane (Offterdinger et al., 2002; Reif et al., 2016; Sak et al., 2012). Once internalized, HER3 is quickly ubiquitinated and transferred to proteasomes for degradation. For this reason, HER3-M expression, as detected by IHC, is not necessarily corresponding to HER3 synthesis at transcriptional level. Accordingly, various methodologies (*e.g.* PCR, Vera Tag®) have been applied for the quantification of total HER3 expression. However, concerning patient selection for novel HER3-targeting therapies, the presence of cell membrane localized HER3 receptors seems rather critical than total HER3 expression since drugs bind to HER3 ECD. Nonetheless, due to constant trafficking between the subcellular loci, evaluation of HER3-T expression profile by IHC may provide rationale for defining biologically meaningful HER3 expression.

Study II indicated that IHC-based detection of HER3 expression, specifically of HER3-M, in breast cancer tissues demands highly sensitive detection with specific antibody. HER3-M expression was detectable only with DAK-H3-IC antibody clone, while other tested antibodies (clone RTJ1, clone SP71, SAB4500793 pAb) produced non-specific, technically unacceptable staining pattern. In many previous studies, cell membrane staining has been ignored and HER3 status has been defined by cytoplasmic (or nuclear) staining reaction that may be difficult to differentiate from non-specific background staining. In Study II, HER3 protein expression profiles of breast cancer cell lines were analyzed by IHC and FACS. Results were compared for the assessment of IHC assay sensitivity and authenticity of qualitative scoring criteria. Flow cytometry was earlier shown technically feasible and reliable method for the assessment of ER, PR, HER2 and EGFR expression levels in breast cancer cells (Lostumbo, Mehta, Setty, & Nunez, 2006). Results achieved with two

different techniques were consistent, although were not shown to correlate in terms of statistical significance. Study II demonstrated low level HER3 expression in basal-like (JIMT-1, MDA-MB-231), and high HER3 expression in luminal or HER2+ (*e.g.* EFM-192A, MDA-MB-453, HCC1419) breast cancer cell lines by IHC and FACS. Consistently with the previous finding of Balko et al. (2012), Study II showed high HER3 expression in luminal cell layer and low level HER3 expression in basal cell layer in histologically normal breast ducts.

HER3 in clinical breast carcinomas

Study III demonstrated that HER3 is predominantly expressed in breast carcinomas and is not dependent on HER2 status, irrespective of its subcellular localization (HER3-M, HER3-C). Approximately 75% of breast cancers were shown to display high HER3-T expression, which provides rationale for HER3-targeted therapies. Low HER3 expression was found to define more aggressively behaving breast cancer types characterized with ER negativity, lymph node invasion, large tumour size, basal-like phenotype, TNBC subtype, and young age at diagnosis. Interestingly, HER3 loss has been suggested to genetically alter breast epithelial cells towards basal phenotype (Balko et al., 2012). Basal-like phenotype has also been shown to determine poor clinical outcome and trastuzumab resistance in HER2+ breast carcinomas (Bagaria et al., 2012; Martin-Castillo et al., 2015). Similarly, HR-HER2+ breast cancers have been shown to relapse more likely than HR+HER2+ carcinomas (Vaz-Luis et al., 2012). In a recent *in vitro* study, HER2+ breast cancer cells exhibiting lower proliferation activity were demonstrared to over-express HER3 (Kirouac et al., 2016). Nevertheless, Studies III-IV did not find any associations between HER3 and proliferation markers Ki-67 and MCM2, which is in line with the recently published clinical breast cancer study (Takada et al., 2018).

Study III confirmed that low HER3 expression is clinically relevant biomarker for predicting reduced RFS in *HER2*-amplified breast cancer. Breast cancers with low HER3-T expression were demonstrated to have two-fold risk for recurrence during the follow-up. In addition to HER3-T, also lymph node involvement and large tumour size were confirmed as independent prognostic factors for the first disease recurrence (multivariate analysis), in accordance with previous study (Geurts et al., 2017). Although low HER3 expression correlated with such clinicopathological features that typically define poor survival, HER3 was not found prognostic among HER2- breast carcinomas. In general, the role of HER3 as a prognostic biomarker remains undefined due to contradictory results presented in

the previous studies (Table 5, p.51). Most of these studies have found association between high HER3 and unfavorable breast cancer outcome. Contrarily, some researchers have associated low HER3 level with poor BCSS, in compliance with the Study III, or did not find any clinically relevant associations between HER3 and breast cancer outcome. Careful review disclosed that these studies were performed with different detection methods and mostly on metastatic breast cancers. The oldest studies were rarely focused on particular breast cancer subtype, although recurrence pattern and HER signaling are certainly dependent on breast cancer subtype (Hynes & MacDonald, 2009; Ribelles et al., 2013).

Previous studies by Takada et al. (2018), Han et al. (2012), and Abd El-Rehim et al. (2004) are in line with Study III by supporting that patients carrying breast carcinomas with high HER2/HER3 co-expression have more favorable prognosis than those with carcinomas displaying over-expression of either receptor type. One explanation clarifying the detrimental impact of low HER3 relates to intensive HER2 signaling due to paradoxical HER2 homodimerization in *HER2*-amplified breast carcinomas. Spears et al. (2012) have confirmed that HER2 homodimerization is common in *HER2*-amplified breast cancer type and relates to reduced RFS, although abundancy of HER2:HER3 heterodimers was also associated with increased risk of recurrence. In addition, absence of HRGβ1 and consequently reduced PI3K/Akt signaling in HER3 over-expressing carcinomas may provide explanation for better prognosis. HER3 expression was not shown to associate with RFS among HER2- breast carcinomas. Accordingly, Study III evidences that the prognostic value of HER3 is restricted to *HER2*-amplified breast cancers although the prevalence of HER3-T expression was shown to be similar both in HER2- and HER2+ breast cancers.

Recently, HER3 has been highlighted as an anti-cancer therapy target and predictive biomarker defining anti-HER therapy response (N. Zhang et al., 2016). HER3 over-expression has been shown to predict poor outcome in HER2+ breast cancer patients receiving adjuvant trastuzumab as a first-line therapy (Adamczyk et al., 2017; Lipton et al., 2013). In contrast, high HER3 mRNA (Baselga et al., 2014) and protein (Takada et al., 2018) expression has been associated with better clinical outcome in HER2+ metastatic breast cancer patients who were treated with adjuvant TPD regimen as a first-line therapy. Recent studies (Barros et al., 2014; Green et al., 2014; Spears et al., 2012) carried out with novel PLA technology have confirmed that the number of HER2:HER3 dimers correlates with expression of HER2 and HER3 proteins in HER2+ breast tumours. Still, the predictive value of HER2:HER3 heterodimers has not been confirmed in adjuvant trastuzumab treated patients.

Study III did not find survival differences among trastuzumab-treated and untreated patients in relation to HER3 expression. To further clarify the prognostic value of HER3, comparison of survival data with expression profiles in primary breast tumour and the corresponding metastasis seems worthwhile.

The focus in Study III was primarily on *HER2*-amplified breast cancer because of tight interaction between HER2 and HER3 receptors and novel therapy approaches aiming at hindering this interplay. Study III demonstrated that HER3 is not particularly associated with HER2 status but was shown to be expressed equally both in HER2+ and HER2- breast carcinomas. Although the predominance of HER2:HER3 dimerization is well established, the significance of intensive HER3 signaling in non-HER2 dependent breast cancer type has been speculated. HER3 over-expression has been attributed to reduced survival in HER2- breast carcinomas (Chiu et al., 2010) and TNBC (Bae et al., 2013). One oncogenic mechanism in these types may be related to high co-expression of EGFR and HER3 receptors. Choi et al. (2012) suggested that HER3 is a key sensor of HER signaling since it has ability to regulate its own expression and activation after perturbation. In this study, HER3:EGFR dimerization and consequent downstream signaling was induced in both low and high HER2 expressing breast cancer cells after exposure to anti-HER2 therapy, even in the absence of HRGβ1. Accordingly, the efficacy of HER3-targeted therapies in HER3-enriched HER2- breast cancer types has been speculated, but not yet clinically confirmed (Campbell & Moasser, 2015; Schneeweiss et al., 2018).

6.4 NEDD4-1 and NRDP1 in *HER2*-amplified breast carcinoma

Experiments performed in Study II with breast cancer cell lines and clinical breast carcinomas demonstrated that HER3 localizes both in cell membrane and cytoplasm, and its expression pattern varies inter-tumorally. Protein degradation processes through ubiquitin-proteasome pathway has been suggested to essentially contribute to HER3 membrane retention (Amin et al., 2012; Carraway, 2010). Consequently, low level expression of HER3 degradation regulators, NEDD4-1 and NRDP1, was hypothetized to inversely contribute to HER3-M expression. For this reason, Study III aimed at clarifying NEDD4-1 and NRDP1 protein expression in relation to HER3-M and HER3-C.

For the first time, Study III demonstrated that NEDD4-1 is predominantly expressed in *HER2*-amplified breast carcinomas; 83% of carcinomas displayed intense cytoplasmic staining for NEDD4-1. No inverse correlation was confirmed

between HER3 and NEDD4-1 expression. By contrast, high NEDD4-1 expression was significantly related to high HER3-M expression. Thus, Study III did not confirm that NEDD4-1 could independently reduce HER3-M expression in breast cancer cells, as shown by IHC. However, this result needs to be confirmed because of relatively small representation of carcinomas characterized with low HER3 and low NEDD4-1. Two previous studies have clarified NEDD4-1 expression in this context and reported that NEDD4-1 is expressed in approximately 50% of breast carcinomas (Y. Chen et al., 2016; Jung et al., 2013). This proportion is considerably lower than in Study III but may be explained by minor representation of HER2+ carcinomas in the previous studies.

Contrary to NEDD4-1, NRDP1 was shown infrequently expressed in *HER2*-amplified breast carcinomas; only 8.3% of carcinomas were characterized with detectable NRDP1 protein expression in Study III. This result confirms earlier finding reported by Yen et al. (2006). They proposed that low NRDP1 in HER2+ breast cancers may provide growth advantage for HER2/HER3-dependent breast cancer cells due to reduced degradation of HER3 receptors. By this mechanism, low NRDP1 may probably facilitate tumour progression. In contrast, another study (Jiao et al., 2015) reported high NRDP1 expression even in half (58%) of breast cancers, and it was associated with poor clinical outcome. Although the analyzing criteria were consistent with those used in Study III, results were not comparable which may be derived from differences in IHC procedures and sample cohorts. In a study of Jiao et al., only 38% of carcinomas represented HER2+ type. In Study III, neither NEDD4-1 nor NRDP1 were shown to have clinically relevant associations and were not predictive of outcome in *HER2*-amplified breast carcinomas.

6.5 MCM2 and Ki-67 in *HER2*-amplified breast carcinoma

Cell proliferation activity is commonly defined by Ki-67 expression, which is recommended method for breast cancer diagnostics as well (Duffy et al., 2017). Nevertheless, the prognostic significance of Ki-67 is controversial, and has been criticised in particularly aggressive HER2+ breast cancer type (Aleskandarany et al., 2012; Kontzoglou et al., 2013; Niikura et al., 2014). Recently, Yousef et al. (2017) suggested that MCM2 is preferable to Ki-67 in predicting BCSS. However, the clinical relevance of MCM2 has not been profoundly studied in distinct breast cancer types, especially in HER2+ subtype, and was therefore clarified in Study IV.

Fluoro-chromogenic double labelling and digital image analysis for MCM2 detection

In Study IV, a novel methodological approach was successfully implemented for the detection of MCM2 expression in breast cancer. This method combines fluoro-chromogenic double labelling IHC on a single tissue section, sequential slide digitization using bright field and fluorescence illumination, and DIA with ImmunoRatio 2.5 on multilayer WSI. In practice, MCM2 labelling was used for the detection of proliferative cells, and CK labelling for cancer cell selection. Nuclear MCM2 label (DAB) was clearly distinguishable from cytoplasmic CK label (Cy2 fluorochrome) and blue counterstaining (hematoxylin). In some breast carcinomas showing particularly high MCM2 expression, strong DAB precipitation diffused partly into cytoplasm and was shown to hinder binding of subsequently applied CK antibody. This caused weaker IF signal in cancer cells but MCM2 staining was still analyzable in all cases. Recently launched CE-IVD validated Virtual Double Staining™ (VDS) (VisioPharm A/S, Hoersholm, Denmark) tool exploits also CK-masking for cancer cell detection, but labelling should be performed on consecutive tissue sections that are separately digitized and afterwards aligned for DIA (Koopman, Buikema, Hollema, de Bock, & van der Vegt, 2018; Roge, Riber-Hansen, Nielsen, & Vyberg, 2016).

By using ImmunoRatio 2.5 with IF correction (brightfield and IF blending mode), non-malignant (CK-) proliferative cells (MCM2+) were successfully excluded from the analysis. This DIA method was shown to clearly increase assay accuracy and reproducibility in comparison with VA. All samples were analyzed independently by two raters (self-defined ROIs), and results were shown to significantly correlate with each other. Preliminary study with Ki-67 stained TMA training set showed that analysis restricted to bright field only yield in slightly lower (~2%) LI than was achieved by analyzing with IF correction mode (*unpublished result*). Utility of the first ImmunoRatio version (no IF correction) in Ki-67 analytics has been shown by several researchers (Gonzalez-Gonzalez et al., 2016; Yeo et al., 2017). In general, various DIA platforms have been demonstrated to improve the reliability of Ki-67 and MCM2 analyses in comparison with VA (Joshi et al., 2015; Koopman et al., 2018; Roge et al., 2016; Stålhammar et al., 2016; Zhong et al., 2016). The method presented in Study IV was shown particularly useful in distinguishing lobular breast cancer cells (CK+) from TAICs and stromal cells (CK-). It has been recently suggested that the prognostic cut-off for Ki-67 should be as low as 4% for ILC (Carbognin et al., 2017), which highlights the importance of accurate and reliable analyzing tool.

Study IV confirmed that a majority of *HER2*-amplified breast cancers were highly proliferative, as was determined by MCM2 and Ki-67 expression. Compared to Ki-67, MCM2 expression level was shown to be considerably higher; median LIs for Ki67 and MCM2 were 33.0% *vs* 63.5%, respectively. Similar results were previously published in breast cancer cohorts unspecified for HER2 status (Gonzalez et al., 2003; Joshi et al., 2015; Wojnar et al., 2010; Yousef et al., 2017). This is perceivable since MCM2 is expressed throughout the cell cycle, and also by non-cycling cells having proliferation capacity (Stoeber et al., 2001), while Ki67 is not present in early G1 phase (Gerdes et al., 1984; Lopez et al., 1991). Expression levels of MCM2 and Ki67 were shown to significantly correlate with each other and with cyclin E1 expression, similarly to earlier result by Bukholm et al. (2003). MCM2 was shown useful in subgrouping *HER2*-amplified breast carcinomas into more aggressive ones, characterized with high histological grade and negative HR status, although was not shown to associate with tumour size nor lymph nodal status. This result is in line with the previous studies (Gonzalez et al., 2003; Joshi et al., 2015; Yousef et al., 2017). Collectively, Study IV confirmed that *HER2*-amplified breast cancers are aggressive, in general, and appear with high proliferation capacity, determined by particularly high expression of Ki-67, MCM2 and cyclin E1 proteins.

Study IV did not demonstrate any prognostic utility for MCM2 in predicting breast cancer recurrence during the mean follow-up of 5.3 years. Thus far, no other studies have been published concerning the prognostic role of MCM2 in HER2+ breast cancer type. Wojnar et al. (2010) did not find any prognostic value for MCM2 in a group of invasive ductal breast carcinomas. In contrast, many previous studies have demonstrated that high MCM2 protein (Bukholm et al., 2003; Gonzalez et al., 2003; Joshi et al., 2015; Loddo et al., 2009; Tokes et al., 2016; Yousef et al., 2017) or mRNA (Kwok et al., 2014; Nieto-Jimenez et al., 2016; Yousef et al., 2017) expression is indicative of reduced clinical outcome in breast cancer cohorts. In the absence of standardization, various cut-off values have been applied to define low and high MCM2 expression, which complicates comparison between the studies. Study IV showed also that Ki-67 is predictive of reduced RFS in *HER2*-amplified breast cancers when the cut-off was set to 34%, which is higher than the consensus cut-off 20% (Duffy et al., 2017). Conclusively, Study IV indicated that cell proliferation markers are of limited prognostic significance in *HER2*-amplified breast cancer. Their role is rather pronounced *e.g.* in distinguishing slowly proliferating luminal A breast carcinomas from more aggressive subtypes, like HER2+ carcinomas.

7 CONCLUSIONS

The main findings and conclusions of this doctoral thesis are described below and recapitulated in Table 15 (p.134):

I. Cyclin E1 was over-expressed in 37% of *HER2*-amplified primary breast cancers, especially in association with HR negativity, poor differentiation, large tumour size, high proliferation activity (Ki-67) and basal-like phenotype. Cyclin E1 was not prognostic of early disease recurrence, irrespective of 9-wk adjuvant trastuzumab therapy. Co-amplification of *CCNE1* and *HER2* was demonstrated in 7.3% of primary breast carcinomas. (**Study I**)

II. IHC-based detection of HER3 in breast carcinomas requires careful antibody selection and highly sensitive detection. HER3 expression pattern was varying in breast cancer cell lines and clinical breast carcinomas. (**Study II**)

III. High HER3-T expression was demonstrated in 75% of breast carcinomas, irrespective of HER2 status. Low HER3 expression associated with clinicopathological characteristics that typically define aggressively behaving and therapeutically unfavorable breast cancers. These features included TNBC subtype, basal cell origin, large tumour size, axillary lymph node invasion, ER negativity, and young age at diagnosis. Low HER3 expression was associated with significantly higher risk of recurrence in *HER2*-amplified breast carcinomas. HER3 status was not shown to predict response to 9-wk adjuvant trastuzumab therapy as a first-line therapy. (**Study III**)

IV. High-level expression of NEDD4-1 and NRDP1 was related to HER3 over-expression, but neither of them was associated with RFS. NEDD4-1 and NRDP1 were not considered useful for sub-categorizing the heterogeneous group of *HER2*-amplified breast carcinomas. (**Study III**)

V. MCM2 was strongly expressed in *HER2*-amplified breast carcinomas, especially in association with high histological grade, HR negativity, and high Ki-67. MCM2 was not prognostic of early recurrence in *HER2*-amplified breast carcinomas, irrespective of adjuvant trastuzumab therapy. Fluoro-chromogenic double labelling combined with sequential scanning and ImmunoRatio 2.5 DIA was shown to be useful method for the assessment of cell proliferation activity by MCM2 expression. (**Study IV**)

VI. On basis of **Studies I-IV**, large tumour size, positive lymph nodal status and low HER3-T expression were the most important factors to predict reduced RFS in *HER2*-amplified breast cancers. Cyclin E1, MCM2 and HER3 were shown applicable biomarkers for sub-categorizing *HER2*-amplified breast cancers into more aggressively behaving types.

Table 15. Summary of studied novel biomarkers with statistically significant clinicopathological associations and prognostic value in *HER2*-amplified breast carcinoma.

Biomarker	Study	Associations with general clinicopathological characteristics							Prognostic implication
		ER	**PR**	**Ki67**	**Grade**	**Size**	**Basal type**	**Nodal status**	**RFS**
Cyclin E1 ↑	I	↓	↓	↑	↑	↑	bp+		
CCNE1 amp	I		↓	↑					
HER3	II, III								
HER3-M ↓		↓							reduced
HER3-C ↓						↑			reduced
HER3-T ↓							bp+	pN+	reduced
NEDD4-1	III								
NRDP1-C ↑	III		↑						
NRDP1-N	III								
MCM2 ↑	IV	↓	↓	↑	↑				

Definitions for abbreviations and symbols:
↓ low expression; ↑ high expression; ER and PR (↓ <10%, ↑ ≥10%); Ki67 (↑ ≥20%); grade (↑ III); size (↑ ≥pT3); bp+: basal phenotype (CK5/14+ with ER-); pN+: positive axillary nodal status; HER3-M/-C/-T: membranous/cytoplasmic/total HER3 expression; amp: *CCNE1* amplification; NRDP1-C/-N: cytoplasmic/nuclear NRDP1. Statistical significance was determined using Mann-Whitney *U* test, Pearson's Chi-Square test and Mantel-Cox univariate test.

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ORIGINAL PUBLICATIONS

PUBLICATION I

Cyclin E amplification, over-expression, and relapse-free survival in HER-2-positive primary breast cancer

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CYCLIN E AMPLIFICATION, OVER-EXPRESSION, AND RELAPSE-FREE SURVIVAL IN HER-2-POSITIVE PRIMARY BREAST CANCER

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ABSTRACT

Cyclin E is a well-characterized cell cycle regulator and an amplified oncogene in breast cancer. Over-expression of cyclin E has generally been associated with poor survival. Recent studies have shown an interaction between HER-2 (*ERBB2*) and cyclin E, but the exact mechanism is unknown. Interestingly, cyclin E over-expression has been associated with trastuzumab resistance. We studied cyclin E over-expression, *CCNE1* amplification and relapse-free survival in HER-2-positive primary breast cancers treated with and without trastuzumab therapy. Formalin-fixed paraffin-embedded tissue samples from 202 HER-2-positive breast carcinomas were studied. Expression levels of cyclin E and proliferation marker Ki-67 were determined using immunohistochemistry. Chromogenic *in situ* hybridization (CISH) with a gene-specific BAC probe was used to analyze presence of *CCNE1* amplification. Majority of HER-2-positive breast carcinomas exhibited nuclear staining for cyclin E protein. Cyclin E was highly expressed (≥50% cells) in 37% of cases. Incidence of *CCNE1* amplification (≥6 gene copies/cell or clusters) was 8%. Cyclin E amplification and over-expression were strongly associated with each other, grade, hormone receptors, and Ki-67. Neither high cyclin E expression nor *CCNE1* amplification was associated with relapse-free survival (RFS) irrespective of short-term (9-week regimen) adjuvant trastuzumab therapy. These results confirm cyclin E and HER-2 gene co-amplification in a fraction of HER-2-positive breast cancers. Cyclin E is frequently over-expressed but appears to have limited value as a prognostic or predictive factor in HER-2-positive breast cancer regardless of trastuzumab therapy.

Keywords: Cyclin E, CCNE1, Immunohistochemistry, Breast cancer, HER-2, Survival

INTRODUCTION

Breast cancer mortality has decreased overall during the last decades [1]. Nevertheless, a remarkable number of breast cancer patients develop recurrent and eventually fatal metastatic disease [2]. Of the several subtypes of breast cancer, those with human epidermal growth factor receptor 2 (HER-2) gene *(ERBB2)* amplification and over-expression are associated with decreased survival [3,4]. Amplification of *ERBB2* gene occurs approximately in 15-20% of primary breast carcinomas [5,6]. Despite of notable advances reached in the treatment of this cancer type with targeted anti-HER2 therapy (trastuzumab and lapatinib), a sizable fraction of patients experience disease recurrence. Treatment of HER-2 positive breast cancer remains a clinical challenge because not all tumors respond to anti-HER2 therapy due to primary or acquired resistance [7,8]. Accordingly, biomarkers that could predict appearing of relapse and treatment response early at diagnosis are certainly needed.

One suggested biomarker is cyclin E (cyclin E1), coded by gene *CCNE1*. In the breast tissue, cyclin E is expressed only in breast malignancies [9,10] and it has been shown co-amplified and over-expressed in HER-2-positive breast cancer [11]. Natrajan et al. [12] characterized the function of 19q12 amplicon and found that *CCNE1* is one of the drivers within this amplicon.

Cyclin E and other cyclins act by forming complexes with their catalytic subunits, cyclin-dependent kinases (CDKs), at various stages in the cell cycle. When over-expressed, cyclins are demonstrated to enhance cell proliferation [13]. More specifically, over-expression of cyclin E accelerates the cell cycle by shortening the G1-S transition and thus promotes cell proliferation and oncogenesis [14,15].

Several studies have demonstrated that cyclin E immunoreactivity has a significant association with poor differentiation, high Ki-67 index and a lack of estrogen receptor in primary breast cancer [16,17]. Over-expression of cyclin E in breast tumors has also been shown to correlate with poor overall and breast

cancer specific survival [18,19]. Still, there are some studies that contradict the prognostic role of cyclin E over-expression. Comparison of earlier studies on prognostics of cyclin E is complicated due to varying breast cancer subtypes studied, methods and cut-off values used to determine cyclin E over-expression [19].

In HER-2-positive breast cancer, cyclin E and HER-2 may interact with each other in a vicarious manner, but the exact mechanism is unknown. Oncogenic effects of HER-2 receptor are based on multiple mechanisms on various signal transduction pathways mediated by this receptor. Specifically, HER-2 affects cell cycle regulation by shortening the G1 phase leading to enhanced proliferation and cell cycle progression. Over-expression of HER-2 has been shown to increase Cdk2 activity [20]. Cyclin E together with Cdk2, regulates G1-S transition in the cell cycle [14].

Mittendorf et al. [21] suggested that HER-2 directly regulates cyclin E function. Level of cyclin E protein, particularly its low molecular weight isoforms (LMW), decrease upon HER-2 down-regulation and HER-2 inhibition. On the contrary, Scaltriti et al. [11] have reported that high cyclin E leads to decreased function of HER-2 receptor. Cyclin E over-expression and *CCNE1* amplification were associated with worse clinical benefit and lower progression-free survival in adjuvant trastuzumab treated breast cancer patients [11]. Presence of acquired *CCNE1* amplifications was also detected in trastuzumab-exposed BT474 cells displaying resistance to trastuzumab [11]. Based on these results, it has been suggested that cyclin E could be a predictive factor of poor response to anti-HER2 therapy.

The aim of the present study was to analyze expression level of total cyclin E and incidence of *CCNE1* amplification to clarify their role in predicting progression of HER-2-positive breast cancer. Earlier data is to some extent contradictory and inadequate. In previous studies the proportion of HER-2-positive tumors has also been quite small. Accordingly, a large sample collection of HER-2-positive breast cancer cases was established for this study. The prognostic role of cyclin E – in terms of protein expression level and *CCNE1* amplification – was analyzed comparatively in adjuvant trastuzumab treated and untreated patient cohorts. In this way we tried to clarify that does cyclin E predict lower survival in adjuvant trastuzumab treated HER-2-positive breast cancer that was hypothesized on ground of interesting finding by Scaltriti et al. [11].

MATERIALS AND METHODS

Case selection

Altogether 202 consecutive patients diagnosed with HER-2-positive primary invasive breast cancer between the years 2003 and 2007 were selected to this study. The inclusion criteria were availability of representative tumor samples and clinical follow-up data. All the tissue samples were fixed overnight with 10% buffered formalin, routinely processed and embedded as paraffin blocks. Information regarding *ERBB2* amplification status (by CISH) and hormone receptor (estrogen ER, progesterone PR) status was already available from the clinical reports. Diagnostic data and follow-up information were collected retrospectively from the medical records. Relapse-free survival in association with cyclin E data was analyzed separately in patients with (n=87) and without (n=106) adjuvant trastuzumab therapy. Adjuvant trastuzumab treated patients were treated according to a nine week trastuzumab regimen [22]. Median follow-up period for relapse-free survival was 5.4 years. This study was approved by the local ethics committee (R07082).

Immunohistochemical stainings

Cyclin E expression was studied using immunohistochemistry which has been shown as a reliable and preferred method [23]. For immunohistochemistry, tumor sample blocks (*n*=202) were cut into 3-4 µm thick sections that were mounted on positively charged Super Frost Plus® slides. Slides were deparaffinized with hexane and dehydrated with graded alcohols. Heat-induced antigen retrieval (HIER) was done in TE-buffer (50 mM Tris 1 mM EDTA, pH 9) at 98°C for 15 minutes. For blocking of endogenous peroxidase slides were treated with 3% H_2O_2 for 5 minutes (Ki-67) or with 0.5% H2O2 in methanol for 10 minutes (cyclin E) at room temperature. Following antibodies were used: mouse monoclonal antibody against Ki-67 (clone MIB-1, Dako, Denmark) and mouse monoclonal antibody against cyclin E (clone 13A3, Novocastra, Leica Biosystems, UK). The Ki-67 antibody was used at a dilution of 1:500 and incubated 30 minutes at room temperature. Cyclin E antibody was used at a dilution of 1:100 and was incubated on the slides for one hour at room temperature.

For cyclin E immunohistochemistry, the Power Vision Plus detection kit (ImmunoLogic, GX Duiven, Netherlands) was used with freshly prepared 3',3-diaminobenzidine tetrahydrochloride ImmPACT DAB (Vector Laboratories Inc., UK). In the Ki-67 staining protocol Histofine® Simple Stain MAXPO (Nichirei Biosciences Inc., Japan) was used as a HRP-conjugated secondary antibody. Slides were counterstained with undiluted Mayer's hematoxylin (Oy FFChemicals Ab, Haukipudas, Finland), dehydrated, cleared and mounted. Staining protocols were carried out with Autostainer 480 (Lab Vision, CA, USA) automated immunostainer. Placenta was used as a known positive tissue control for cyclin E staining [24]. Positive and negative (primary antibody omitted) controls were included in each staining batch.

Hormone receptor status was determined earlier using monoclonal antibodies against estrogen receptor ER (clone 6F11, Novocastra, Leica Biosystems, UK) and progesterone receptor PR (clone 312, Novocastra, Leica Biosystems, UK), both at a dilution of 1:500. For detection we used Power Vision Plus kit (ImmunoLogic, GX Duiven, Netherlands). Information on HER-2 status was readily available and was determined using chromogenic in situ hybridization (CISH) with a digoxigenin-labeled in-house probe (human BAC clone RP11-94L15) used in routine clinical diagnostics.

Scoring of immunohistochemical stainings

Staining pattern was analyzed in the cancerous areas showing the strongest staining intensity. Scoring was performed by microscopically reviewing the slides to estimate the percentage of cancer cells displaying with clearly detectable brown nuclear staining. Cytoplasmic staining without any nuclear staining was regarded as negative finding.

Staining by Ki-67 antibody was analyzed with Olympus System Microscope BX43 from three visual fields (×200) using ImmunoRatio software [25]. For analysis of cyclin E staining, slides were scanned with Aperio ScanScope XT virtual microscope (Aperio Technologies, Vista, USA) and examined on computer screen by calculating 100 cells from five distinct areas covering invasive carcinoma. ImageJ image analysis software was used for cell calculations. Results were announced as a mean value.

Ki-67 staining was considered low if less than 20% of cells were stained in nuclei and high if ≥ 20% cells were stained. This cut-off point has been shown to yield excellent prognostication when Ki-67 (clone MIB-1) staining and ImmunoRatio software are used [25].

Cyclin E staining was classified to low category when <50% cells were stained in nuclei and high when ≥50% cells were stained. In spite of extensive previous immunohistochemical studies on cyclin E expression in breast cancer, there is no standardized cut-off to be used in determination of cyclin E positivity and has therefore widely differed in earlier studies. We ended up in using 50% cut-off point. No remarkable differences were seen in results when the cut-off point between low and high values was set

either at 30%, 35% (median) or 50%. Figure 1 shows the distribution of cyclin E immunoreactivity in the current study.

ER and PR were considered positive when >10% of tumor cell nuclei were stained according to a widely used consensus cut-off. For HER-2 CISH, amplification was considered when six or more gene copies were found per cell or due to presence of typical gene copy clusters.

Chromogenic *in situ* hybridization (CISH)

Chromogenic *in situ* hybridization (CISH) method was utilized for determination of *CCNE1* amplification in HER-2-positive breast tumors (*n*=185). Genomic probe for *CCNE1* was made using the human BAC clone RPCI-11-104J24 (Invitrogen Corp., UK), which was purified, amplified and labeled with digoxigenin (DIG) using nick translation. Labeled probe was precipitated and resuspended in hybridization mixture (containing 15% dextran sulfate, 70% formamide, 20X SSC, placental DNA and cot-1 DNA).

In CISH hybridization protocol, slides were deparaffinized with hexane, dehydrated and pretreated by boiling them in TE buffer (50 mM Tris 1 mM EDTA, pH 9) at +98°C for 15 minutes. The slides were first treated with 0.2M HCl for five minutes and afterwards washed with distilled water. Samples were digested with ready-to use pepsin solution (Digest-All™ 3, Invitrogen, UK) for 20 minutes at room temperature following PBS wash. Samples were post-fixed in 5% formalin for 10 minutes, washed and dehydrated in graded alcohols. Ten to twenty microliters of probe mixture was applied onto the slides, which were coverslipped and sealed with rubber cement. Slides were denatured at 95ºC for 5 minutes and hybridized at 42ºC for 48 hours in a humid chamber of StatSpin® ThermoBrite slide hybridizer (IRIS International Inc., CA, USA). After the hybridization step, unbound probe was washed by immersing the slides in wash buffer (0.4 × SSC / 0.3% NP-40) for three minutes at 72ºC followed by one minute wash in another wash buffer (2 × SSC / 0.1% NP-40) at room temperature.

DIG-labeled hybridized probe was detected using monoclonal anti-digoxin antibody (Jackson ImmunoResearch Laboratories Inc., UK) at a dilution of 1:10000. Histofine® Simple Stain MAXPO (Nichirei Biosciences Inc., Japan) and freshly prepared ImmPACT DAB (Vector Laboratories Inc., UK) were used for detection. Slides were lightly counterstained with diluted (1:2) Mayer's hematoxylin, dehydrated, clarified and mounted. The staining protocol was carried out with Autostainer 480 (Lab Vision, CA, USA) immunostainer. Human breast carcinoma cell line MDA-MB-157 (obtained from ATCC) is previously shown to carry *CCNE1* amplification [26,12] and was used as a positive control in each hybridization batch.

Analysis of chromogenic in situ hybridization results

CISH slides were examined thoroughly with Olympus System Microscope BX43 using magnification of 400-600. *CCNE1* amplification status was determined according to the number of signals (gene copy number) seen in cancer cells. Criteria for determining *CCNE1* amplification were following: copy number ≥6 or presence of gene copy clusters.

Follow-up of patients and determination of relapse pattern and survival

Survival analyses were performed on primary breast cancer patients with no distant metastases (n=193, T1-3, N0-3, M0) at diagnosis. In order to determine relapse-free survival (RFS), patients were followed from the date of surgery to progression of the disease. Patients who did not relapse were censored at the time of cancer-unrelated death or the date of the last clinical follow-up visit.

Statistical data analysis

Associations between cyclin E expression, *CCNE1* amplification and other variables were tested using Pearson's chi-square test, Fisher's two-tailed exact test (whenever applicable) and two-tailed Mann-Whitney test. Survival analyses were performed using Kaplan-Meier method. Multivariate log-rank test (Mantel-Cox) was used to compare survival curves to assess the prognostic effects of clinicopathological features and other covariates on survival. All statistical tests were performed using BMDP version 4.0 (BMDP Statistical Software Inc.) or GraphPad Prism version 4.0 (GraphPad Software Inc.). Generally, *p*-values below 0.05 were considered statistically significant.

RESULTS

Clinicopathological characteristics

Patients' oncological treatment followed current clinical practice at the time of diagnosis. In general, patients diagnosed between January 2003 and September 2005 did not receive adjuvant trastuzumab based chemotherapy. Patients diagnosed after September 2005 received nine week trastuzumab-based treatment administered according to the schema by Joensuu et al. [22]. Nine cases were diagnosed with distant metastatic disease (pM1) and were excluded from the disease-free survival analyses. Altogether, there were 87 patients (45.1%) that were treated with adjuvant trastuzumab and 106 patients (54.9%) who were treated with other adjuvant chemotherapy. Characteristics and clinicopathological features of patient cohorts were compared. In trastuzumab untreated patient cohort there were more ER positive tumors (Fisher's exact test, p=0.0066), while in trastuzumab treated cohort there were more patients with family history of breast or ovarian cancer (Fisher's exact test, p=0.0035). No other statistically significant differences were found between these patient
cohorts.

Analysis of cyclin E over-expression

Cyclin E expression was determined with immunohistochemistry. Staining for cyclin E was predominantly nuclear, displaying moderate or strong intensity. Inter- and intratumoral differences were seen in staining pattern and faint cytoplasmic staining was seen in some tumors. The proportion of cyclin E positive cells ranged from 0 to 100%. The frequency histogram of the tumors by cyclin E in 10% unit classes is shown in Figure 1. Majority of the tumors were clearly cyclin E positive. Only 3.5% (7 of 202) of all samples stained weakly (<5% of cancer cells) by cyclin E antibody. Representative examples of the cyclin E stainings are shown in Figure 2A. Cyclin E immunoreactivity was classified to low (<50%) and high (≥50%) categories according to the percentage of stained nuclei in cancer cells. Nearly two-thirds (63.4%, n=128 of 202) of tumors were classified as low and 36.6% (n=74 of 202) as high with respect to the cyclin E expression level.

Statistically significant correlations were found between cyclin E expression and negative estrogen (p=0.0007) and progesterone receptor status (p=0.0003), high Ki67 staining index (p=0.0001), tumor grade (p=<0.0001) and size (p=0.0197, p=0.0310). Correlations with other variables were not found (see Figure 3). Table I depicts patients' clinicopathological characteristics according to cyclin E expression level.

Analysis of *CCNE1* amplification

Overall 8.1% (15 of 185) of HER-2-positive breast cancer cases were shown to harbor *CCNE1* amplification. Tumor samples with *CCNE1* amplification showed significantly higher cyclin E expression levels (Mann-Whitney, two-tailed $p=<0.0001$, $U=442.0$). An example of *CCNE1* amplification detected by CISH is shown in Figure 2B. Correlations between *CCNE1* amplification and high Ki-67 staining index were also statistically significant (Mann-Whitney, two-tailed $p=0.0014$, $U=640.0$). No statistically significant association was found between *CCNE1* amplification and tumor size, hormone receptor status, grade or nodal involvement. The correlations between *CCNE1* amplification and studied clinicopathological parameters are shown in Figure 4 (A-G).

In addition to clinical FFPE-samples, cyclin E expression and *CCNE1* amplifications were studied also in breast cancer cell lines. Variation in staining pattern was seen also in this material. Interestingly, we found that trastuzumab resistant JIMT-1 cell line, established in our laboratory [27], display cyclin E over-expression and carry *CCNE1* amplification.

Analysis of patients' survival and factors prognostic to relapse

Relapse-free survival (RFS) was the primary outcome and was determined from the date of surgery (or date of diagnosis if not operated) to the date of relapse or ending of follow-up. Median follow-up period for RFS was 65 months, ranging from two to 108 months. RFS curves were estimated by the Kaplan-Meier method, adjusted for treatment and clinicopathological features and compared by log-rank test (Mantel-Cox).

Neither cyclin E expression level (log-rank test, $p = 0.4939$, hazard ratio 1.269, 95% CI of ratio 0.6512 to 2.432) nor *CCNE1* amplification (log-rank test, $p = 0.9801$, hazard ratio 0.9820, 95% CI of ratio 0.2376 to 4.059) were shown to predict appearing of relapse and can not be considered as prognostic factors to RFS (Figures 5A, 5B). Ki67 staining index and steroid receptor status were not either found as prognostic factors to relapse (log-rank test, p-values for Ki67 0.5863, ER 0.0865 and PR 0.1311).

Survival analysis shows significantly increased risk of relapse associated with lymph nodal involvement (HR 0.2459, 95% CI of ratio 0.1263 to 0.4827, $p=<0.0001$, log-rank test). Overall 32.6% (28 out of 86) of breast cancer patients diagnosed with positive axillary lymph nodes were relapsed, while only 10.3% (11 out of 107) of node-negative patients had relapse. Tumor size (pT status) correlated positively with occurring of relapse. Patients with bigger breast tumor relapsed significantly more probably (log-rank test, $p=0.0001$) than patients with minor tumors.

Performed analyses did not demonstrate that adjuvant trastuzumab treatment (9-week regimen) is associated with a longer relapse-free survival (log-rank test, $p=0.4204$). During the follow-up period, 17.2% of (15 out of 87) adjuvant trastuzumab treated patients were suffering from relapse. Consistently, 21.7% (23 out of 106) of patients that were untreated with adjuvant trastuzumab were relapsed. Cyclin E expression level was not shown prognostic to relapse in HER2-amplified breast cancer patients treated with adjuvant trastuzumab therapy (Figure 5D) or in patients treated with other therapy options (Figure 5C).

DISCUSSION

We undertook a retrospective immunohistochemical analysis of cyclin E protein expression in HER-2-positive breast carcinomas and correlated expression levels with tumor histopathological characteristics, patient clinical features and pattern of relapse. We found that cyclin E was expressed in a majority of

cancer cases analyzed. Nearly 40% of HER-2-positive breast tumors were shown to have high (>50% stained nuclei) cyclin E expression level. According to earlier studies [10,17,28], over-expression of cyclin E is more common in HER-2-positive breast cancers than in HER-2-negative cancers. It has been shown that cyclin E immunoreactivity is found only in breast malignancies and is therefore associated with more aggressive features [9]. In cancer cells, cyclin E is constitutively expressed due to disturbed cell cycle regulation that appears eg. as active cyclin E-Cdk2 complexes throughout the cycle [29]. Amplification of the cyclin E coding gene *CCNE1* leads also to increased cyclin E mRNA and protein expression levels [26,12].

In the current study cyclin E expression level was shown to positively correlate with tumor grade and size, high Ki67 staining index and negative steroid receptor (ER and PR) status. These results are comparable to the findings made in earlier studies [16,30,17]. But, also opposite results have been published [31]. Cyclin E was shown to be significantly over-expressed at protein level when the coding gene *CCNE1* was amplified, as has been recently demonstrated also by other researchers [12].

According to our study, prevalence of *CCNE1* amplification in HER-2-positive breast cancer was quite low (8.1%). Totally 185 breast tumors were analyzed by *CCNE1* CISH. This result is comparable to earlier study by Moelans et al. [32]. They detected co-amplification of *ERBB2* and *CCNE1* in 7% of studied invasive breast carcinomas (n=104). Natrajan et al. [12] have recently determined amplification frequency of 19q12 locus (location of its driver gene *CCNE1*) in grade III primary breast cancers using microarray comparative genomic hybridization (aCGH). In their study only 3% (n=2/64) of HER-2 positive patients was shown to have 19q12 amplification. In the same study, *CCNE1* amplification was found exclusively in one of 64 examined HER-2-positive breast tumors and was not significantly associated with HER-2 status. According to a recent study [11] performed on 55 HER-2-positive breast cancers, the incidence of *CCNE1* amplification or over-expression was 35%. The proportion of clear amplifications was not specified. Scaltriti et al. [11] analyzed also aCGH data set of 595 breast cancers [33] and estimated that *CCNE1* amplification occurs in 20% of HER-2-positive breast cancers.

In other studies prevalence of *CCNE1* amplification in primary breast cancer cases that were not sorted out by HER-2 status varied from 1.6% [12] to 12% [32]. In a study of Callagy et al. [34] *CCNE1* amplifications were seen in 6% of studied primary breast cancer cases (n=187). Although cyclin E protein over-expression has previously been associated with positive HER-2 status, it is not clearly indicated that *CCNE1* amplification is more common in HER-2-positive breast cancer than in primary breast cancer with negative HER-2 status.

Despite of extensive studies on significance of cyclin E expression in prediction of breast cancer progression into relapse, its role from clinical perspective is still ambiguous due to the conflicting data. According to a study by Kim et al. [31], cyclin E over-expression in primary breast cancer can independently predict the risk of distant, especially visceral relapse after curative breast surgery. Mittendorf et al. [21] demonstrated that breast cancer patients with HER-2 over-expression and high levels of cyclin E had decreased 5-year disease specific survival compared with those expressing low levels of cyclin E. In another study survival was worse in patients with tumors over-expressing cyclin E together with a lack of steroid receptors in spite of nodal status [17].

In a meta-analysis comprising of over 2500 breast cancer patients and 12 independent studies [18] it was shown that high levels of cyclin E appears to be an independent prognostic factor to overall and breast cancer specific survival, but not to relapse-free survival (RFS). Similar results were obtained also in a recent meta-analysis comprising of 7759 patients from 23 eligible studies [19]. Also Lindahl et al. [35] found correlation between cyclin E over-expression and poor overall survival. However, not all researchers confirm the prognostic significance of cyclin E over-expression [36,16,37]. In a study of Peters et al. [38] it was conversely shown that cyclin E over-expression was associated with a longer recurrence-free survival. Additionally, Berglund et al. [39] pointed out in their wide study that cyclin E expression correlates

with less infiltrative growth, which is in contradiction with studies linking high cyclin E with more aggressive tumor behavior.

Cyclin E expression is significantly associated with metastasis-free survival in lymph node-negative breast cancer [40]. Sieuwerts et al. [41] have got similar results by measuring mRNA levels with quantitative real-time PCR to evaluate the prognostic value of cyclin E mRNA in lymph-node negative breast cancer. According to Potemski et al. [42], high expression of cyclin E is a significant factor of poor prognosis, especially in the node-positive group (n=174). In this study the cut-off level for cyclin E over-expression was remarkably lower; samples with ≥2% of stained tumor cell nuclei were considered cyclin E overexpressing. In other studies the percentage of cells showing nuclear staining by cyclin E antibody have been varied from 5 to 50% when the tumors were considered cyclin E over-expressing ones. In some studies the cut-off level is not even reported.

Based on the results of the current study, expression level of cyclin E does not correlate with relapse-free survival (RFS) in HER-2-positive breast cancer. Differences were not seen in adjuvant trastuzumab treated (9-week regimen) and non-trastuzumab treated patient cohorts. But, it is noteworthy that in non-trastuzumab treated patient cohort there were more ER-positive cancer cases, which could have influenced on our results. It is well known that patients with HER-2 and ER-positive breast cancer have better prognosis when compared to ER-negative HER-2-positive cases [43]. According to a recent study by Vaz-Luis et al. [44] patients diagnosed with hormone receptor positive HER-2-positive breast cancer were less likely to experience recurrence. However, in the present study Kaplan Meier curves did not shown better RFS for ER-positive breast cancer cases when the adjuvant chemotherapy was not taken into account. Statistically significant difference in relapsing rate of ER-positive and ER-negative breast cancer cases was not either seen in the log-rank test (p=0.1266, HR 1.645, 95% CI of ratio 0.8606 to 3.360).

In this study *CCNE1* amplification showed no prognostic role in HER-2-positive breast cancer. *CCNE1*-amplified HER-2-positive tumors were those with high Ki-67 index and high cyclin E expression level. Callagy et al. [34] detected also correlation between *CCNE1* amplification and cyclin E over-expression. Contrary to the findings of Callagy et al. [34] and Moelans et al. [32], present study claims that *CCNE1* amplification is not significantly associated with ER negativity. But, in contrast to the current study, in these two earlier studies both HER-2 positive and negative breast cancer cases were included. In a study of Callagy et al. [34], *CCNE1* amplification was not either found to be associated with survival in breast cancer (n=232, median follow-up 4.8 years). Scaltriti et al. [11] claim that cyclin E amplification/over-expression is associated with a lower progression-free survival in trastuzumab-treated HER-2-positive breast cancer patients. But in their study only few patients were included (n=34). Other studies on prognostics of *CCNE1* in breast cancer were not found.

Taken together, our results suggest that cyclin E expression and *CCNE1* amplification have no prognostic relevance in HER-2-positive primary breast cancer. Progression of this cancer type could be predicted only by the presence of positive axillary lymph nodes and bigger tumor size. In contrast, earlier study [45] claims that high level of cyclin E is more important prognostic factor than the presence of positive lymph nodes in primary breast cancer.

Low-molecular weight isoforms (LMW) of cyclin E are formed as a result of alternative posttranslational cleavage of full-length cyclin E. LMW forms are detected significantly more frequently in breast tumor tissue than in adjacent normal breast tissue due to differences in cyclin E processing. These forms have more efficient binding ability to Cdk2 and are shown to have enhanced oncogenic activity. (Harwell et al., 2000; Wingate et al., 2009) [46,47] According to Porter et al. [48], LMW forms can more readily induce G1-S transition than the full-length form of cyclin E does. Over-expression of LMW isoforms is suggested to induce chromosome instability in the cell, but the exact mechanism is still to be elucidated [49].

Consequently, over-expression of these forms is associated with decreased survival in breast cancer [45,21]. However, also opposite results have been published. According to a study by Tokai et al. [50], presence of LMW isoforms of intact cyclin E has no prognostic role in breast cancer. In multivariate analysis patient survival was not correlated with either form of cyclin E. LMW fragments are shown to preferentially accumulate in the cytoplasm of cancer cells [51]. In the current study we focused on analyzing of nuclear staining by cyclin E antibody so we could not define which staining was due to presence of intact full-length cyclin E and which due to LMW fragments.

CONCLUSIONS

According to this study cyclin E expression in the HER-2-positive breast cancer correlates with negative hormone receptor status, Ki-67 labeling index, tumor grade and size. Incidence of *CCNE1* amplification in HER-2-positive breast cancer was quite low: 8.1%. *CCNE1* amplification was shown to correlate positively with cyclin E expression and Ki-67 labeling index.

Expression level of cyclin E and *CCNE1* amplification were not identified as independent prognostic factors of relapse in HER-2-positive breast cancer. Differences were not seen in adjuvant trastuzumab treated or non-trastuzumab treated patient cohorts. Cyclin E is not recommended to be used in clinical practice as prognostic marker in HER-2-positive breast cancer.

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Compliance with ethical standards: This study was approved by the local ethics committee (R07082).

Conflicts of interest: None

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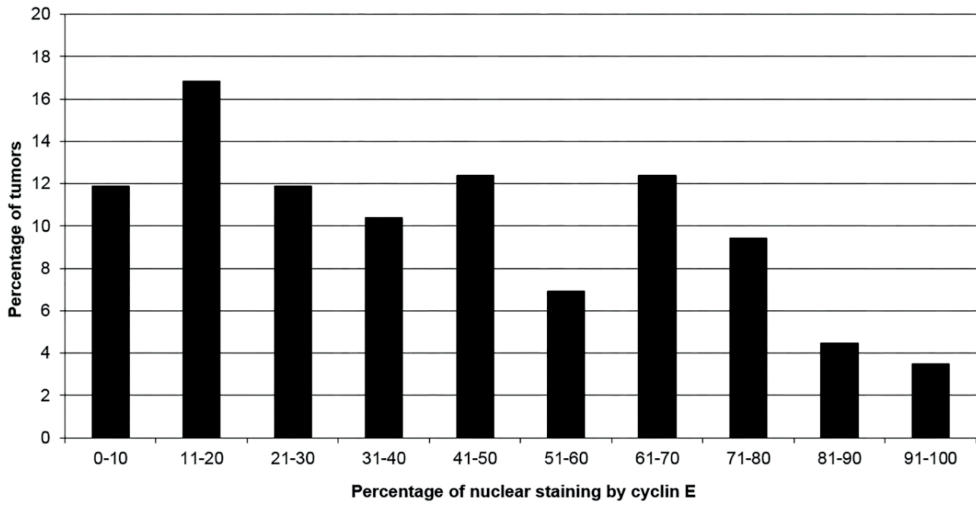

Figure 1. Distribution of cyclin E immunoreactivity in the HER-2-positive breast cancer (*n*=202).

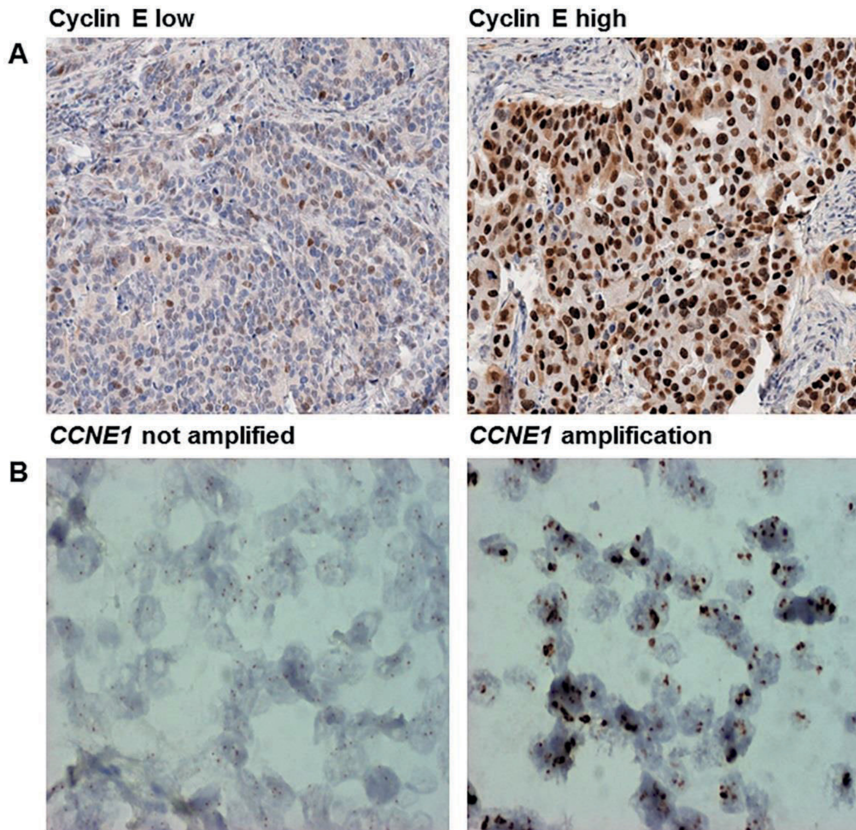

Figure 2. Examples of cyclin E immunohistochemistry (A) and corresponding chromogenic in-situ hybridization of *CCNE1* gene (B). Counterstaining with Mayer's hematoxylin. Magnifications: 200X (A) and 400X (B).

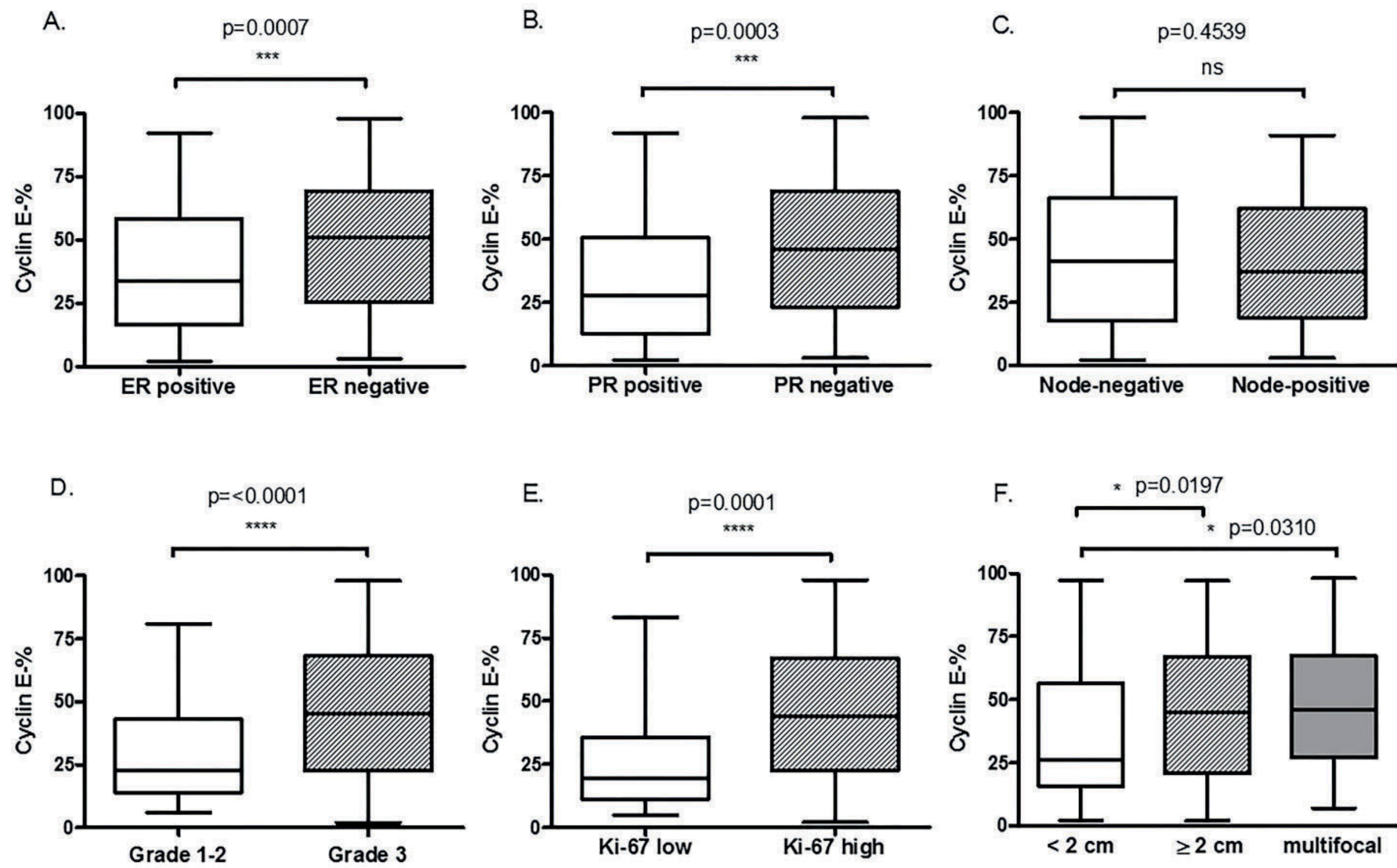

Figure 3. Correlations between cyclin E immunoreactivity and hormone receptor status (A, B), nodal involvement (C), tumor grade (D), Ki-67 index (E) and tumor size (F). Abbreviations: * = statistically significant result ($p \leq 0.05$), *** = statistically significant result ($p \leq 0.001$), **** = statistically significant result ($p \leq 0.0001$), ns = no statistical significance ($p > 0.05$).

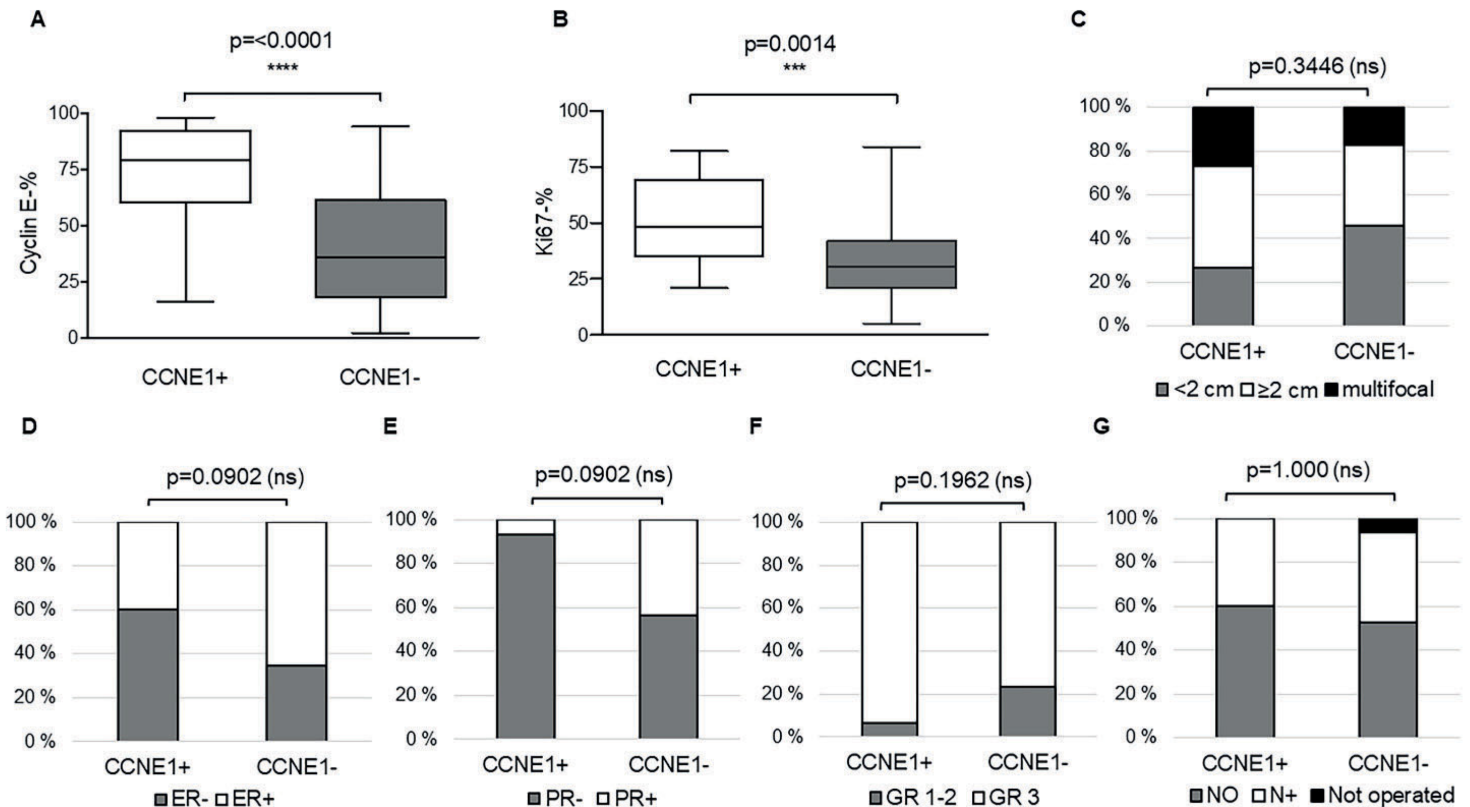

Figure 4. Correlations between *CCNE1* amplification and cyclin E immunoreactivity (A), Ki-67 index (B), tumor size (C), hormone receptor status (D, E), grade (F) and nodal involvement (G). Abbreviations: CCNE1+ = *CCNE1* amplification, CCNE1- = no *CCNE1* amplification, **** = statistically significant result ($p \leq 0.001$), *** = statistically significant result ($p \leq 0.001$), ns = no statistical significance.

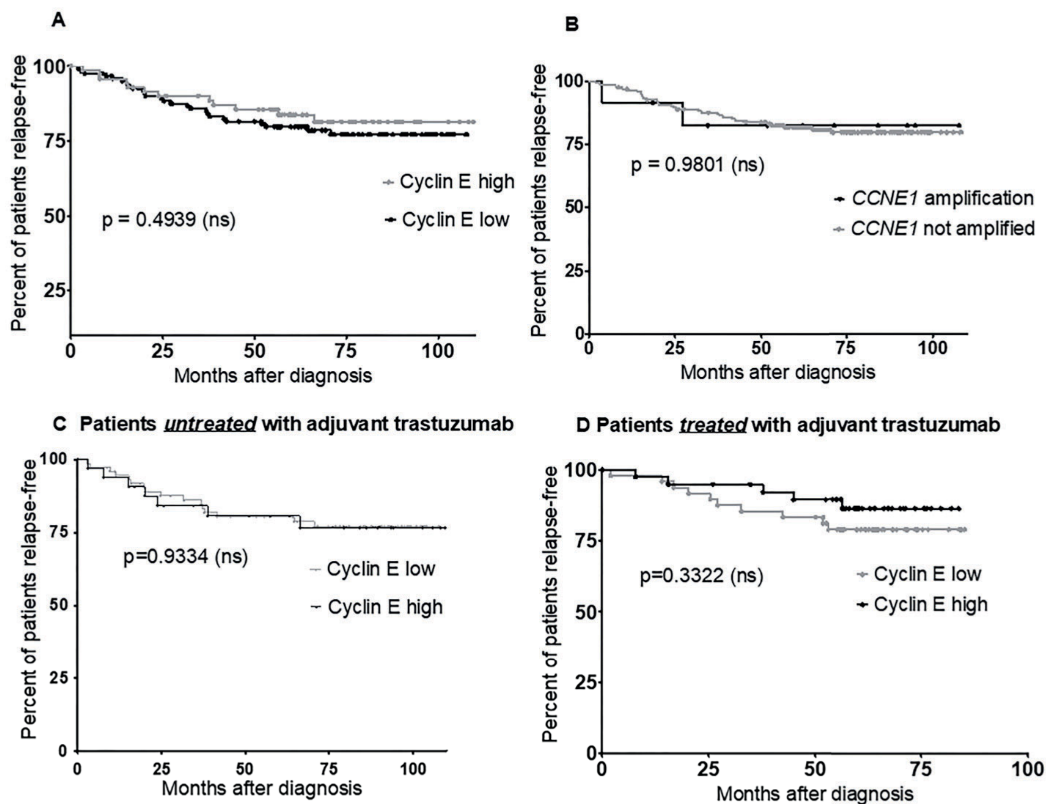

Figure 5. Kaplan-Meier survival plots for relapse-free survival (RFS) of HER-2-positive breast cancer patients stratified according to cyclin E expression (A) and *CCNE1* amplification (B). RFS of HER-2-positive breast cancer patients presented according to cyclin E expression and adjuvant trastuzumab therapy: patients treated without (C) and with (D) trastuzumab. Abbreviation: ns = no statistical significance.

Table I Patient characteristics according to cyclin E expression level.

Patient characteristic	*n*	Cyclin E low *n* (%)	Cyclin E high *n* (%)
Age			
<50 years	48	34 (70.8%)	14 (29.2%)
≥50 years	154	94 (61.0%)	60 (39.0%)
Menopausal status			
Pre-menopausal	47	35 (74.5%)	12 (25.5%)
Post-menopausal	154	93 (60.4%)	61(39.6%)
Tumor grade			
I	2	2 (100.0%)	0 (0%)
II	45	38 (84.4%)	7 (15.6%)
III	150	83 (55.3%)	67 (44.7%)
DCIS + microinvasion	1	1 (100%)	0 (0%)
Tumor size			
<2 cm	84	58 (69.0%)	26 (31.0%)
2-5 cm	70	39 (55.7%)	31 (44.3%)
>5 cm	5	4 (80.0%)	1 (20.0%)
≥10 cm	1	1 (100%)	0 (0%)
Multifocal	34	20 (58.8%)	14 (41.2%)
Tumor histological type			
Ductal	176	109 (61.9%)	67 (38.1%)
Lobular	15	12 (80.0%)	3 (20.0%)
Other	11	7 (63.6%)	4 (36.4%)
Nodal involvement			
Node-negative	107	62 (57.9%)	45 (42.1%)
Node-positive	84	58 (69.0%)	26 (31.0%)
Not known (not operated)	11	8 (72.7%)	3 (27.3%)
Estrogen receptor (ER)			
Negative	77	38 (49.4%)	39 (50.6%)
Positive	125	90 (72.0%)	35 (28.0%)
Progesterone receptor (PR)			
Negative	122	68 (55.7%)	54 (44.3%)
Positive	80	60 (75.0%)	20 (25.0%)
Ki-67 status			
Low	43	35 (81.4%)	8 (18.6%)
High	159	93 (58.5%)	66 (41.5%)
Distant metastases at diagnosis			
No	152	92 (60.5%)	60 (39.5%)
Yes	9	5 (55.6%)	4 (44.4%)
Not known	37	28 (75.7%)	9 (24.3%)
***CCNE1* amplification**			
Not amplified	170	112 (65.9%)	58 (34.1%)
Amplified	15	3 (20.0%)	12 (80.0%)

PUBLICATION
II

Comparison of Antibodies for Immunohistochemistry-based Detection of HER3 in Breast Cancer

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COMPARISON OF ANTIBODIES FOR IMMUNOHISTOCHEMISTRY-BASED DETECTION OF HER3 IN BREAST CANCER

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ABSTRACT

Background: Growth factor receptor HER3 (*ErbB3*) lacks standardized immunohistochemistry (IHC) -based methods for formalin-fixed paraffin-embedded (FFPE) tissue samples. We compared 4 different anti-HER3 antibodies to explain the differences found in the staining results reported in the literature.
Materials and Methods: Four commercial HER3 antibodies were tested on FFPE samples including mouse monoclonal antibody clones, DAK-H3-IC and RTJ1, rabbit monoclonal antibody clone SP71, and rabbit polyclonal antibody (SAB4500793). Membranous and cytoplasmic staining patterns were analyzed and scored as 0, 1+, or 2+ according to the intensity of the staining and completeness of membranous and cytoplasmic staining. A large collection of *HER2*-amplified breast cancers (n=177) was stained with the best performing HER3 antibody. The breast cancer cell line, MDA-453, and human prostate tissue were used as positive controls. IHC results were confirmed by analysis of flow cytometry performed on breast cancer cell lines. Staining results of FFPE samples were compared with samples fixed with an epitope-sensitive fixative (PAXgene).
Results: Clear circumferential cell membrane staining was found only with the HER3 antibody clone DAK-H3-IC. Other antibodies (RTJ1, SP71, and polyclonal) yielded uncertain and nonreproducible staining results. In addition to cell membrane staining, DAK-H3-IC was also localized to the cytoplasm, but no nuclear staining was observed. In *HER2*-amplified breast cancers, 80% of samples were classified as 1+ or 2+ according to the HER3 staining on the cell membrane. The results from FFPE cell line samples were comparable to those obtained from unfixed cells in flow cytometry. IHC conducted on FFPE samples and on PAXgene-fixed samples showed equivalent results.
Conclusions: We conclude that IHC with the monoclonal antibody, DAK-H3-IC, on FFPE samples is a reliable staining method for use in translational research. Assessment of membranous HER3 expression may be clinically relevant in selecting patients who may most benefit from pertuzumab or other novel anti-HER3 therapies.

Key Words: HER3, ErbB3, DAK-H3-IC, immunohistochemistry, antibody, breast cancer

INTRODUCTION

HER3 is a transmembrane receptor tyrosine kinase encoded by the *ERBB3* gene at chromosome band 12q13. Along with the homologous receptors EGFR (*HER1*, *ErbB1*), HER2/neu (*ErbB2*) and HER4 (*ErbB4*), HER3 is a member of the type I growth factor receptor family[1]. ErbB receptors are important signaling molecules that are closely associated with cancer development and progression[2]. HER3 typically interacts with HER2 via heterodimerization[3,4]. Dimerization induces subsequent activation of PI3K/Akt and MAPK signaling cascades[1]. HER2-positive breast cancer is characterized by constitutive signaling of 2 key oncogenes, *HER2* and *HER3*[5,6] , and therefore the role of overexpressed HER3 has recently been highlighted in the pathogenesis of this type of breast cancer[4,6]. The contribution of HER3 in signaling cascades is regulated through diverse mechanisms, including transcriptional, posttranscriptional, translational and posttranslational control[5]. Unlike *HER2*, *HER3* is not known to undergo gene amplification[7].

HER3, in association with HER2, has a critical role in the control of breast cancer growth and invasion. Both receptor types are needed to drive breast tumor cell proliferation[4,8]. In anticancer therapy, HER3 is thought to play a role in resistance to HER2-directed breast cancer therapies, such as trastuzumab[9,10] and trastuzumab emtansine (T-DM1)[11]. *In vitro* studies have implied that breast cancers driven primarily by HER2 overexpression and HER2 homodimers are more susceptible to trastuzumab than tumors driven by HER2-HER3 heterodimers[12,13]. In this context, HER3 can be regarded as a novel, potential target in breast cancer therapy. Moreover, it has been proposed that dual-blocking of HER2 and HER3 may be valuable in breast cancers driven by elevated expression of HER2 and HER3, even in the absence of *HER2* amplification[14]. In the context of dual anti-HER2 therapy, it becomes necessary to identify prognostic and predictive factors that can be used to designate therapy options for HER2-positive breast cancer[15]. This forms the basis for translational studies of HER3 expression.

Expression of HER3 in breast cancer has been widely studied by performing immunohistochemistry (IHC) with a multitude of antibodies and staining protocols (Table I, supplemental material). Currently, there are no standardized or universally accepted methods for the detection of HER3 expression by IHC. Comparison of published studies reveals almost no consistency in the reported results. Our aim was to study and optimize an IHC method for the evaluation of membranous and cytoplasmic HER3 in breast carcinoma. We concentrated on using HER2-positive breast cancer as study material because of the relevant association with HER3 in the dimerization process.

MATERIALS AND METHODS

Sample Material and Antibodies

The training set used in preliminary antibody testing consisted of archival formalin-fixed paraffin-embedded (FFPE) breast carcinomas and FFPE samples prepared from pelleted breast cancer cell cultures. The study set consisted of 177 HER2-positive primary breast carcinomas (FFPE) and 10 breast cancer cell lines. Gene amplification status of *HER2* was confirmed by chromogenic *in situ* hybridization (CISH). Breast carcinoma sample pairs (*n*=13) consisting of FFPE and PAXgene-fixed (PreAnalytiX GmbH, Hombrechtikon, Switzerland) tumor samples were also studied to assess the effects of fixation on preservation of HER3 antigens. Ethical approval for the use of clinical samples was obtained from the local ethics committee (R07082).

Adjacent sections (4 to 5 μm thick) were stained with four different HER3 antibodies using various protocols. The antibodies tested included mouse monoclonal antibody clones DAK-H3-IC (Dako, Denmark) and RTJ1 (Leica Biosystems, UK), rabbit monoclonal antibody clone SP71 (Spring Bioscience,

CA) and rabbit polyclonal antibody SAB4500793 (Sigma-Aldrich). DAK-H3-IC, RTJ1 and SP71 antibodies were raised against the intracellular (C-terminal) domain of HER3, whereas polyclonal antibody (SAB4500793) is raised against the extracellular (N-terminal) domain of HER3. All these antibodies are specified for application in IHC. Characteristics of tested antibodies are listed in the Table 1.

IHC Staining Protocol

Slides were deparaffinized with hexane (2×5 min) and dehydrated with absolute ethanol. Heat-induced antigen retrieval (HIER) was performed by boiling slides in TE-buffer (50 mM Tris 1 mM EDTA, pH 9) at +98°C for 15 minutes. Ultra Vision Protein Block (Lab Vision Corporation, Fremont CA, USA) was used to reduce background staining. Slides were then incubated with HER3 antibody (30 min, RT) diluted in Normal Antibody Diluent (ImmunoLogic, AD Duiven, Netherlands). Bright Vision Plus kit (ImmunoLogic) was used for detection according to the manufacturer's instructions. 3',3-diaminobenzidine tetrahydrochloride ImmPACT DAB (Vector Laboratories Inc., UK) was used as a chromogen and applied on the slides for 5 minutes (RT). Counterstaining was performed with Mayer's hematoxylin. Staining reactions were conducted using a LabVision Autostainer with TBS-Tween (0.05%) as washing buffer. Slides were dehydrated, cleared with xylene and mounted using DePeX mounting medium.

Assay reproducibility was tested by including breast cancer samples of known staining pattern in every staining batch. Breast cancer cell line MDA-453[10] and normal human prostate[16] are known to overexpress HER3 and were used as positive controls.

Optimization of the Staining Protocol

The staining protocol was optimized by applying various antibody concentrations, incubation times and HIER-methods. In addition to the Bright Vision Plus detection system (ImmunoLogic), two other polymer-based systems named Histofine® Simple Stain MAXPO (Nichirei Biosciences Inc., Japan) and UltraVision™ Quanto Detection System HRP DAB (ThermoScientific) were tested. Furthermore, an ultra-sensitive tyramide-based kit CSA II Biotin-free Tyramide Signal Amplification System K1497 (Dako) was also tested.

Microscopic Analysis

Using a light microscope (Olympus BX43), HER3 staining was evaluated within the cancerous area displaying the strongest staining intensity. Samples were classified according to the staining intensity and proportion of specifically stained cancer cells, as displaying negative or low (0), intermediate (1+) or high (2+) HER3 expression. Membranous and cytoplasmic staining patterns were evaluated separately. Criteria for HER3 membrane staining were as follows: (0) no staining or low staining (<10% of cancer cells), (1) >10% of cancer cells display clear circumferential membrane staining, and (2) >30% of cancer cells display clear circumferential membrane staining. Score 1+ on membrane staining was set as a threshold to define HER3-positive tumor. Criteria for HER3 cytoplasmic staining were as follows: (0) no staining at all or only faint staining, (1) overall cytoplasmic staining (low intensity), and (2) high intensity cytoplasmic staining covering most of the cancer cells. Dual-viewing (S.L. and J.I.) of samples was also performed to confirm the results. Staining results were evaluated blindly without reference to other clinicopathologic characteristics of a patient's tumor. For analysis of HER3 membrane staining, ImmunoMembrane software version 1.0i (http://jvsmicroscope.uta.fi/

immunomembrane/) was also used to obtain a semi-quantitative IM (ImmunoMembrane) score (0 to 20 points) on IHC stainings. IM score is formed on the basis of completeness (0 to 10 points) and intensity (0 to 10 points) of cell membrane staining[17].

Flow Cytometric Analysis of Breast Cancer Cell Lines

Cell Lines and Culture Conditions

The intensity of DAK-H3-IC staining was compared with HER3 expression as measured by flow cytometry. Eight HER2-positive breast cancer cell lines (MDA-453, SKBR3, BT-474, HCC1419, JIMT-1, UACC812, MDA-361, EFM-192), one esophageal adenocarcinoma cell line (OE19) and one HER2-negative breast cancer (MDA-231) cell line were selected for this study. Cells were cultured according to instructions given by the cell line suppliers. The JIMT-1 cell line was established in our laboratory[18]. OE19 cells were supplied by Health Protection Agency Culture Collections (HPACC, UK) and EFM-192 cells by Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany). Other cell lines were purchased from American Type Culture Collection (ATCC).

Immunofluorescence Staining for Flow Cytometric Analysis

For the fluorescent staining procedure, pelleted cells harvested from the culture flasks were washed with phosphate-buffered saline (PBS) and blocked using 3% BSA-PBS. Cells were stained with mouse monoclonal HER3 antibody clone H3.90.6 against the extracellular domain of HER3 (Thermo Scientific, Fremont, CA, USA) at a concentration of 20 µg/ml. After a 20-minute incubation, cells were washed with PBS and 3% BSA-PBS. Secondary antibody Alexa Fluor® 488-conjugated Goat Anti-Mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) was used at a concentration of 30 µg/ml. After 40 minutes of incubation time and PBS washes, samples were fixed with 1% paraformaldehyde (diluted in PBS). During the procedure, cells were kept on ice and ice-cold PBS was used for the washes to prevent receptor internalization. After adding the secondary antibody, samples were kept in the dark to prevent the fluorochrome from fading.

Fluorescence intensity data were acquired using the BD Accuri™ C6 flow cytometer (BD Biosciences) by analyzing 20 000 events (cells). Data analysis was performed with cells gated on a dot plot FSC (forward scatter) against SSC (side scatter). Median fluorescence values (PE) were determined from the FL1 histogram. Background fluorescence values were subtracted from the sample values to obtain the exact fluorescence intensity.

RESULTS

Comparison of 4 HER3 Antibodies by IHC

The DAK-H3-IC antibody showed staining for HER3 antigens localized in breast cancer cell membranes and cytoplasm. Staining results by DAK-H3-IC antibody showed negligible variation from batch to batch. Other tested antibodies, RTJ1, SP71 and polyclonal SAB4500793 yielded nonspecific and nonreproducible staining. Staining with RTJ1 was predominantly cytoplasmic with weak or nonexistent membranous staining. SP71 showed nonspecific staining especially in leucocytes surrounding the cancer cells without any membranous or cytoplasmic staining in the adjacent epithelial cells. The polyclonal HER3 antibody displayed strong cytoplasmic and stromal background staining with faint nuclear staining of cancer cells but no membranous staining.

Intratumoral heterogeneity and sample-to-sample variability was observed in staining patterns with all tested HER3 antibodies. In some samples, cell membranes showed intense staining with the DAK-H3-IC antibody, but other tested HER3 antibodies did not show any membranous staining (Figure 1). In most cases, samples classified as HER3 low or negative showed a consisted staining pattern regardless of the antibody being used. Paired samples fixed with 10% neutral-buffered formalin or PAXgene were evaluated to assess the effects of formalin fixation on HER3 antigens. Comparable staining results were obtained with both methods of fixation (data not shown). The staining reaction intensity could not be further enhanced by using a tyramide-based detection system.

Staining differences were also observed in breast cancer cell lines. MDA-453 and HCC1419 cells display high membranous and cytoplasmic HER3 staining, whereas JIMT-1 and SKBR3 cells show notably weaker HER3 staining (Figure 2).

HER3 Analysis of Breast Cancer Cell Lines Using Flow Cytometry

Analysis of flow cytometry was used to obtain quantitative data on HER3 expression in breast cancer cell lines. Fluorescent staining of cells caused an increase in fluorescence intensity when compared to unstained cell controls. In the staining protocol, cells were not permeabilized; only HER3 receptors localized to the cell membrane were detectable. For comparison, paraffin-embedded cell line samples were stained with DAK-H3-IC antibody against the HER3 receptor. Membranous IHC staining of the cells was analyzed using ImmunoMembrane software to obtain a semiquantitative IM Intensity Score that describes the intensity and completeness of cell membrane staining. The correlation between flow cytometry and IHC-based measurements of HER3 expression is shown in Figure 3.

HER3 Staining in Breast Tumors

A large sample collection of *HER2*-amplified primary breast cancers (n=177) was studied using the DAK-H3-IC antibody clone with the optimized staining protocol. Samples show predominantly intermediate or high HER3 membrane staining (80.2%, 142 of 177); low HER3 membrane status was found only in 19.8% (35 of 177) of cases. The cytoplasmic staining reflected a similar pattern (Table 2). In normal human breast tissue, HER3 staining was observed mainly in the luminal epithelial cell layer (Figure 4).

Clinicopathologic Correlations of HER3

High membranous expression of HER3 was strongly associated with positive estrogen receptor (ER) status and a higher patient age (>50 years) at diagnosis. Low cytoplasmic expression of HER3 correlated significantly with a lower patient age, premenopausal status and a bigger tumor size. No other HER3-related clinicopatholologic correlations were found (Table 3).

DISCUSSION

HER3 gene and protein expression has been widely studied using IHC (Table 1, supplemental material), VeraTaq® assays[10], reverse-transcriptase PCR[19,20] and proximity ligation assays (PLA)[21,13]. Despite extensive utilization of IHC, there is currently no universally accepted method for the determination of HER3 status. A significant problem in HER3 expression studies is the lack of validation of HER3 antibodies. We noticed that staining protocols and staining results of earlier HER3-related studies are commonly insufficiently reported (Table I, supplemental material). Because of the variability in methods used for detection of HER3 expression, interpretation of clinical significance should be made with caution.

There is no consensus for analytic criteria (cutoff values) in determining biologically meaningful HER3 overexpression.

Some researchers have evaluated HER3 staining localized to the membrane, because it is the putative site of the receptor tyrosine kinases. Others have also included intracytoplasmic staining without noticeable staining of membranes. In several studies, the HER3 staining pattern is characterized mostly as cytoplasmic (Table I, supplemental material). Using light microscopy to analyze immunohistochemical staining, it is challenging to distinguish between background staining, a recently synthesized receptor trafficked to the outer membrane, or a receptor internalized upon ligand binding. However, one can assume that HER3 receptors localized only to the cell membrane are therapeutically essential because of HER2-HER3 dimerization[1]. Lack of membranous staining may be due to low sensitivity of the antibody or because of artifact-inducing staining conditions. However, when considering the fact that HER3 receptors are constitutively internalized in a clathrin-dependent manner[22], cytoplasmic staining may be reasonable to take into account. In addition, nuclear expression of HER3 has been described[23,24], but its biological relevance is unknown.

A noteworthy difference compared with HER2 expression is that there are no clear criteria for definition of HER3 overexpression. In nonmalignant breast epithelium, HER3 expression is relatively weak. We compared 4 HER3 antibodies and found that specific circumferential membrane staining was obtained with only one of the tested antibodies (DAK-H3-IC). Other tested antibodies demonstrated mainly cytoplasmic and stromal background staining. To our knowledge, the DAK-H3-IC antibody has been used in a few earlier published breast cancer studies (Table I, supplemental material), but other HER3 antibody clones have been more commonly applied.

HER3, in association with *HER2*, is considered a key oncogene in breast cancer and is therefore hypothesized to be a potential target for dual therapy approaches[25,26]. A recent study by Lipton et al.[10] suggests that HER3 is an informative biomarker of clinical outcomes on trastuzumab therapy. According to Lipton et al.[10], patients with HER2-positive and HER3 overexpressing (verified by Vera-Tag® assay) breast cancers were significantly associated with poor response to trastuzumab-based therapy. Thus, low HER3 expression may indicate better trastuzumab responsiveness. However, comparison of results by VeraTag® assays and HER3 IHC are lacking.

It is known that trastuzumab is not able to block the formation of HER2-HER3 heterodimers because it binds to a HER2 domain that is not involved in receptor dimerization[27]. As for pertuzumab, it binds to another HER2 domain and prevents HER2-HER3 dimerization blocking signaling mediated by both of these receptor types[28]. Consequently, pertuzumab, in addition to trastuzumab, is currently preferred for the treatment of metastatic HER2-positive breast cancer[28]. Several *in vitro* studies have shown significant inhibition of HER3-mediated signaling in breast cancer cells, even in cells that are resistant to anti-HER2 antibodies[29]. Antisense oligonucleotides that inhibit cancer cell growth by specific downregulation of HER3 expression have also been developed, along with bispecific antibodies that target both HER2 and HER3 receptors[26]. These new approaches may be preferable for the treatment of breast cancer characterized by *HER2* amplification and HER3 overexpression.

In breast cancer diagnostics, IHC is a standard method for the detection of hormone receptor status, HER2 expression and proliferation index via Ki-67 expression[30]. On the basis of the results of this study, we consider IHC as a technically feasible method for determining HER3 expression in current translational research and for use in the future to select patients who are most likely to benefit from HER3-related therapies.

The role of HER3 as a prognostic factor has remained controversial (Table I, supplemental material). A likely explanation is the variability in HER3 antibodies and IHC protocols, as well as selection of an optimal threshold for overexpression. Despite the biological interaction between HER2 and HER3, only

a few studies have concentrated on determining the prognostic role of HER3 in HER2-positive breast cancer.

CONCLUSIONS

On the basis of our experience, we prefer the DAK-H3-IC antibody clone for determination of HER3 expression by IHC. This antibody demonstrated clear circumferential membrane staining with less cytoplasmic and stromal background staining compared with the other tested antibodies. A reliable IHC method for staining against can be used to define subtypes of HER2-positive breast cancer and may be of clinical importance in identifying patients eligible for treatments with dual anti-HER2 and anti-HER3 therapy approaches.

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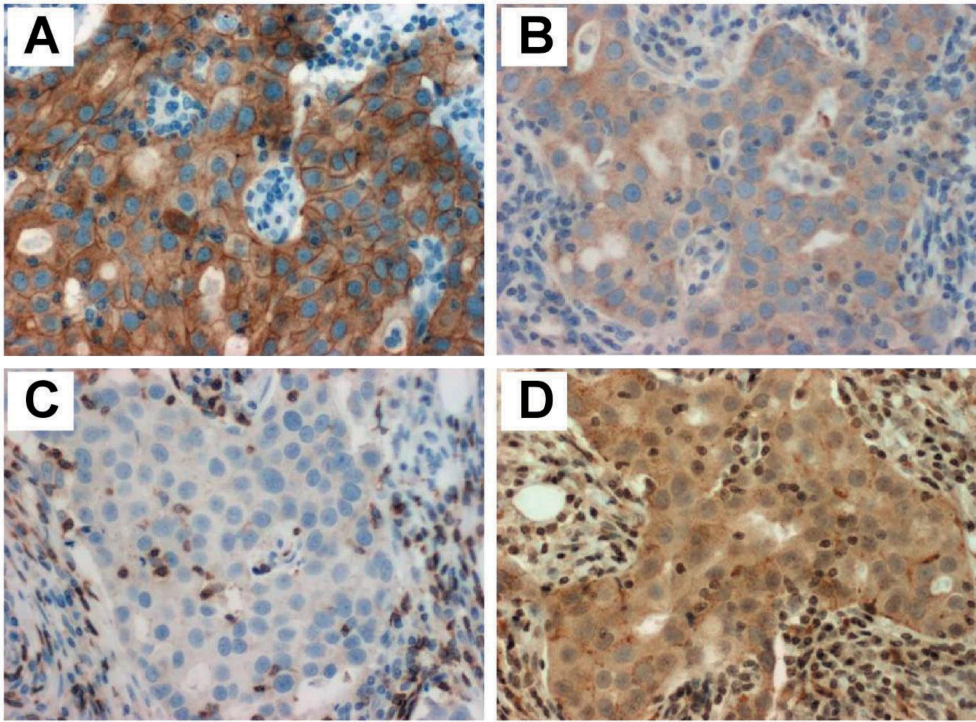

FIGURE 1 Comparison of IHC staining results obtained with different HER3 antibodies: DAK-H3-IC (A), RTJ1 (B), SP71 (C) and polyclonal SAB4500793 (D) on consecutive sections of primary breast cancer sample. According to staining performed with DAK-H3-IC, membranous HER3 staining was 2+ and cytoplasmic staining was 1+. Magnification ×200.

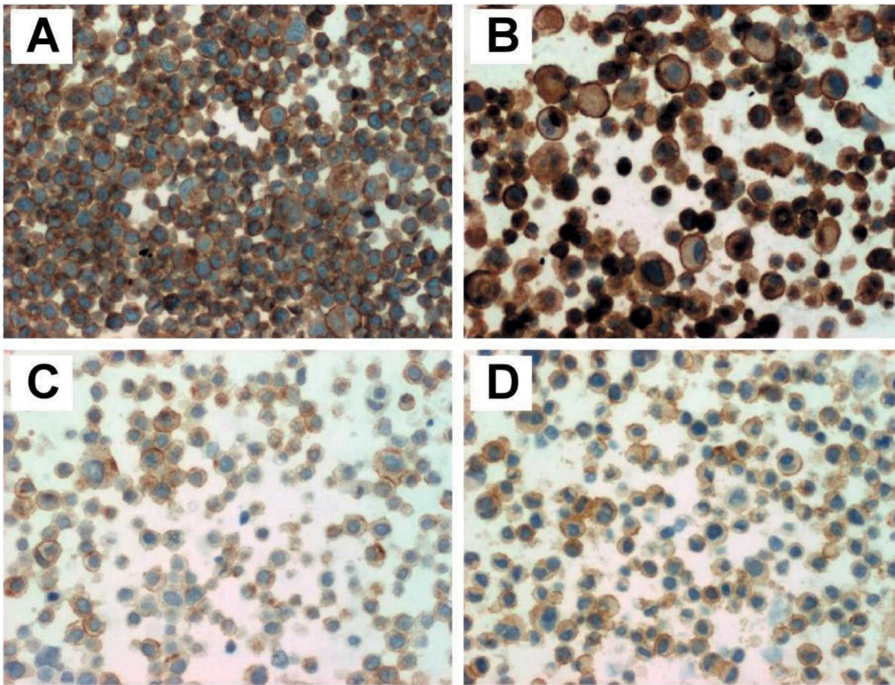

FIGURE 2 Breast cancer cell lines with different HER3 expression statuses. MDA-453 (A) and HCC1419 (B) display high membrane and cytoplasmic staining. JIMT-1 (C) shows low membrane and cytoplasmic staining. SKBR3 (D) shows intermediate staining. IHC was performed using DAK-H3-IC antibody (1:100, 1h incubation time). Magnification ×200.

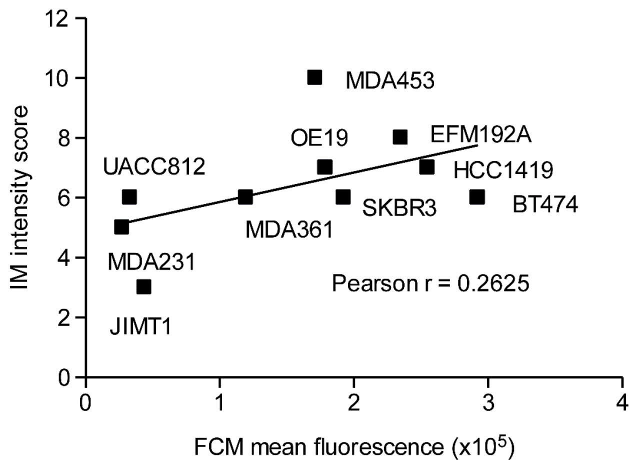

FIGURE 3 Correlation (Pearson, r=0.2625) between IM Intensity Score (ImmunoMembrane) of HER3 IHC staining and mean fluorescence intensity measured with flow cytometry in cancer cell lines. The HER3 antibody clone used in flow cytometry was H3.90.6 and clone DAK-H3-IC was used for determining IM Intensity Scores.

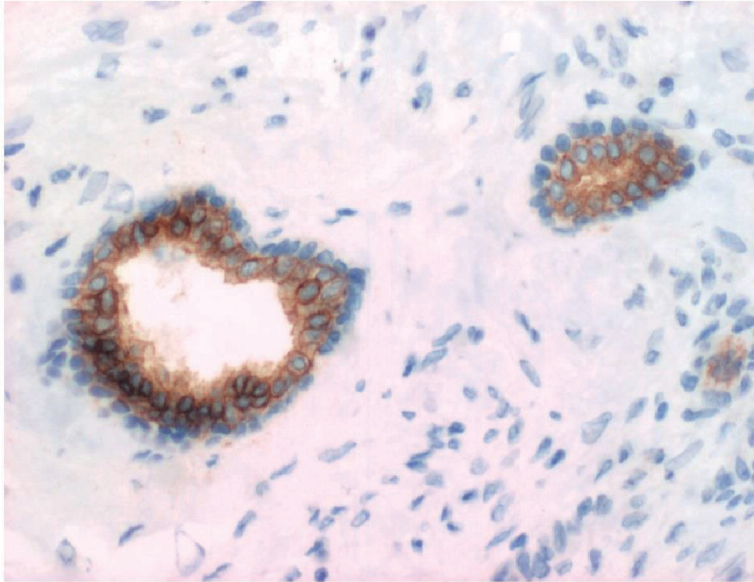

FIGURE 4 High membranous HER3 expression is observed in normal luminal epithelial cells in human breast tissue. IHC was performed using DAK-H3-IC antibody (1:100, 1h incubation time). Magnification ×200.

TABLE I (Supplemental Digital Content I, available at http://links.lww.com/AIMM/A121)

TABLE 1 Characteristics of compared primary antibodies for ErbB3 (HER3).

Clone	Host/Isotype	Conc. (mg/L)	Source
DAK-H3-IC	Mouse/IgG2a, kappa	122	DAKO A/S, Glostrup, Denmark
SP71	Rabbit/IgG	*	Spring Bioscience, Pleasanton, CA
RTJ1	Mouse IgM	*	Leica Biosystems, Newcastle Upon Tyne, UK
	Polyclonal(SAB4500793)	1	Sigma-Aldrich, St. Louis, MO

*Information not provided by the manufacturer

TABLE 2 HER3 expression in *HER2*-amplified breast cancers (*n*=177) according to the cellular localization of a positive IHC reaction.

	Low	Intermediate	High
Cell membrane staining	19.8 %	31.1 %	49.1 %
Cytoplasmic staining	8.5 %	28.8 %	62.7 %

TABLE 3 Clinicopathological correlations of membranous and cytoplasmic HER3 staining in *HER2*-amplified breast cancers (*n*=177) (Pearson's chi-square test).

	Membranous HER3	**Cytoplasmic HER3**
Estrogen receptor (ER)	*P*=0.0127	NS
Progesterone receptor (PR)	NS	NS
Ki-67 proliferation index	NS	NS
Tumor grade	NS	NS
Tumor histological type	NS	NS
Tumor size	NS	*P*=0.0000*
Nodal involvement	NS	NS
Patient's age at diagnosis	*P*=0.0216	*P*=0.0001
Patient's menopausal status	NS	*P*=0.0002
Family history (breast ca.)	NS	NS

* Inverse association

NS indicates not statistically significant

PUBLICATION
III

Clinicopathological and prognostic correlations of HER3 expression and its degradation regulators, NEDD4-1 and NRDP1, in primary breast cancer

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RESEARCH ARTICLE Open Access

Clinicopathological and prognostic correlations of HER3 expression and its degradation regulators, NEDD4–1 and NRDP1, in primary breast cancer

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Abstract

Background: Human epidermal growth factor receptor HER3 (ErbB3), especially in association with its relative HER2 (ErbB2), is known as a key oncogene in breast tumour biology. Nonetheless, the prognostic relevance of HER3 remains controversial. NEDD4–1 and NRDP1 are signalling molecules closely related to the degradation of HER3 via ubiquitination. NEDD4–1 and NRDP1 have been reported to contribute to HER3-mediated signalling by regulating its localization and cell membrane retention. We studied correlations between HER3, NEDD4–1, and NRDP1 protein expression and their association with tumour histopathological characteristics and clinical outcomes.

Methods: The prevalence of immunohistochemically detectable expression profiles of HER3 (n = 177), NEDD4–1 (n = 145), and NRDP1 (n = 145) proteins was studied in primary breast carcinomas on archival formalin-fixed paraffin-embedded (FFPE) samples. Clinicopathological correlations were determined statistically using Pearson's Chi-Square test. The Kaplan-Meier method, log-rank test (Mantel-Cox), and Cox regression analysis were utilized for survival analysis.

Results: HER3 protein was expressed in breast carcinomas without association with *HER2* gene amplification status. Absence or low HER3 expression correlated with clinically aggressive features, such as triple-negative breast cancer (TNBC) phenotype, basal cell origin (cytokeratin 5/14 expression combined with ER negativity), large tumour size, and positive lymph node status. Low total HER3 expression was prognostic for shorter recurrence-free survival time in *HER2*-amplified breast cancer (p = 0.004, p = 0.020 in univariate and multivariate analyses, respectively). The majority (82.8%) of breast cancers demonstrated NEDD4–1 protein expression - while only a minor proportion (8. 3%) of carcinomas expressed NRDP1. NEDD4–1 and NRDP1 expression were not associated with clinical outcomes in *HER2*-amplified breast cancer, irrespective of adjuvant trastuzumab therapy.

Conclusions: Low HER3 expression is suggested to be a valuable prognostic biomarker to predict recurrence in *HER2*-amplified breast cancer. Neither NEDD4–1 nor NRDP1 demonstrated relevance in prognostics or in the subclassification of *HER2*-amplified breast carcinomas.

Keywords: HER3, ErbB3, NEDD4–1, NRDP1, FLRF, RNF41, Prognostic biomarker, Survival, Breast cancer

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Background

Human epidermal growth factor receptor HER3 (ErbB3), a cell membrane-associated protein encoded by the *ERBB3* gene, is a promising target for cancer therapy, especially in HER2-positive (carrying *ERBB2*/*HER2* gene amplification) breast carcinoma [1]. Both HER3 and HER2 belong to a family of epidermal growth factor receptor (EGFR, HER) tyrosine kinases that activate after receptor dimerization. This culminates in the initiation of signal transduction pathways that markedly regulate cellular viability [1]. When catalytically defective, HER3 is unable to homodimerize and orchestrate its own activation [2, 3]. HER3 is known to interact most preferably with its structurally homologous relative HER2 once bound with its ligand heregulin (HRG), also called neuregulin-1 [4–6]. Heterodimerization between HER2 and HER3 induces subsequent PI3K/AKT and Ras/Raf/MAPK signalling cascades [7]. The presence of HER3, as an allosteric activator, is required to maintain active HER2-mediated signalling [8, 9], and aberrantly intensified HER2-HER3 signalling is hence critically associated with breast carcinogenesis and tumour cell proliferation [4, 10–12].

HER3 protein overexpression has been shown to commonly co-occur with *HER2* gene amplification and HER2 overexpression, therefore, HER3 is thought to contribute markedly to the pathogenesis of *HER2*-amplified breast cancer subtype [4, 13, 14]. The co-expression of HER2 and HER3 proteins [15, 16] and abundance of HER2-HER3 heterodimers in situ have also been associated with adverse clinical outcomes in breast cancer [17–19]. The formation of HER2-HER3 heterodimers also inhibits HER3 downregulation [20]. Due to the close interaction between HER2 and HER3, dual inhibitory therapy is preferred and clinically relevant treatment for carcinomas with altered HER2 signalling [8, 11, 21]. In addition to HER2-positive breast carcinomas, therapeutic targeting of HER3 receptors has been suggested also in the treatment of HER3-dependent, HER2-negative breast cancers to prevent cell growth-promoting signalling triggered by intensified HER3-HER1 heterodimerization [22]. Several HER3-targeting molecules have been developed as therapeutics, and many of them are currently being tested in clinical trials [23, 24].

After a careful survey of the literature, it appears that the prognostic value of HER3 expression (at the protein or mRNA level) in breast cancer is controversial (Table 1). Overexpressed HER3 is mostly associated with a worse survival [16, 25–35], but conflicting results have also been published [36–40]. Many studies did not find any demonstrable relationships between HER3 and patient survival [15, 41–53]. Studies focusing on HER3 specifically in *HER2*-amplified breast cancer [16, 25, 26, 29, 31, 32, 37, 38, 41, 44, 45, 48, 49, 52, 54] have not drawn conclusive results either. Interestingly, HER3 activation has been implicated as a molecular mechanism inducing inherent or acquired de novo resistance to anti-HER2 therapy [19, 31, 55, 56]. Continuous inhibition of HER2 signalling may lead to compensatory HER3 activation, which results from heterodimerization between HER3 and its alternative dimerization partner HER1 [57, 58].

The exact mechanisms behind aberrant HER3 protein expression have not been fully elucidated [13]. Unlike HER2, HER3 does not undergo gene amplification during breast carcinogenesis [16, 59, 60]. Cancer-related *ERBB3* mutations are relatively uncommon, except for colon and gastric carcinomas [59, 61]. One hypothesis is that excessive cellular HER3 expression may be due to defects in downstream signalling mechanisms that regulate HER3 membrane trafficking [13]. Aberrant expression of HER3 degradation regulators may lead to an abnormal accumulation or deficit of membrane-bound HER3 receptors, consequently influencing HER3 signalling efficiency. Here, we studied the expression of two proteins, NEDD4–1 (*ne*ural precursor cell expressed *d*evelopmentally *d*ownregulated *4–1*) and NRDP1 (*n*euregulin *r*eceptor *d*egradation *p*rotein *1*, also known as FLRF and RNF41), which are known to be necessary for HER receptor quantity control [62]. NEDD4–1 [63] and NRDP1 [64–67] are both E3 ubiquitin protein ligases suggested to crucially downregulate HER3 and its subcellular localization by mediating HER3 receptors to degradation via the ubiquitin-proteasome-pathway. Defects in ubiquitination are critical and lead to aberrant receptor activity and signalling [68]. Hypothetically, HER3 overexpression may be associated with the concurrent absence of its ubiquitination regulators, NEDD4–1 and NRDP1.

Low NEDD4–1 expression due to *NEDD4–1* knockdown has been demonstrated to activate HER3 and increase cancer cell proliferation in vivo and in vitro [63]. Conversely, NEDD4–1 overexpression has resulted in decreased HER3 expression and increased HER3 ubiquitination [63]. Aberrant expression of NEDD4–1 has been implicated in the pathogenesis and adverse prognosis of several human malignancies [69–72]. Despite the frequent overexpression in breast cancer [73, 74], the prognostic value of NEDD4–1 remains unclear in the clinical context.

NRDP1, in turn, is less frequently overexpressed than NEDD4–1 in breast carcinoma [75, 76]. NRDP1 overexpression has been shown to cause a decrease in HER3 expression and an inhibition of breast cancer cell growth in vitro [75]. Conversely, a loss of NRDP1 followed by *NRDP1* knockdown suppressed HRG-induced HER3 ubiquitination and degradation in MCF7 breast cancer cells [64]. An inverse correlation between NRDP1 and HER3 expression in situ has been demonstrated in breast tumours derived from *ERBB2* transgenic mice

Table 1 Literature review of studies relating to HER3 prognostics in human breast cancer

Publication by	Laboratory Methodology	Cohort Characteristics	Prognostic Implications
Takada et al. [91]	IHC (RTJ2)	met-HER2+ BCA (*n* = 29), TPD	↓ Low HER3 expression was associated with shortened PFS
Adamczyk et al. [25]	IHC (SP71)	HER2+ BCA (*n* = 97), Adj.T	↑ High HER3 expression (only with concurrent PTEN negativity) was associated with shorten MFS
Duchnowska et al. [44]	VeraTag assay	HER2+ BCA (*n* = 189), Adj.T	- No correlation between HER3 expression and OS in advanced stage HER2 + BCA
Nishimura et al. [54]	VeraTag assay	met-HER2+ BCA (*n* = 47), T	- HER3 expression did not has any influence on PFS in trastuzumab-refractory advanced HER2 + BCA
Koutras et al. [39]	qRT-PCR	BCA (*n* = 663, HER2 + BCA *n* = 143)	↓ Low HER3 mRNA (only with concurrently high EGFR, high HER2, low HER4 mRNA) was associated with worse DFS
Baselga et al. [38]	qRT-PCR*, IHC** (DAK-H3-IC)	HER2+ BCA (*n* = 740*/497**), Adj.T	↓ High HER3 mRNA was associated with better prognosis in metastatic HER2 + BCA
Berghoff et al. [16]	IHC (DAK-H3-IC)	met-BCA (*n* = 110, met-HER2 + BCA *n* = 34)	↑ High HER3 expression was associated with shorter OS in initially metastatic HER2 + BCA subgroup
Park et al. [31]	IHC (DAK-H3-IC)	met-HER2+ BCA (*n* = 125), T	↑ High HER3 expression was associated with worse PFS in initially metastatic HER2 + BCA
Bae et al. [26]	IHC (DAK-H3-IC)	HR-BCA (*n* = 886, HER2 + BCA *n* = 221)	↑ High HER3 expression was associated with poorer DFS in HER2 + BCA subgroup and poorer DFS and OS in TNBC
Czopek et al. [48]	IHC (DAK-H3-IC)	HER2+ BCA (*n* = 35)	- No correlation between HER3 expression and DFS or OS
Lipton et al. [29]	VeraTag assay	met-HER2+ BCA (*n* = 89), T	↑ High HER3 expression was associated with shorter PFS in initially metastatic HER2 + BCA
Gori et al. [41]	IHC (RTJ1)	met-HER2+ BCA (*n* = 61), T	- HER3 was not significantly associated with clinical outcome in initially metastatic HER2 + BCA
Han et al. [37]	VeraTag assay	met-HER2+ BCA (*n* = 50), T	↓ High HER3 expression was related to longer TTP in advanced HER2 + BCA
Larsen et al. [43]	IHC (DAK-H3-IC)	ER+ BCA (*n* = 1062)	- HER3 expression did not shown any association to DFS
Chiu et al. [27]	IHC (Ab-10 pAb)	BCA (*n* = 3123)	↑ High HER3 expression was associated with decreased BCSS
Yonemori et al. [45]	IHC (DAK-H3-IC)	HER2+ BCA (*n* = 44), neoAdj.T	- HER3 expression did not significantly correlate with pCR
Giltnane et al. [28]	AQUA	BCA (*n* = 550)	↑ High HER3 expression was associated with decreased survival
Haas et al. [42]	IHC (SGP1)	HER2- BCA (*n* = 171)	- No prognostic value for HER3
Sassen et al. [50]	IHC (5A12), FISH	BCA (*n* = 173)	- No prognostic value for HER3 expression, *HER3* gene amplification was related to decreased DFS

Table 1 Literature review of studies relating to HER3 prognostics in human breast cancer *(Continued)*

Publication by	Laboratory Methodology	Cohort Characteristics	Prognostic Implications
Giuliani et al. [52]	IHC (RTJ1)	met-HER2+ BCA (*n* = 103), T	- No prognostic value for HER3
Lee et al. [36]	IHC (pAb)	BCA (*n* = 378)	↓ High HER3 expression correlated with longer DFS
Bianchi et al. [53]	IHC (RTJ1)	BCA (*n* = 145)	- No prognostic value for HER3 expression singly, but high co-expression of HER2/3/4 predicted worse prognosis
Fuchs et al. [34]	IHC (C-17 pAb)	BCA (*n* = 48)	↑ High HER3 expression singly and in co-expression with high HER1 and HER2 was associated with poor prognosis
Robinson et al. [32]	IHC (polyclonal)	met-HER2+ BCA (*n* = 104), T	↑ High HER3 expression was associated with worse OS
Wiseman et al. [33]	IHC (2-18C9)	BCA (*n* = 242)	↑ High HER3 expression independently and with high HER1 and/or HER2 was associated with decreased DSS
Abd El-Rehim et al. [15]	IHC (RTJ1)	BCA (*n* = 1499)	- No prognostic value for HER3 singly, but in co-expression with high HER2 predicted unfavorable DFS and OS
Smith et al. [49]	IHC	met-HER2+ BCA (*n* = 77), T	- No prognostic value for HER3
Bièche et al. [35]	qRT-PCR	BCA (*n* = 130)	↑ High HER3 mRNA was associated with shorten RFS
Witton et al. [30]	IHC (H3.105.5)	BCA (*n* = 220)	↑ High HER3 expression was associated with reduced BCSS survival
Suo et al. [47]	IHC (sc-415), RT-PCR	BCA (*n* = 100)	- High HER3 expression was predictive for reduced DFS or BCSS only in co-overexpression with HER2 or HER1 + HER2
Pawlowski et al. [40]	qRT-PCR	BCA (*n* = 365)	↓ Elevated HER3 mRNA expression was associated with a better prognosis in terms of OS, but did not relate to RFS
Travis et al. [46]	IHC (RTJ1)	BCA (*n* = 346), met-BCA (*n* = 145)	- No prognostic value for HER3 expression neither in primary nor metastatic breast cancer
Lemoine et al. [51]	IHC (49.3 pAb)	BCA (*n* = 195)	- No demonstrable relationship between HER3 expression and survival

Abbreviations: Adj.T = adjuvant trastuzumab therapy; BCA = primary breast cancer; BCSS = breast cancer-specific survival; DFS = disease-free survival; DSS = disease-specific survival; ER+ BCA = oestrogen receptor-positive breast cancer; HER2- BCA = HER2-negative breast cancer; HER2+ BCA = HER2-positive primary breast cancer; HR- BCA = hormone receptor-negative breast cancer; IHC = immunohistochemistry (antibody clone); met- = breast cancer diagnosed at advanced stage; MFS = metastasis-free survival; neoAdj.T = neoadjuvant trastuzumab therapy; n = number of patients being determined for HER3 status and followed for survival; OS = overall survival; pAb = polyclonal antibody; PFS = progression-free survival; pCR = pathologically complete response; qRT-PCR = quantitative reverse transcription polymerase chain reaction; RFS = recurrence-free survival; T = trastuzumab therapy after metastasis; TNBC = triple-negative breast cancer; TPD = trastuzumab, pertuzumab, docetaxel regimen; TTP = time to progression; ↑ = high HER3 mRNA or protein expression associated with worse clinical outcome; ↓ = low HER3 mRNA or protein expression associated with worse clinical outcome

[75] and in human breast carcinomas [76]. The prognostic and clinical significance of NRDP1 remains unknown. In the current study, we studied the association between HER3, NEDD4–1, and NRDP1 protein expression, clinicopathological characteristics and clinical outcomes in primary breast cancer, especially in the *HER2*-amplified subtype.

Methods

Clinical sample material

Two separate archival sample collections of formalin-fixed paraffin-embedded (FFPE) primary breast carcinomas were used for biomarker analyses conducted in compliance with the REMARK guidelines [77]. The first sample collection, “the BCA cohort”, consisted of

308 primary, invasive breast carcinomas that were diagnosed in the area served by Tampere University Hospital between 1990 and 1999. Of these carcinomas, 47 (15.3%) were characterized as HER2-positive based on HER2 protein overexpression. Lobular carcinomas were overrepresented in this cohort compared to the overall prevalence of this type of carcinoma (Table 2). This sample set was prepared as tissue microarray (TMA) sections and was originally established for another study, which has been described in more detail in publications by Korhonen et al. [78, 79]. Primary treatment for patients was conducted according to the existing clinical practice: surgery, post-operative radiotherapy, adjuvant cytotoxic chemotherapy (mostly CMF) and endocrine therapy (Table 3).

The other sample collection, specified as the "HER2+ BCA cohort", consisted exclusively of 177 *HER2*-amplified invasive breast carcinomas diagnosed during the years 2003–2007 in the Pirkanmaa Hospital District. The status of hormone receptors, oestrogen receptor (ER) and

Table 2 Clinicopathological characteristics of primary breast cancer patients in BCA cohort and HER2+ BCA cohort

Characteristic	*n*	BCA cohort, n (%)	*n*	*HER2*-amplified BCA cohort, n (%)
Follow-up period for RFS (range)		*Mean 10.4 yr. (1 mo.-22 yr.)*		*Mean 5.3 yr. (1 mo.-9 yr.)*
Age (range)	308	*Median 61 yr. (32–93 yr.)*	177	*Median 60 yr. (29–91 yr.)*
< 50 years		64 (20.8)		36 (20.3)
≥ 50 years		244 (79.2)		141 (79.7)
HER2 status	308		177	
Positive		47 (15.3)		177 (100.0)
Negative		261 (84.7)		0 (0.0)
ER status	307		177	
Positive (≥10%)		248 (80.8)		113 (63.8)
Negative (< 10%)		59 (19.2)		64 (36.2)
PR status	307		177	
Positive (≥10%)		201 (65.5)		74 (41.8)
Negative (< 10%)		106 (34.5)		103 (58.2)
Triple negativity	307		177	
TNBC (HER2−/ER-/PR-)		30 (9.8)		0 (0.0)
No TNBC		277 (90.2)		177 (100.0)
Histological grade	232		174	
I-II		179 (77.2)		41 (23.6)
III		53 (22.8)		133 (76.4)
Ki67 proliferation index	230		177	
Low (< 20%)		165 (71.7)		33 (18.6)
High (≥20%)		65 (28.3)		144 (81.4)
Histological type	304		168	
Ductal		173 (56.9)		156 (92.9)
Lobular		131 (43.1)		12 (7.1)
Tumour size	177		142	
< 2 cm		57 (32.2)		68 (47.9)
≥ 2 cm		120 (67.8)		74 (52.1)
Tumour size	308		172	
pT1-pT2		282 (91.6)		161 (93.6)
pT3-pT4		26 (8.4)		11 (6.4)
Lymph nodal spread	286		169	
Positive pN+		114 (39.9)		73 (43.2)
Negative pN0		172 (60.1)		96 (56.8)

Number of patient cases with available data (*n*) for each character is marked within the columns

Table 3 Primary treatments of patients in BCA and HER2+ BCA study cohorts

Primary treatment	BCA cohort (*n* = 308)		*HER2*-amplified BCA cohort (*n* = 177)	
	n	%	*n*	%
Breast surgery				
Mastectomy (*ablation*)	161	52.4	101	57.1
Conservative surgery (*resection*)	146	47.6	72	40.7
No operation			3	0.6
Unknown	1			
Post-operative radiotherapy	198	65.3	110	62.1
No	105	34.7	67	37.9
Unknown	5			
Adjuvant endocrine therapy	97	32.1	104	58.8
No	205	67.9	73	41.2
Unknown	6			
Adjuvant chemotherapy	40	13.4	133	75.1
No	259	86.6	44	24.9
Unknown	9			
Adjuvant trastuzumab			82	46.3
No	308	100.0	95	53.7

progesterone receptor (PR), *HER2* gene amplification, and Ki67 proliferation index were determined during the diagnostic procedure, and related data were retrieved from the clinical records. *HER2* gene amplification status was previously determined by the chromogenic in situ hybridization (CISH) technique. This sample set was prepared as whole tissue sections. Approximately half (*n* = 82) of the carcinomas, primarily patients diagnosed after June 2005, were treated with conventional chemotherapy combined with adjuvant trastuzumab during 9-wk schema as a first-line therapy [80] for primary disease. The remaining patients (*n* = 95) did not receive any adjuvant HER2-targeted therapy for primary disease. In addition to surgery and adjuvant cytotoxic chemotherapy (mostly consisting of taxanes, CEF), post-operative radiotherapy and adjuvant endocrine therapy were given when necessary (Table 3).

Samples were selected for the current study according to the following inclusion criteria: availability of representative tumour tissue (FFPE), adequate pathological characterization, and clinical follow-up data. Clinicopathological data and follow-up information were collected, retrospectively. The mean follow-up period for recurrence-free survival (RFS) in the HER2+ BCA cohort was 5.3 years (range: 1 month to 9 years) and 10.4 years (range: 1 month to 22 years) for the BCA cohort. NEDD4–1 and NRDP1 expression was studied in a smaller fraction of the HER2+ BCA cohort representing available *HER2*-amplified cases (*n* = 145). Table 2 describes the clinicopathological characteristics of the study cohorts.

Immunohistochemical stainings

For immunohistochemistry (IHC), serial four-μm-thick sections were cut from FFPE sample blocks and mounted on Super Frost Plus® slides followed by deparaffinization and dehydration. Heat-induced epitope retrieval (HIER) was performed in TE buffer (50 mM Tris 1 mM EDTA, pH 9) at 98 °C for 15 min. To determine HER3 protein expression, we used the optimized IHC staining protocol described in our earlier study [81]. We used a mouse monoclonal (clone DAK-H3-IC) antibody against the human HER3 protein at a dilution of 1:100.

Table 4 Details of antibodies used in the IHC-protocols of the current study

Antibody	Host species	Catalog No.	Clonality	Dilution	Manufacturer/distributor
Anti-Human HER3	Mouse	M7297	DAK-H3-IC	1:100	DAKO A/S, Glostrup, Denmark
FLRF/RNF41 Antibody	Rabbit	A300-049A	polyclonal	1:3000	Bethyl Laboratories, Inc., Montgomery, Texas, USA
Anti-Nedd4, WW2 domain	Rabbit	#07–049	polyclonal	1:750	Merck KGaA, Darmstadt, Germany
Cytokeratin 5 Antibody	Mouse	NCL-L-CK5	XM26	1:150	Leica Biosystems Newcastle Ltd., Newcastle Upon Tyne, UK
Cytokeratin 14 Antibody	Mouse	NCL-L-LL0022	LL0022	1:150	Leica Biosystems, Newcastle Ltd., Newcastle Upon Tyne, UK
Anti-human Ki67	Mouse	BSH-7302	BS4	1:100	Nordic BioSite AB, Täby, Sweden

The expression of basal epithelium cytokeratins 5 and 14 was determined using the same IHC protocol with an antibody cocktail composed of anti-human mouse monoclonal antibodies CK14 (clone LL002) and CK5 (clone XM26), both diluted at 1:150. Ki-67 expression was determined similarly in BCA cohort samples with mouse monoclonal Ki-67 antibody (clone BS4) at a dilution of 1:100.

For NEDD4–1 IHC, we used rabbit polyclonal anti-NEDD4 WW2 domain antibody (dilution 1:750) to detect NEDD4–1 proteins. Bright Vision+ Poly-HRP-Anti-mouse/rabbit IgG kit (ImmunoLogic, AD Duiven, the Netherlands) and 3,3′-diaminobenzidine tetrahydrochloride DAB-2V kit (Nichirei Biosciences Inc., Tsukiji, Chuo-ku, Tokyo, Japan) were used for the detection of immunoreactivity according to manufacturers' instructions. To detect the NRDP1 protein, we used rabbit polyclonal FLRF/RNF41 antibody (dilution 1:3000), EnVision™ FLEX High pH HRP and EnVision™ FLEX DAB + reagents (Dako, Glostrup, Denmark), according to manufacturers' protocols. After staining, slides were counterstained with Mayer's Hematoxylin (Oy FF-Chemicals Ab, Haukipudas, Finland) with 1:4 addition of 2% copper sulfate to intensify the DAB reaction. Slides were then dehydrated, cleared with xylene and sealed with DePeX mountant.

All staining reactions were conducted using the LabVision™ Autostainer 480S platform. As positive control samples, we used human FFPE tissues known to express the specified proteins: normal prostate ductal cells for HER3 [82], kidney proximal tubule cells for NEDD4–1 [83], testicular cells in seminiferous ducts and mononuclear blood cells for NRDP1 [84]. A negative staining control was prepared by omitting and replacing the primary antibody with diluent reagent and was included in each staining batch. An additional file 1 and Table 4 present detailed information on antibodies and IHC-staining protocols used in the current study.

Microscopic analysis and interpretation of immunoreactivity

Samples stained for HER3, NEDD4–1 and NRDP1 were scanned with SlideStrider (Jilab Inc., Tampere, Finland) into digital images that were examined virtually with JVSview JPEG2000 [85] and SlideVantage 1.2 (Jilab Inc., Tampere, Finland) viewer applications. The ImmunoRatio 2.5 application was used for automated cell counting of distinct cancer cells with nuclear immunoreactivity [86]. Staining patterns were analysed within the invasive cancerous tissue area displaying the most intense brown DAB reaction (region of interest, ROI).

For HER3 appearance, both membranous and cytoplasmic staining reactions were inspected on a computer screen. Samples were classified according to the staining intensity and proportion of specifically stained cancer cells as previously described [81]. Briefly, HER3 staining localized to the cancer cell outer membrane was considered 'membranous' and was scored according to the following criteria: (0) absent/low staining (< 10% of cells), (1+) intermediate circumferential staining (10–30% of cells) and (2+) strong circumferential staining (> 30% of cells). The staining reaction observed in the cancer cell cytoplasm was considered 'cytoplasmic' and was categorized as (0) no/faint staining, (1+) overall low-intensity staining, and (2+) prevalent high-intensity staining covering most of the cancer cells. Score 1+ was set as a threshold to define HER3 positivity both for membranous and cytoplasmic staining. Total HER3 staining was designated as negative for cases with low (0/1+) membranous staining concurrently with low (0/1+) cytoplasmic staining and as positive for cases with high (2+) membranous and/or (2+) cytoplasmic staining.

The NEDD4–1 protein expression pattern was analysed by scoring the staining intensity as follows: 0 (no staining), 1+ (weak), 2+ (moderate), and 3+ (strong). Samples with scores < 3+ were seen as NEDD4–1 negative 'low expressing' and samples with score 3+ as NEDD4–1 positive 'high expressing'. Overall, the NEDD4–1 staining pattern in cancerous areas was homogenous, and therefore, the percentage of stained cells was not evaluated.

NRDP1 staining was analysed by applying a scoring system presented in a study by Jiao et al. [76]. We analysed nuclear and cytoplasmic staining separately. Staining intensity was scored accordingly: 0 (no staining), 1 (weak), 2 (moderate), and 3 (strong). Based on the percentage of stained cancer cell nuclei, samples were classified as 0 (< 1%), 1 (1–24%), 2 (25–49%), 3 (50–74%), and 4 (75–100%). The grades were then multiplied to determine a score for low and high nuclear expression. Cases with scores ≤3 were defined as 'low expressing' and those with scores ≥4 as 'high expressing'. Cytoplasmic NRDP1 expression was categorized as high if the staining intensity in the tumour cells was moderate or strong. Expression patterns of basal epithelium cytokeratins 5 and 14 and Ki67 protein were analysed with Olympus System Microscope BX43. Carcinomas were interpreted as positive for CK5 and CK14 expression if more than 20% of the malignant cells displayed clear cytoplasmic staining [87]. For Ki67 protein expression, we used a 20% cut-off value to determine low (< 20%) and high (≥20%) cell proliferation activity [86].

Statistical analysis

All statistical data analyses were performed using IBM® SPSS® Statistics version 23 (IBM Corp.). Generally, *p*-values < 0.05 were considered statistically

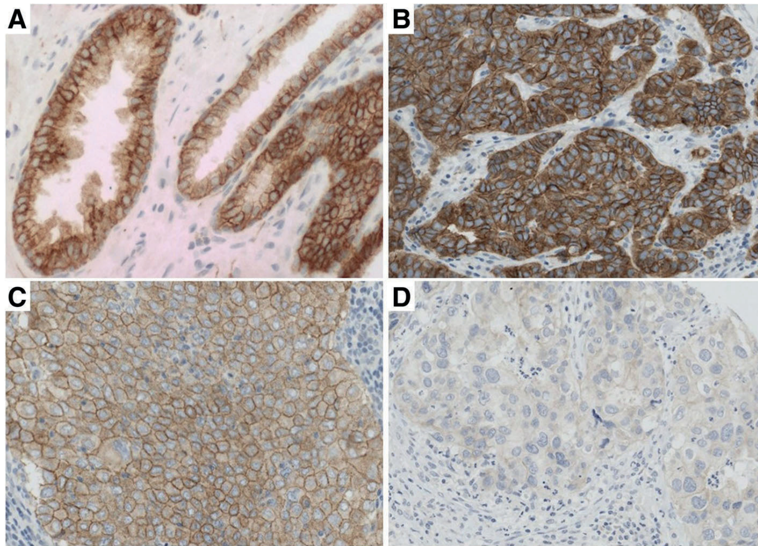

Fig. 1 HER3 immunohistochemistry. **a** Positive control (prostate), **b** Concurrently high (score 3+) membranous and cytoplasmic HER3 expression (breast carcinoma), **c** High (score 3+) membranous HER3 expression with negative/low (score 0) cytoplasmic HER3 status, **d** Negative/low total cellular HER3 staining. Mayer's Hematoxylin used as a counterstain

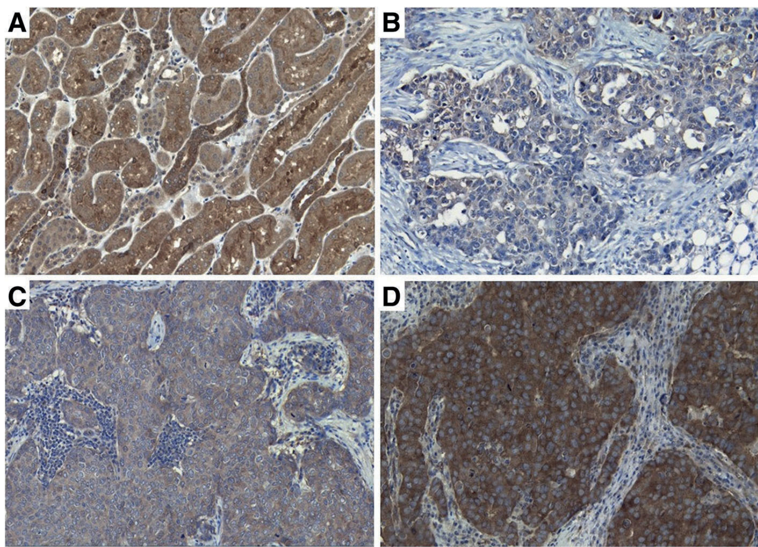

Fig. 2 NEDD4–1 immunohistochemistry. **a** Positive control (kidney), **b** Negative/low NEDD4–1 expression (score 1+, breast carcinoma), **c** Moderate NEDD4–1 expression (score 2+, breast carcinoma), **d** High NEDD4–1 expression (score 3+, breast carcinoma). Mayer's Hematoxylin used as a counterstain

significant for any relationship being considered. Proportions among categorical variables were compared using Pearson's Chi-Square test to determine clinicopathological correlations. Kaplan-Meier survival analysis and log-rank test (Mantel-Cox) were used to compare survival differences for each categorical variable. RFS time was chosen as the endpoint for the current study. To determine RFS, patients were followed from the date of surgery for initial diagnosis to the date of disease progression as local recurrence or distant metastasis. Patients who did not experience recurrence during the follow-up were censored at the time of death or last date of medical record inspection.

Results

HER3 protein expression in breast carcinomas

In the BCA sample set consisting of HER2-positive and -negative breast carcinomas (BCA cohort), high membranous HER3 expression was observed in half of the cases (51.9%, 160 of 308). Nearly all (95.8%, 295 of 308) carcinomas showed HER3 protein expression localized in the cancer cell cytoplasm. When the total cellular HER3 expression pattern was evaluated, the majority (75.3%, 232 of 308) of carcinomas were classified as HER3-positive, 'high total HER3 expressing'. One-fourth of the carcinomas (24.7%, 76 of 308) were determined to be HER3-negative, 'low total HER3 expressing'. Figure 1 shows examples of membranous and cytoplasmic HER3 IHC staining patterns observed in the present study.

HER3, NEDD4–1, and NRDP1 protein expression in *HER2*-amplified breast carcinomas

To determine whether HER3 protein expression is common in *HER2*-amplified breast cancer subtype, we also studied HER3 expression in the HER2+ BCA cohort established for this purpose. We noticed that 80.2% (142 of 177) of *HER2*-amplified breast carcinomas showed complete circumferential membrane staining for HER3. Cytoplasmic HER3 staining was more common, since only a small fraction (8.5%, 15 of 177) of these carcinomas were completely unstained. High total HER3 expression was demonstrated in 75.7% of cases (134 of 177), and one-fourth of carcinomas were designated as HER3-negative. Overall, HER3 protein was heterogeneously expressed within the cancerous areas represented in whole tissue sections. The HER3 staining pattern was, therefore, equally evaluated from the ROI showing the most intense DAB reaction (Fig. 1).

Next, we studied NEDD4–1 and NRDP1 protein expression in a cohort of *HER2*-amplified breast carcinomas. Most of the cases (82.8%, 120 of 145) demonstrated strong-to-moderate NEDD4–1 staining localized predominantly in the cytoplasmic region (Fig. 2). Approximately one-fifth (17.2%, 25 of 145) of the cases were categorized as NEDD4–1 low expression based on faint IHC staining reaction. The staining intensity and subcellular localization of NEDD4–1 protein were homogenous within the cancerous areas. Cells in histologically normal breast ducts were also positive for NEDD4–1. NRDP1 protein expression was uncommon in *HER2*-amplified breast carcinomas. NRDP1 localization in carcinoma cells was clearly nuclear or cytoplasmic (Fig. 3). The high presence of nuclear or cytoplasmic NRDP1 protein was observed in a minor proportion (8.3%, 12 of 145) of samples, while the majority of carcinomas (91.7%, 133 of 145) were classified as low for NRDP1 expression.

Association of HER3, NEDD4–1 and NRDP1 with clinicopathological characteristics

In the BCA cohort, we noticed that HER3 protein expression was not dependent on HER2 status

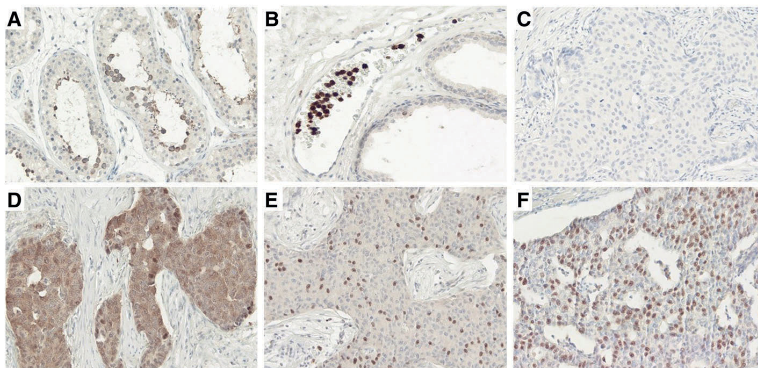

Fig. 3 NRDP1 immunohistochemistry. **a** Positive control (testis, cells in seminiferous ducts), **b** Positive control (mononuclear blood cells), **c** Absent NRDP1 expression (breast carcinoma), **d** Cytoplasmic NRDP1 expression (breast carcinoma), **e** and **f** Nuclear NRDP1 expression (breast carcinoma). Mayer's Hematoxylin used as a counterstain

Table 5 Associations between HER3 protein expression and clinicopathological characteristics

Characteristic	*BCA cohort*									*HER2-amplified BCA cohort*								
	HER3-m (%)		*p*	HER3-c (%)		*p*	HER3-t (%)		*p*	HER3-m (%)		*p*	HER3-c (%)		*p*	HER3-t (%)		*p*
	-	+		-	+		-	+		-	+		-	+		-	+	
HER2 status			0.615			0.990			0.882									
Negative	85.8	83.8		84.6	84.7		84.2	84.9										
Positive	14.2	16.2		15.4	15.3		15.8	15.1										
Estrogen receptor status			0.057			0.280			0.839			0.013*			0.376			0.104
Negative	23.6	15.1		30.8	18.7		18.4	19.5		54.3	31.7		46.7	35.2		46.5	32.8	
Positive	76.4	84.9		69.2	81.3		81.6	80.5		45.7	68.3		53.3	64.8		53.5	67.2	
Progesterone receptor status			0.002**			0.368			0.443			0.888			0.882			0.716
Negative	43.2	26.4		46.2	34.0		38.2	33.3		57.1	58.5		60.0	58.0		55.8	59.0	
Positive	56.8	73.6		53.8	66.0		61.8	66.7		42.9	41.5		40.0	42.0		44.2	41.0	
Triple-negativity (HER2-/ER-/PR-)			0.000***			0.099			0.798									
No	83.8	96.2		76.9	90.8		89.5	90.5										
Yes	16.2	3.8		23.1	9.2		10.5	9.5										
Histological grade			0.121			0.855			0.705			0.435			0.767			0.956
I-II	72.8	81.4		75.0	77.3		75.4	77.8		28.6	22.3		26.7	23.3		23.3	23.6	
III	27.2	18.6		25.0	22.7		24.6	22.2		71.4	77.7		73.3	76.7		76.7	76.4	
Ki-67 proliferation index			0.597			0.985			0.852			0.475			0.213			0.658
Low	73.4	70.2		71.4	71.7		72.7	71.4		22.9	17.6		6.7	19.8		20.9	17.9	
High	26.6	29.8		28.6	28.3		27.3	28.6		77.1	82.4		93.3	80.2		79.1	82.1	
Histological type			0.629			0.359			0.204			0.055			0.940			0.960
Ductal	55.5	58.2		69.2	56.4		63.2	54.8		85.3	94.8		93.3	92.8		92.7	92.9	
Lobular	44.5	41.8		30.8	43.6		36.8	45.2		14.7	5.2		6.7	7.2		7.3	7.1	
Lymph nodal status			0.531			0.637			0.716			0.232			0.169			0.035*
Negative pN0	58.3	61.9		66.7	59.9		62.0	59.5		65.7	54.5		40.0	58.4		42.9	61.4	
Positive pN+	41.7	38.1		33.3	40.1		38.0	40.5		34.3	45.5		60.0	41.6		57.1	38.6	
Tumor size (TNM stage)			0.840			0.921			0.781			0.173			0.001***			0.368
pT1-pT2	91.9	91.3		92.3	91.5		90.8	91.8		88.6	94.9		73.3	95.5		90.7	94.6	
pT3-pT4	8.1	8.7		7.7	8.5		9.2	8.2		11.4	5.1		26.7	4.5		9.3	5.4	
Tumor size (cm)			0.003**			0.834			0.357			0.643			0.014*			0.143
<2cm	21.6	42.7		28.6	32.4		26.7	34.1		51.7	46.9		15.4	51.2		37.1	51.4	
≥2cm	78.4	57.3		71.4	67.6		73.3	65.9		48.3	53.1		84.6	48.8		62.9	48.6	
Patient age at diagnosis			0.726			0.624			0.217			0.069			0.000***			0.156
<50 years	21.6	20.0		15.4	21.0		15.8	22.4		31.4	17.6		60.0	16.7		27.9	17.9	
≥50 years	78.4	80.0		84.6	79.0		84.2	77.6		68.6	82.4		40.0	83.3		72.1	82.1	
Cytokeratin 5/14 expression												0.583			0.561			0.006**
Negative										85.3	88.7		92.9	87.6		76.2	92.0	
Positive										14.7	11.3		7.1	12.4		23.8	8.0	
Basal phenotype (CK5/14+, ER-)												0.191			0.801			0.001***
No										85.3	92.5		92.9	90.8		78.6	95.2	
Yes										14.7	7.5		7.1	9.2		21.4	4.8	

p-values from Pearson's Chi-Square test, statistically significant values are underlined and marked with symbols $*p<0.05$, $**p\leq0.01$, and $***p\leq0.001$. Percentages of breast carcinomas presented according to membranous (−m), cytoplasmic (−c), and total (−t) HER3 expression; −/+ means low/high HER3 expression by IHC

irrespective of its cellular localization (membranous $p = 0.615$, cytoplasmic $p = 0.990$, total $p = 0.882$). In addition, we found that low membranous HER3 protein expression was associated with an aggressive triple-negative breast cancer (TNBC) phenotype ($p = 0.000$), defined as concurrently negative ER, PR, and HER2 statuses. Similarly, negative PR receptor status alone ($p = 0.002$) and larger tumour size ≥2 cm ($p = 0.003$) were related to low membranous HER3. Cytoplasmic or total cellular HER3 expression were not

Table 6 Associations between NEDD4–1 and NRDP1 protein expression and clinicopathological characteristics in *HER2*-amplified breast cancer cohort

Characteristic		Cytoplasmic NRDP1 expression *n* (%)			Nuclear NRDP1 expression *n* (%)			Cellular NEDD4–1 expression *n* (%)		
	n	NRDP1-	NRDP1+	*p*	NRDP1-	NRDP1+	*p*	NEDD4–1-	NEDD4–1+	*p*
Cases	145	133 (91.7)	12 (8.3)		133 (91.7)	12 (8.3)		25 (17.2)	120 (82.8)	
Estrogen receptor				0.057			0.206			0.421
Positive	97	86 (64.7)	11 (91.7)		87 (65.4)	10 (83.3)		15 (60.0)	82 (68.3)	
Negative	48	47 (35.3)	1 (8.3)		46 (34.6)	2 (16.7)		10 (40.0)	38 (31.7)	
Progesterone receptor				0.006**			0.125			0.053
Positive	66	56 (42.1)	10 (83.3)		58 (43.6)	8 (66.7)		7 (28.0)	59 (49.2)	
Negative	79	77 (57.9)	2 (16.7)		75 (56.4)	4 (33.3)		18 (72.0)	61 (50.8)	
Histological grade				0.953			0.446			0.134
I-II	35	32 (24.2)	3 (25.0)		31 (23.5)	4 (33.3)		9 (36.0)	26 (21.8)	
III	109	100 (75.8)	9 (75.0)		101 (76.5)	8 (66.7)		16 (64.0)	93 (78.2)	
Ki-67 proliferation index				0.228			0.228			1.000
Low	29	25 (18.8)	4 (33.3)		25 (18.8)	4 (33.3)		5 (25.0)	24 (20.0)	
High	116	108 (81.2)	8 (66.7)		108 (81.2)	8 (66.7)		20 (75.0)	96 (80.0)	
Histological type				0.022*			0.880			0.403
Ductal	128	119 (93.7)	9 (75.0)		118 (92.2)	10 (90.9)		22 (88.0)	106 (93.0)	
Lobular	11	8 (6.3)	3 (25.0)		10 (7.8)	1 (9.1)		3 (12.0)	8 (7.0)	
Lymph nodal status				0.120			0.277			0.516
Positive pN+	60	58 (45.3)	2 (20.0)		53 (42.1)	7 (58.3)		9 (37.5)	51 (44.7)	
Negative pN0	78	70 (54.7)	8 (80.0)		73 (57.9)	5 (41.7)		15 (62.5)	63 (55.3)	
Tumor size (cm)				0.669			0.820			0.715
< 2 cm	57	52 (48.1)	5 (55.6)		52 (49.1)	5 (45.5)		9 (45.0)	48 (49.5)	
≥ 2 cm	60	56 (51.9)	4 (44.4)		54 (50.9)	6 (54.5)		11 (55.0)	49 (50.5)	
Tumor size (TNM stage)				0.511			0.341			0.177
pT1-pT2	138	127 (96.2)	11 (100.0)		127 (96.9)	11 (91.7)		23 (92.0)	115 (97.5)	
pT3-pT4	5	5 (3.8)	0 (0.0)		4 (3.1)	1 (8.3)		2 (8.0)	3 (2.5)	
Patient age at diagnosis				0.856			0.004**			0.771
< 50 years	27	25 (18.8)	2 (16.7)		21 (15.8)	6 (50.0)		4 (16.0)	23 (19.2)	
≥ 50 years	118	108 (81.2)	10 (83.3)		112 (84.2)	6 (50.0)		21 (84.0)	97 (80.8)	
HER3 membrane expression				0.905			0.505			0.002**
Low	26	24 (18.0)	2 (16.7)		23 (17.3)	3 (25.0)		10 (40.0)	16 (13.3)	
High	119	109 (82.0)	10 (83.3)		110 (82.7)	9 (75.0)		15 (60.0)	104 (86.7)	
HER3 cytoplasmic expression				0.300			0.215			0.360
Low	11	11 (8.3)	0 (0.0)		9 (6.8)	2 (16.7)		3 (12.0)	8 (6.7)	
High	134	122 (91.7)	12 (100.0)		124 (93.2)	10 (83.3)		22 (88.0)	112 (93.3)	
HER3 total cellular expression				0.041*			0.942			0.620
Low	35	35 (26.3)	0 (0.0)		32 (24.1)	3 (25.0)		7 (28.0)	28 (23.3)	
High	110	98 (73.7)	12 (100.0)		101 (75.9)	9 (75.0)		18 (72.0)	92 (76.7)	
Cytokeratin 5/14 expression				0.199			0.199			0.578
Negative	127	115 (87.8)	12 (100.0)		115 (87.8)	12 (100.0)		23 (92.0)	104 (88.1)	
Positive	16	16 (12.2)	0 (0.0)		16 (12.2)	0 (0.0)		2 (8.0)	14 (11.9)	

p-values were calculated using Pearson's Chi-Square test, statistically significant values are underlined and marked with *$p<0.05$, **$p\leq0.01$, and ***$p\leq0.001$

associated with any particular clinicopathological characteristics (Table 5). HER3 expression was not related to neither cellular proliferation activity (Ki67) nor lymph nodal status. When the BCA cohort was analysed and stratified for HER2 status, we noticed that clinicopathological correlations were statistically significant only in HER2-negative carcinomas. In this group, low membranous HER3 expression was strongly associated with negative ER ($p = 0.003$) and negative PR ($p = 0.002$) statuses, high (III) grade ($p = 0.008$) and larger (≥2 cm) tumour size ($p = 0.006$).

In a cohort of 177 *HER2*-amplified breast carcinomas, low HER3 expression was related to clinicopathological characteristics known to predict poor clinical outcome, with the exception of the cell proliferation marker Ki67, which was not shown to associate with HER3 (Table 5). Low membranous HER3 expression was associated with negative ER status ($p = 0.013$). Low cytoplasmic HER3 expression, in turn, was related to large tumour size (≥2 cm, $p = 0.014$ or pT3-pT4, $p = 0.001$), young patient age (< 50 years) at diagnosis ($p = 0.000$), and premenopausal status ($p = 0.000$). Carcinomas with low total cellular HER3 expression were associated with lymph nodal infiltration ($p = 0.035$), cytokeratin proteins 5 and 14 expression ($p = 0.006$), and basal phenotype ($p = 0.001$). Basal phenotype was determined by concurrent cytokeratin 5/14 expression and negative ER status [87].

For NEDD4–1 and NRDP1, we found few clinicopathological correlations (Table 6). High NEDD4–1 expression was shown to correlate with high expression of the cell membrane-located HER3 protein ($p = 0.002$). The majority (87.4%, 104 of 119) of carcinomas showing high membranous HER3 expression were demonstrated to co-overexpress NEDD4–1 protein. In a group of carcinomas with low membranous HER3 expression, NEDD4–1 was negative in 38.5% (10 of 26) of carcinomas. High cytoplasmic NRDP1 expression was observed mainly in PR-positive breast carcinomas ($p = 0.006$) and correlated with total HER3 expression ($p = 0.041$). Low nuclear NRDP1 expression was observed mostly in carcinomas diagnosed in patients aged ≥50 years ($p = 0.004$). Neither nuclear nor cytoplasmic NRDP1 protein expression was associated with NEDD4–1.

Prognostic implications of HER3, NEDD4–1 and NRDP1 in breast cancer

In the BCA cohort, approximately one-third (36.4%, 112 of 308) of breast carcinomas developed metastatic disease recurrence during the long-term follow-up period lasting up to 22 years (mean 10.4 years). Lymph nodal infiltration pN+ ($p = 0.000$), tumour size of pT3-pT4 ($p = 0.009$), TNBC phenotype ($p = 0.006$), histological grade III ($p = 0.007$), and PR negativity ($p = 0.035$) were shown to predict breast cancer recurrence in univariate analysis (log-rank Mantel-Cox). Of these, only lymph nodal spread was of prognostic utility ($p = 0.002$, Exp (B) 2.145) for shorter RFS in multivariate Cox regression analysis. HER3, in turn, was not associated with the clinical outcome of breast cancer.

During the mean follow-up time of 5.3 years (range 1 month to 9 years), 20.3% (36 of 177) of *HER2*-amplified breast cancer cases experienced recurrence of the disease. Distantly located metastases (61.1%, 22 of 36) were more common than local relapses (38.9%, 14 of 36). Altogether, 18.3% of patients receiving adjuvant trastuzumab therapy experienced relapse, while 22.1% of patients treated without trastuzumab were relapsing during the follow-up ($p = 0.573$). According to the univariate log-rank analysis, we found lymph nodal infiltration ($p = 0.000$), tumour size of pT3-pT4 ($p = 0.000$), and low total cellular HER3 protein expression ($p = 0.004$) as strong indicators of shortened RFS in *HER2*-amplified breast cancer (Table 7, Fig. 4). The estimated mean RFS time was shortened as follows: RFS for pN+ (vs. pN0) carcinomas was 6.7 (8.4) years, for pT3-pT4 -sized tumours (vs. pT1-pT2) 4.2 (7.9) years, and for low (vs. high) total HER3 expressing carcinomas 6.3 (8.0) years. We also found statistical significance for low membranous ($p = 0.025$) and cytoplasmic ($p = 0.010$) HER3 expression in predicting breast cancer recurrence during the follow-up period (Table 7, Fig. 4). Low total cellular HER3 expression was demonstrated to find relapsing *HER2*-amplified breast carcinomas most efficiently; 41.7% (15 of 36) of cases with recurrence were shown to demonstrate low total cellular HER3 expression. Correspondingly, one-third of relapsing carcinomas (33.3%, 12 of 36) were classified as low for membranous HER3 expression, and one-fifth (19.4%, 7 of 36) were classified as low for cytoplasmic HER3 expression. When survival analyses were performed and stratified according to adjuvant trastuzumab therapy, we observed that low total cellular and cytoplasmic HER3 expression were of prognostic utility only in a cohort treated without adjuvant trastuzumab. Based on that data, we do not see HER3 as a useful biomarker to predict the effectiveness of adjuvant trastuzumab, at least when complied with the 9-wk regimen represented in a fraction of patients in the HER2+ BCA cohort.

Based on univariate analyses, lymph nodal involvement (pN+), tumour size of pT3-pT4 and low total cellular HER3 expression were consequently tested for their prognostic value in multivariate Cox regression analysis. All of these categorized variables were independent negative prognostic factors of *HER2*-amplified breast cancer. Low total cellular HER3 protein expression was shown to

Table 7 Univariate and multivariate Cox regression analysis for prognostic value of study variables to predict RFS in *HER2*-amplified breast cancer

Characteristic	Univariate analysis Multivariate analysis						
	n	*p*	Mean RFS	95% CI for RFS	*p*	Exp (B)	95% CI for Exp (B)
Estrogen receptor status	177	0.090					
Progesterone receptor status	177	0.176					
Histological grade	174	0.831					
Ki-67 proliferation index	177	0.171					
Histological type (lobular/ductal)	168	0.774					
Lymph nodal status pN+ (vs pN0)	169	0.000***	6.7 (8.4)	5.9 (8.0) – 7.5 (8.7)	0.002**	3.486	1.608, 7.555
Tumor size TNM stage ≥ pT3 (vs < pT3)	172	0.000***	4.2 (7.9)	2.3 (7.5) – 6.2 (8.3)	0.001***	4.016	1.703, 9.468
Patient age at diagnosis	177	0.118					
Menopausal status	176	0.082					
Cytokeratin 5/14 expression	167	0.447					
Basal phenotype (CK5/14+, ER-)	167	0.955					
Total cellular HER3 low (vs high)	177	0.004**	6.3 (8.0)	5.3 (7.6) – 7.3 (8.4)	0.020*	2.305	1.143, 4.648
Membranous HER3 low (vs high)	177	0.025*	6.6 (7.9)	5.6 (7.4) – 7.7 (8.3)			
Cytoplasmic HER3 low (vs high)	177	0.010*	5.9 (7.8)	4.2 (7.4) – 7.6 (8.2)			
NEDD4–1 expression	145	0.261					
NRDP1 nuclear expression	145	0.689					
NRDP1 cytoplasmic expression	145	0.711					

Significant p-value (marked as *p<0.05, **p≤0.01, ***p≤0.001) means prognostic value of the variable to predict shorter RFS-time. Mean follow-up period for HER2 + BCA cohort was 5.3 years. Estimated mean RFS time is announced in years for each significant character

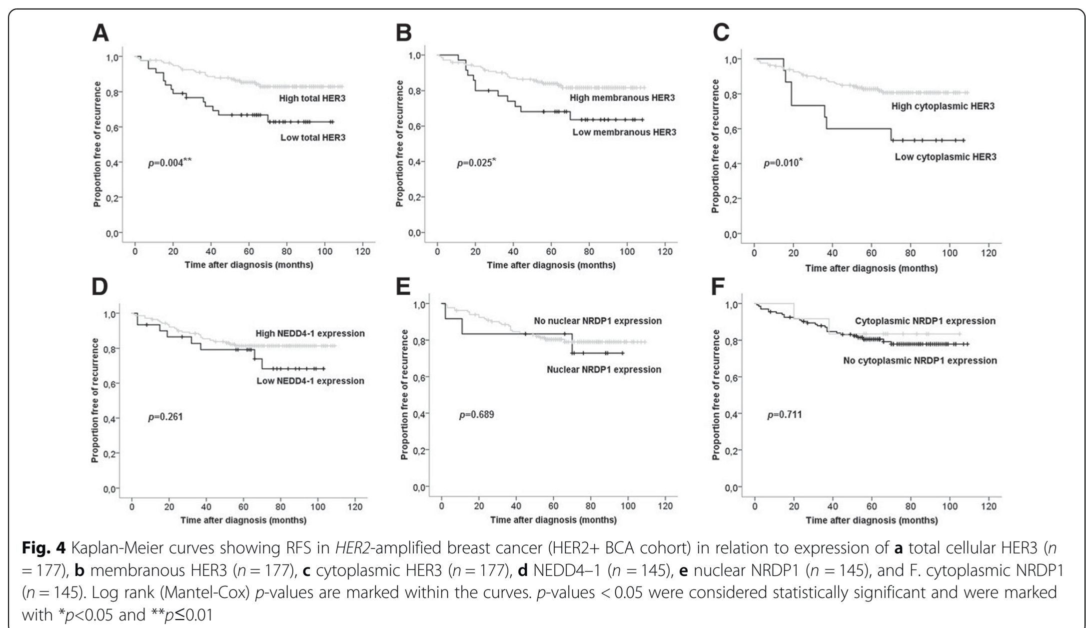

Fig. 4 Kaplan-Meier curves showing RFS in *HER2*-amplified breast cancer (HER2+ BCA cohort) in relation to expression of **a** total cellular HER3 (n = 177), **b** membranous HER3 (n = 177), **c** cytoplasmic HER3 (n = 177), **d** NEDD4–1 (n = 145), **e** nuclear NRDP1 (n = 145), and F. cytoplasmic NRDP1 (n = 145). Log rank (Mantel-Cox) *p*-values are marked within the curves. *p*-values < 0.05 were considered statistically significant and were marked with *p<0.05 and **p≤0.01

increase the risk of breast cancer recurrence by 2.3-fold relapse risk, positive lymph nodal status 3.5-fold, and tumour size of pT3-pT4 by 4.0-fold (Table 7).

NEDD4–1 and NRDP1 expression did not show any prognostic value for predicting the outcome of *HER2*-amplified breast cancer in terms of recurrence-free survival (Fig. 4). Additionally, neither NEDD4–1 nor NRDP1 expression was predictive of the efficiency of short-term (9-wk schema) adjuvant trastuzumab therapy.

Discussion

The role of HER3 in breast cancer biology has been extensively studied, especially in the context of personalized cancer therapy [1]. The current study confirmed the predominance of HER3 protein expression in primary breast cancer, as detected by IHC. The majority (75%) of breast carcinomas were shown to display intense HER3 expression irrespective of HER2 status. From a therapeutic perspective, this provides a rationale for HER3-targeted pharmaceuticals, which are defining the state of the art in breast cancer therapy, especially for *HER2*-amplified subtype. The role of anti-HER3 therapy in the treatment of HER3-dependent, non-*HER2*-amplified breast carcinomas has also been speculated recently [88]. However, e.g. lumretuzumab, in combination with pertuzumab and paclitaxel, was not confirmed clinically relevant therapy for patients with HER3-positive, HER2-low breast cancer [89], although was demonstrated effective in HER2-low/ER+ mouse xenograft model in vivo when combined with pertuzumab and endocrine (fulvestrant) therapy [90].

Interestingly, we found that low HER3 expression was associated with features that commonly define breast cancer aggressiveness: large size (≥pT3), axillary lymph nodal infiltration (pN+), negative ER status, triple-negativity (ER-, PR-, HER2-) and basal phenotype (CK5/14+, ER-). However, we were not able to find a statistically significant association between low HER3 expression and high proliferation activity (indicated by the Ki-67 proliferation index), which supports the recently published result by Takada et al. [91]. On the contrary, Kirouac et al. [92] reported earlier that HER2-positive breast cancer cells showing lower proliferation activity in vitro have concomitantly higher HER3 expression levels.

Our results demonstrate that low HER3 protein expression is indicative of shorter RFS in *HER2*-amplified breast carcinomas. Negative or low HER3 status was shown to independently increase the risk of breast cancer recurrence by two-fold. In the multivariate analysis, low membranous HER3 and low total cellular HER3 expression were prognostic factors for relapse occurrence, with well-known poor outcome determinants lymph nodal infiltration (pN+) and large tumour size (≥pT3). Despite extensive research focusing on HER3 over the past twenty years, its clinical utility in cancer prognostics - specifically in breast cancer - remains undefined [93], as has been reviewed within the current study (Table 1). When focusing on breast cancer, there are studies linking HER3 overexpression to unfavourable outcome, and others, such as the current study, that adversely associate low HER3 (mRNA or protein) expression with worse prognosis. However, some studies did not find any association between HER3 and breast cancer outcome. In addition, only some of the studies have focused on the *HER2*-amplified breast cancer subtype, in which HER signalling is specifically different from other subtypes [7]. Considering survival data, one should remember that the pattern of recurrence is already dependent on the intrinsic subtype [94], which for we have inspected our results stratified for HER2 status.

One explanation to elucidate the HER3 survival context in *HER2*-amplified breast cancer subtype could be related to intensified HER2 signalling because of paradoxical HER2 homodimerization in carcinomas with concurrently low HER3 but high HER2 expression due to amplified *HER2*. It has been previously confirmed that HER2 homodimerization is frequent, especially in breast carcinomas characterized by *HER2* gene amplification, and is related to reduced RFS [17]. In the present study, we did not find any survival differences when HER2-negative breast carcinomas (BCA cohort) with normal HER2 signalling were stratified for HER3. Earlier studies [15, 37] support that patients having both high HER2 and HER3 expression have significantly longer time to disease progression compared to patients having either high HER2 or HER3 expression in their carcinomas. Based on these observations, HER3 cannot be considered an independent prognostic factor in breast cancer overall because its clinical impact is mostly dependent on the co-expression of other HER receptors, such as HER2. Accordingly, we suggest that the HER2-HER3 interaction and its effects on growth-promoting signalling in HER2-dependent carcinomas are biologically different from carcinomas with low HER2 expression. For this reason, the prognostic applicability of HER3 should be analysed separately in breast cancers stratified for HER2 status. Additional intrinsic factors, such as the absence of HRG in HER3-overexpressing carcinomas, may also explain the finding of favourable outcomes in carcinomas characterized by high HER3 protein expression.

HER3 activation is suggested as one mechanism to account for inherent or acquired resistance to anti-HER2 therapies [19, 31, 55, 56]. The high presence of HER3 mRNA has been related to a better prognosis in patients carrying HER2-positive breast carcinoma treated with adjuvant pertuzumab therapy [38]. HER3 protein overexpression, for its part, has been shown to predict poor outcome in a group of HER2-positive breast cancer patients receiving

adjuvant trastuzumab as a first-line therapy [25, 29]. In contrast, a recently published study [44] postulates that HER3 is not an informative biomarker to predict trastuzumab sensitivity. Overall, it seems that the expression profile of any single HER protein, in addition to HER2, is insufficient to predict the trastuzumab response. This is due to a complicated signalling network involving interacting HER receptors, their ligands and downstream signalling proteins [38, 49, 95].

We also analysed HER3 expression and RFS in a subgroup of patients who received adjuvant trastuzumab therapy. In the current study, HER3 expression was not shown to be predictive for adjuvant short-term (9-wk regimen) trastuzumab therapy as a first-line therapy. The recurrence rate and relapse-free survival time during the follow-up were not markedly different when stratified according to adjuvant trastuzumab therapy. Presently, one year is the recommended standard for trastuzumab therapy duration, which is based on clinical proof of prolonged survival compared to a shorter administration regimen [96–98]. This may have affected the observed recurrences in HER2+ BCA cohort, and is considered as a limitation of this study when applying these results in the current clinical practice.

The expression of HER3 receptors differs specifically from its close relative HER2. Unlike HER3, HER2 tightly attaches to the cell membrane when trafficked from the Golgi apparatus to its putative membranous location, remaining there for prolonged periods [99, 100]. This enables reliable detection and localization of HER2 protein by IHC. In contrast, HER3 receptors are unstable and constitutively internalized from the cell membrane into the cytoplasm and nucleus [101–103], which complicates the detection of this receptor type by IHC. Once internalized, HER3 is quickly ubiquitinated and transferred to proteasomes for degradation. Due to the continuous trafficking of HER3 receptors, the appearance of membrane-bound HER3 receptors does not necessarily conform the efficiency of HER3 protein synthesis machinery at the transcriptional level. There are many mechanisms in distinct facets of HER3 protein synthesis that can be disabled when HER3 is down- or up-regulated [13]. In addition, abnormal cellular HER3 receptor quantity or localization may be due to altered HER3 degradation mechanisms or the presence of exogenous stimuli with regulatory capacity on HER3 [102, 104, 105].

In the current study, we also demonstrated the expression of two regulatory proteins, NEDD4–1 and NRDP1, both of each contribute to the maintenance of HER3 receptors by mediating the degradation process via ubiquitination. We demonstrated that NEDD4–1 protein was predominantly over-expressed in *HER2*-amplified breast carcinomas; herein, 83% of carcinomas were positive for NEDD4–1. Only one earlier study clarified the NEDD4–1 protein expression pattern in breast cancer and demonstrated NEDD4–1 expression in 55% of studied cases [74]. This earlier finding is not fully comparable with the current result because of the minor representation of HER2-positive breast carcinomas. To the best of our knowledge, this is the first study to clarify the relationship between HER3 and NEDD4–1 proteins in primary breast cancer tissue in situ. In contrast to our expectation from the theoretical perspective [63], HER3 protein expression was not negatively associated with NEDD4–1 expression. In fact, we found a statistically significant parallel correlation between membranous HER3 and NEDD4–1 expression. Based on our data, we hypothesize that HER3 trafficking out from the cell membrane preceding its degradation is under more complicated controlling mechanisms than NEDD4–1 expression alone.

NRDP1 protein expression was infrequent and did not show any clinically meaningful correlations or prognostic potential to predict the outcome of *HER2*-amplified breast cancer. The absence of cytoplasmic NRDP1 expression was more common in carcinomas characterized by low cellular HER3 expression but was not otherwise associated with HER3. However, only 8.3% of carcinomas in all were shown to display nuclear or cytoplasmic NRDP1 protein expression in our *HER2*-amplified breast cancer cohort. Consequently, frequent HER3 expression in HER2-dependent breast cancer subtype does not seem to inversely associate with NRDP1 expression, but the result needs to be confirmed in a larger sample cohort because of relatively low NRDP1 expression observed in the current study. We speculate that low NRDP1 expression in *HER2*-amplified breast cancers could be mechanistically explained by the previous study of Yen et al. [75], in which NRDP1 loss was shown to enhance HER2/HER3-dependent breast tumour cell growth and tumour progression. We found only one earlier study focusing on NRDP1 expression in clinical breast cancer cohort. In this study [76], absent or low NRDP1 protein expression (approximately 42% of carcinomas) was related to worse breast cancer outcome during the ten-year follow-up period. NRDP1 expression was shown more common (approximately 58% of carcinomas) than we indicated in the current study. Comparable criteria for determining the NRDP1 expression was applied in both studies, but the IHC staining procedures and sample cohort characteristics, especially for HER2 status, were not similar and may explain the difference.

To further clarify the biological and prognostic relevance of HER3 in the therapy context of *HER2*-amplified breast cancer, many continuing research objects seem necessary. The determination of HER3 expression in metastatic lesions of breast carcinomas treated with

anti-HER2 therapy, such as trastuzumab, would elucidate the concept of intensified HER3 signalling due to HER2 downregulation. HER3 upregulation has been related to trastuzumab resistance in studies [19, 106] showing that breast cancers driven primarily by HER2 homodimerization are more susceptible to trastuzumab therapy than tumours with a predominance of HER2-HER3 heterodimers. From this context, it would be interesting to determine HER3 expression in breast carcinomas that are confirmed intrinsically resistant to trastuzumab. To elucidate the therapeutic predictive potential of HER3, one intriguing thought is to clarify HER3 expression retrospectively in breast cancer patients who were subsequently treated with adjuvant pertuzumab or novel HER3-targeting antibodies.

Conclusions

The results of the current study suggest HER3 as a novel versatile biomarker to predict recurrence of *HER2*-amplified breast cancer. Irrespective of its subcellular localization, absent or low HER3 expression was associated with shorter RFS time when compared to HER3-overexpressing breast carcinomas. Low HER3 expression was associated with clinicopathological characteristics related to more aggressive and therapeutically unfavourable breast cancer types, such as axillary lymph nodal infiltration, larger tumour size, young patient age, negative ER status, triple-negative subtype, and basal phenotype. HER3 did not show any predictive value for the benefit of short-term (9-wk) adjuvant trastuzumab therapy as a first-line therapy. The HER3 degradation regulators NEDD4–1 and NRDP1 did not show any clinically meaningful correlations or predictive or prognostic applicability in *HER2*-amplified breast cancer subtype.

Additional file

Additional file 1: IHC-staining protocols provide detailed information on reagents used in the current study to demonstrate HER3, NEDD4–1, NRDP1, and Cytokeratin 5/14 protein expression on FFPE breast cancer tissues. (PDF 283 kb)

Abbreviations

ER: Oestrogen receptor; FFPE: Formalin-fixed paraffin-embedded tissue; FLRF: Fetal liver ring finger; HER1/2/3: Human epidermal growth factor receptor 1/2/3; HIER: Heat-induced epitope retrieval; HRG: Heregulin; IHC: Immunohistochemistry; NEDD4–1: Neural precursor cell expressed developmentally downregulated 4–1; NRDP1: Neuregulin receptor degradation protein-1; PR: Progesterone receptor; RFS: Recurrence free survival; RNF41: RING finger protein 41; ROI: Region of interest; TMA: Tissue microarray; TNBC: Triple-negative breast cancer

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Availability of data and materials

The datasets used and analyzed in the current study are available from the corresponding author on reasonable request. The datasets supporting the conclusions of this article are included within the article and its additional files.

Authors' contributions

SL, MT and JI designed the study. SL and SS harvested retrospectively clinical follow-up data and diagnostic information from the pathological review of cancer samples. AK provided the BCA sample set and derived clinical data. SL conceived the study in practice, performed data analysis and wrote the manuscript. All authors contributed to data interpretation, read and approved the final manuscript.

Ethics approval and consent to participate

The use of the tumour samples and patient records in this study was approved by the Ethics Committee of Pirkanmaa Hospital District (references no. R07082, R00143), the Ministry of Social Affairs and Health, and the National Authority for Medicolegal Affairs in Finland. The need for informed consent was waived by the aforementioned authorities due to the large number of samples and the fact that a sizeable portion of the patients were already deceased. Individual patient data has not been made available and the dataset has been handled anonymized.

Consent for publication

Not applicable.

Competing interests

None of the authors declares any conflicts of interest.

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